DIETARY INTAKE, B VITAMINS AND HEALTH OUTCOMES IN THE VERY OLD: ANALYSIS OF THE NEWCASTLE 85+ STUDY

A thesis submitted to Newcastle University for the degree of *Doctor of Philosophy*

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Dedication

I dedicate this PhD thesis to my grandmother, Ana Rosa, who turned 85 last March. This would also not have been possible without the unconditional support from my mother, brother, stepfather, stepmother and friends.

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Declaration of Authorship

As it is normal with secondary data analysis, published and submitted manuscripts in this thesis did not only include myself but also supervisors and co-authors who were in a way or another, involved with the Newcastle 85+ Study. My independent contribution was in food coding and assignment of foods to food groups, designing and conducting the study, database management, performing analyses, interpreting the findings and writing the manuscripts with punctual feedback from supervisors and co-authors. The thesis was written in its entirety by me with feedback from my supervisors, Tom Hill, Carol Jagger and Chris Seal. I report no conflict of interest.

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Abstract

The very old (aged 85 and over) are the fastest growing age group in the UK and most western societies. High incidence of disability and chronic diseases, financial constraints, polypharmacy, hospitalisation, body composition, sensory and gastrointestinal changes place very old adults at increased risk of malnutrition. However, very little is known about the dietary habits and health trajectories in this age group. Further, because one-carbon (1-C) metabolism biomarkers are largely modifiable and have been associated with cognition, cardiovascular disease (CVD) and all-cause mortality, its modulation is of special interest. The overall aim of this PhD thesis was to provide an accurate snapshot of the dietary habits of the very old and examine health trajectories with respect to 1-C metabolism biomarkers in a unique cohort such as the Newcastle 85+ Study. Specifically, we aimed to explore the dietary habits of the very old; to investigate the association between folate, vitamin B12 and its status; and to investigate the cognitive decline and mortality trajectories with respect to 1-C metabolism biomarkers. The Newcastle 85+ Study is a longitudinal population-based study of health trajectories and outcomes over 5 years in 845 eighty-five year olds in North East England. Dietary intake was assessed at baseline on two non-consecutive occasions by a 24 hour Multiple Pass Recall. Baseline red blood cell folate (RBC folate), plasma vitamin B12 and total homocysteine (tHcy) concentrations were determined by immunoassays. Cognitive function was assessed at baseline, 1.5, 3 and 5 years with the standardized mini-mental state examination and a battery of attention tests. Mortality was obtained from the Health and Social Care Information Service, UK. A high percentage of the participants did not meet the dietary reference values for energy, non-starch polysaccharides and several micronutrients. Cereals and cereal products were the top contributors for energy, most macronutrients and several micronutrients, including folate. RBC folate and tHcy were associated with better global cognition at baseline but were not predictive of the rate of decline over 5 years. Higher concentrations of tHcy in all participants and plasma vitamin B12 in women were associated with increased risk of all-cause and cardiovascular mortality. This thesis highlights the paucity of data and uncertainties in this age group. Furthermore, it demonstrates a link between 1-C metabolism biomarkers and age-related diseases.

List of Abbreviations

1-C	One carbon
25(OH)D	25-Hydroxyvitamin D
AC	Activation coefficient
AdoCbl	Adenosylcobalamin
AFRD	Agriculture, food and rural development
AOAC	Association of official analytical chemists
Apo A1/B	Apolipoprotein A1/B1
ARH1	Heterogeneous first-order autoregressive
ARUK	Alzheimer's research UK
BMI	Body mass index
BMR	Basal metabolic rate
BP	Blood pressure
Ca	Calcium
Carb	Carbohydrate
CBS	Cystathionine β-synthase
CCP	Cereals and cereal products
CD320	Cluster of differentiation 320
CDR	Cognitive drug research
CI	Confidence interval
CoA	Continuity of attention
COMA	Committee on medical aspects of food policy
CRT	Choice reaction time
CUBN	Cubilin
CVD	Cardiovascular diseases
DBP	Vitamin D binding protein
DHFA	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DNFCS	Dutch national food consumption survey
DNMT	DNA methyltransferase
DRV	Dietary reference values
DVT	Digit vigilance task
EDTA	Ethylenediamine tetraacetic acid
EFSA	European food safety authority
EI	Energy intake
EPHA10	Ephrin receptor A10
EPIC	European prospective investigation into cancer and nutrition

ESPEN	European society for clinical nutrition and metabolism
Est	Estimated
FAD	Flavin adenine dinucleotide
FAS	Fatty acid synthase
FFQ	Food frequency questionnaire
FQH	Food quality and health
FUT2	Fucosyltransferase 2
Gam	Generalized additive model
GP	General practitioner
HAND2	Heart and neural crest derivatives expressed 2
Hba1c	Glycated haemoglobin
HDL	High density lipoprotein
HNRC	Human nutrition research centre
HOXD4	Homeobox D4
HR	Hazards ratio
HSCIC	Health and social care information centre
IF	Intrinsic factor
IL-6	Interleukin 6
IoM	Institute of medicine
IQR	Interquartile range
LC/MS-MS	Liquid chromatography with mass spectrometry
LDL	Low density lipoprotein
LIDNS	Low income national diet and nutrition survey
LINE-1	Long interspersed nuclear elements-1
LRNI	Lower reference nutrient intake
MAF	Minor allele frequency
MAT	Methionine adenyltransferase
MeCbl	Methylcobalamin
MMP	Meat and meat products
mo	Months
MPR	Multiple pass recall
MTHFR	Methylenetetrahydrofolate reductase
MTR	Methionine synthase
MTRR	Methionine synthase reductase
MUFA	Monounsaturated fatty acids
NDNS	National diet and nutrition survey
NHANES	National health and nutrition examination survey
NHS	National health service
NMES	Non-milk extrinsic sugars

NSP	Non-starch polysaccharides
NS-SEC	National statistics socioeconomic classification
OECD	Organisation for economic co-operation and development
OR	Odds ratio
P:S	PUFA:SFA ratio
PA	Pyridoxic acid
PCFT1	Proton-coupled folate transporter
PLP	Pyridoxal phosphate
PoA	Power of attention
PON1	Paraoxonase 1
PPI	Proton pump inhibitors
PUFA	Polyunsaturated fatty Acids
RBC	Red blood cell
RCS	Restricted cubic splines
RCT	Randomized controlled trial
RE	Retinol equivalents
RTV	Response time variability
SACN	Scientific advisory committee on nutrition
SAH	S-adenosyl homocysteine
SD	Standard deviation
SENECA	Survey in Europe on nutrition and the elderly: a concerted action
SES	Socio-economic
SFA	Saturated fatty acids
SMMSE	Standardized mini-mental state examination
SPSS	Statistical package for the social sciences
SRT	Simple reaction time
Stand	Standard
TCA	Tricarboxylic acid cycle
TCN1	Transcobalamin 1
tHcy	Total homocysteine
THF	Tetrahydrofolate
TNF-α	Tumor necrosis factor α
TS	Thymidylate synthase
TUSC3	Tumor suppressor candidate 3
TWIST2	Twist family basic helix-loop-helix transcription factor 2
UK	United Kingdom
VIF	Variation inflation factor
Vit.	Vitamin
VIU	Venice international university

WW2	World war 2

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CHAPTER ONE

1. Introduction

1.1. Demographics of ageing

The Western world is experiencing a major demographic shift and the United Kingdom (UK) does not escape this trend [1,2]. Adults aged 65 and over comprise, on average, 18% of the total population of the European Union (EU) [3]. A similar figure to what is now observed in the UK, where the number of adults aged 65 and over increased by 17.3% in the last decade and now account for 17.4% of the 64.1 million people [4]. As of 2014, there were 22 countries in Europe with a life expectancy at birth higher than 80 years and 17 countries with a life expectancy at age 65 higher than 20 years [5]. Life expectancy has not only been rising from birth but also at age 65. In the UK, since 1980-82, life expectancy at age 65 has increased from 13.0 years to 18.5 years for men and from 16.9 years to 20.9 years in women in 2013-2015 [6]. This steady rise in life expectancy and decrease in later life mortality make very old people (those aged 85 and over) the fastest growing age segment of western societies [1,2]. In the UK, there are now more than 1.5 million very old people (2.5% of total population) and the number is projected to rise to 3.3 million or 5% over the next 20 years [7].

However, the increase in healthy life expectancy (number of years an individual can expect to live "disease free") has not been as fast as life expectancy. UK's life expectancy at birth increased by 6.2 years in men (from 72.9 to 79.1) and 4.3 years in women (from 78.5 to 82.8) from 1990 to 2013 while healthy life expectancy increased by only 4.7 years for men (from 63.8 to 68.5) and 3.3 years for women (from 67.3 to 70.6) in the same period [8].

Maintaining health in old age is of utmost importance and lifestyle, which include dietary habits, might be responsible for some of the discrepancy between life expectancy and healthy life expectancy.

Large cohorts of ageing from the Health and Retirement Study (HRS) [9] family of studies in Europe such as the Survey of Health, Ageing and Retirement in Europe (SHARE) with 120000+ participants aged 50 and over from continental Europe and Israel [10], the English Longitudinal Study of Ageing (ELSA) which includes 10000+ individuals aged 50 and over [11], the Irish Longitudinal Study on Ageing (TILDA) with more than 8000 participants aged 50 and over [12], and the Northern Ireland Cohort for the Longitudinal Study of Aging (NICOLA) with more than 8000 adults aged 50 and over [13] are essential to better understand the ageing process and, its socioeconomic and psychobiological implications, as well as the societal demands. More specialized cohorts such as the Newcastle 85+ Study and the European Prospective Investigation into Cancer and Nutrition (EPIC) [14] can be of even greater importance to understand specific research questions, including nutritional, in older adults but also the very old.

1.2. The role of nutrition in health and disease

Food is central to the physical, psychological and social wellbeing at all life-stages, including in very old age. Diet is a major determinant in the development and management of a range of conditions and illnesses including type 2 diabetes, coronary heart disease (CHD), atherosclerosis, stroke, and cancer which are frequently the leading of causes of death in western societies and elsewhere [15,16].

Obesity is primarily caused by an imbalance between energy intake and energy expenditure. Obesity is strongly associated with health complications such as cardiovascular disease [17], hypertension, hyperlipidaemia, type 2 diabetes, arthritis, urinary incontinence [18] and several types of cancer [19] in older adults [20]. Dietary changes are important in all stages of life but also in older adults [21]. An increase of 1-2 portions of fruit and vegetables per day could cut cardiovascular diseases (CVD) risk by 30% [22]. The consumption frequency of foods or drinks high in sugar is a major causal factor for the development of dental caries in the absence of good dental hygiene [23]. Further, high consumption of saturated and trans-fat increases low density lipoprotein (LDL) cholesterol and total cholesterol and decreases high density lipoprotein (HDL)

cholesterol, which is a risk factor for heart disease and may persist into very old age [24]. Indeed, a 10% reduction in serum total cholesterol could reduce the risk of CHD by 25-30% [25]. Higher fat consumption is also associated with several types of cancer, mainly in the gastrointestinal tract [26]. Several studies also point to a possible protective role of omega-3 fatty acids in relation to cardiovascular disease and dementia [27]. High consumption of salt and alcohol are associated with high blood pressure [24]. Indeed, a reduction of 6 mm Hg of systolic blood pressure is estimated to reduce the risk of heart attack by 15% and stroke by 40% [28]. Micronutrient deficiencies have also been repeatedly associated with health and diseases. For example, a low vitamin D status may increase the risk of mortality [29], cognitive decline [30], several age-related diseases and low mood and depression [31]; Low vitamin K intake and status was associated with low bone mass and increased fracture risk [32] and; low status of B vitamins, especially folate, B12 and B6 were associated with increased risk of stroke and cognitive decline [33].

1.3. Nutritional guidelines and current challenges in very old age

No specific nutritional guidelines for people aged 85 years and over are provided by major national authorities in the UK, EU (EFSA), USA or Australia. The Food and Nutrition Board, Institute of Medicine in the USA and the Scientific Advisory Committee on Nutrition (SACN) in the UK offer recommendations for those aged 70+ years and 75+ years, respectively, but only for specific aspects and, to a large extent, based on evidence from younger adults with unknown relevance for the very old [34,35]. The British Nutrition Foundation (BNF) issued easy to follow health ageing tips for older adults that synthetize evidence-based recommendations [20,36]:

1) To diversify the diet and make it enjoyable;
2) To frequently monitor weight and waist size;
3) To maintain a good state of hydration by drinking 6-8 glasses (1.2L) of fluid per day on top of the water that is provided from food;
4) To keep alcohol consumption to ≤14 units per week;
5) To increase the dietary fibre intake with wholegrain cereals, legumes, vegetables and fruit**;**
6) To reduce salt intake;

7) To reduce intake of saturated fatty acids and replace them, if needed, for mono and polyunsaturated fatty acids;
8) To increase fish intake (two portions per week) and include oily fish, such as sardines, mackerel, tuna and salmon;
9) To include calcium-rich foods in their diet;
10) To maintain an optimum status of B vitamins;
11) To take a vitamin D supplement per day if aged 65 and over;
12) To stay physically (at least 150 minutes of moderate-intensity aerobic activity or 75 minutes of vigorous intensity aerobic activity per week) and mentally active;
13) To maintain a good dental health and;
14) To avoid smoking.

However, apart from the recommendation to take a daily vitamin D supplement, all other recommendations are applicable to the general population as well as older adults. This stresses the need to better understand the nutritional needs of older adults and the very old.

The very old are a very heterogeneous population group ranging from healthy and active individuals with few disabilities to those with multiple diseases and multimorbidity [37]. In the cross-sectional baseline assessment of the Newcastle 85+ Study, none of the 851 participants were completely free of chronic diseases and the median number of diseases was 5 with an interquartile range (IQR) of 3-6 [37]. This heterogeneity leads to both practical and conceptual difficulties in determining the nutritional needs of the very old and issuing public health recommendations with wide applicability. For example, there is limited understanding and agreement of the outcome measures that could be used to derive nutritional guidelines and nutritional adequacy in this age group. The vast majority of physical and cognitive functions tend to decline with age but the great inter-individual variability of when it happens and how rapid the decline is creates difficulties (but also opportunities) to assess biomarkers of healthy ageing [38,39]. Further, the relationships between biomarkers and health outcomes are frequently considerably different between the very old and their younger counterparts. For example, whilst hypertension is a well-established risk factor for cardiovascular disease (CVD) and mortality among younger adults, the same relationship was not observed in those aged 85 years and over [40]. Indeed, among those aged 80 and over resident in nursing homes, low blood pressure was predictive of higher mortality [41]. High blood pressure was also associated with greater cognitive

impairment in those younger than 75 but with better cognitive function in those aged 85 and over [42]. Analyses of the Newcastle 85+ Study also found that low systolic blood pressure was associated with greater risk of cognitive impairment and disability count [43]. Another good example is the relationship between telomere length and ageing. Several studies based on younger populations showed that telomeres shorten with age [44] and it was proposed that white blood cells' telomere length acted as a biomarker of cumulative oxidative stress [45]. However, the same relationship was not present in the very old as telomere length was not associated with morbidity and mortality in the Newcastle 85+ Study [43]. Given the lack of data on dietary intake, nutritional status and associations with health trajectories in the very old, filling in this gap should take priority.

1.4. Dietary assessment in the very old

To have an accurate record of the habitual food intake of an individual or group of individuals and understand nutrition-related outcomes, collection of robust dietary intake data is essential. Dietary assessment at any life stage presents challenges. Progress in the development of biomarkers could mean that, in the future, such challenges can be addressed by analysis of biological samples [46], but meanwhile dietary assessment remains labour intensive and costly. Methods at researchers' disposal include weighed dietary intakes, estimated weight food diaries, food records and food frequency questionnaires (FFQ), with each method requiring varying levels of commitment, time and cognitive ability from the respondent, as well as the researcher's time and skill. The question to be answered but also the population group to be assessed must be decisive factors when choosing the dietary assessment method.

Assessing food choice and/or nutrient intake in older people, particularly in the very old (85 years and over), presents challenges. The respondent may have little or no involvement in food purchasing or preparation, cognitive impairment may restrict his/her ability to recall intake, and ability to record intake may be limited by physical limitations, sensory impairment and communication difficulties. The interviewer may need to rely on a carer as a proxy reporter of dietary intake which may then be compounded by the fact that a variable number of individuals or carers may be involved in the care of the respondent on any given day. Thus, in this age group, it is important that the chosen retrospective dietary assessment method is not dependent on self-recording of intake by participants. No

retrospective methods of dietary assessment had been used previously in this age group in the UK prior to the Newcastle 85+ Study (all the NDNS surveys used either 4 or 7 day weighed dietary intakes which is very resource intensive and imposes a large burden on subjects). A review of NDNS surveys showed that those of lower socio economic status and minority ethnic groups were under-represented [47]. Therefore a survey to increase the response rate of those living on a low income was planned and a 24hr Multiple Pass Recall (24hr-MPR) method was used in the Low Income National Diet and Nutrition Survey (LIDNS) on 4 different occasions [48].

1.5. Dietary intake in the very old in Europe

Given the challenges that dietary assessment presents in the very old, it is not surprising that dietary intake data in this age group are scarce. Only a reduced number of population based studies have assessed dietary intake in the very old. However, some representative and non-representative studies have attempted to measure energy and nutrient intake of ≥80 or 85 year olds in Europe. The National Diet and Nutrition Survey (NDNS) of people aged 65 years old and over was carried out during 1994-95 in the United Kingdom and included two nationally representative samples drawn from adults aged 65 and over, free-living and institutionalised. Health background questionnaires, 4-d weighed diet record and blood/urine samples were collected. Four hundred and fifty nine free-living adults (172 men and 287 women) aged 85 and over completed the 4-d weighed diet record [49]. The European Prospective Investigation into Cancer and Nutrition (EPIC)-Oxford study is a prospective cohort study that started in 1993 in Oxford, UK and was designed to investigate how diet influences the risk of cancer. A food frequency questionnaire (FFQ), lifestyle questionnaire and blood samples were collected. By the time of the third follow-up in 2010-2014, 1283 adults (411 men and 872 women) aged 80 and over had completed the FFQ [14]. The Dutch National Food Consumption Survey of free-living older adults (DNFCS-Older adults) was carried out in the Netherlands in 2010-2012 and included one nationally representative sample of ≥70 year olds. Two 24-hour recalls, anthropometric measures and background questionnaires were collected. In the DNFCS-Older adults, 225 adults ≥80 (103 men and 122 women) completed both 24-hour recalls [50]. The InCHIANTI Study was conducted in 1998 in Tuscany, Italy and included people aged 21–103 years old. Data were collected on dietary intakes (FFQ), sociodemographic, lifestyle and functional characteristics.

Of the total 1436 that completed the FFQ, 170 (60 men and 113 women) were aged 85 and over [51]. A German nationally-representative study was conducted on behalf of the German Ministry of Health in 1998 to describe the energy and nutrient intakes of older free-living adults. Sociodemographic, lifestyle and dietary assessment (3-d dietary record) were collected. Two hundred and eighty seven adults ≥85 (89 men and 198 women) had complete dietary records for the 3 days [52]. The 2003 Austrian Nutrition Survey collected dietary data, using 3-d food records, on adults aged ≥85 and included 22 men and 93 women [53].

Nutrient intake comparisons between these studies should be done cautiously as the dietary assessment methods, data collection period, sample size, food composition tables used and nutrient definitions are seldom the same. Of the six European studies with considerable numbers of ≥80 or 85 older adults, one used 24h recalls, two used FFQs and three used different forms of diet records. EPIC-Oxford had the largest number of ≥80 (n=1283) of the six. The energy and selected nutrient intakes in European very old adults (≥80 or 85) is shown in **Table 1.1**. Men and women of the German Nutrition Survey and EPIC-Oxford had the highest energy intakes (> 9.25 for men and 8.0 MJ for women). Overall, 41-50% of the energy intake came from carbohydrates, 31-40% from fat and 14-16% from protein. Dietary fibre intake varied considerably between country and study and depended largely on the dietary assessment method (FFQ vs 24h recall) and analysis method [Englyst or the Association of Official Agricultural Chemists (AOAC)]. Vitamin and mineral intakes in the EPIC-Oxford were generally higher than in any other study but the choice of dietary assessment method and participants' age have to be taken into account. The very old adults participating in the Dutch National Food Consumption Survey (DNFCS) had higher intakes of vitamin B12 and calcium than others (except EPIC-Oxford) [50]. This was likely a reflection of the higher contribution of dairy products to calcium and vitamin B12 [50].

Table 1.1. Intake of energy and selected nutrients intake in very old Europeans.

Cohort	Men										Women									
	Energy MJ/d	Carb %	Fat %	Protein %	Fibre g/d	Folate µg/d	B12 µg/d	D µg/d	Ca mg/d	Iron mg/d	Energy MJ/d	Carb %	Fat %	Protein %	Fibre g/d	Folate µg/d	B12 µg/d	D µg/d	Ca mg/d	Iron mg/d
NDNS 65+[49]	6.99[2]	48.5	36.3	15.2	11.4[3]	219	3.8	2.8	717	9.7	5.60[2]	48.4	36.8	14.5	9.4[3]	170	2.9	2.0	619	7.5
EPIC[1] [14]	9.84	49.7	31.4	15.5	24.5[3]	466	7.5	4.2	1157	18.1	9.02	50.3	31.5	16.3	24.0[3]	461	7.5	4.0	1147	17.0
DNFCS[50]	7.40	41.4	34.0	16.4	20.0	46[4]	4.9	3.9	1016	9.6	7.30	41.0	35.0	15.6	16.2	34[4]	4.4	2.9	2030	8.3
InCHIANTI[1] [51]	7.38	50.0	29.0	16.0	17.2	228	-	-	778	11.5	6.36	50.0	32.0	16.0	15.3	200	-	-	701	9.6
German Nutrition Survey[52]	9.34	44.2	33.2	16.3	23.7	123[5]	-	3.8	721	13.3	8.07	42.6	35.0	16.2	19.9	106[5]	-	2.7	729	12.6
Austrian Nutrition Survey[1] [53]	7.40	44.0	40.0	14.0	15.0	174[5]	4.0	3.4	642	10.0	7.10	43.0	40.0	16.0	16.0	166[5]	3.9	3.1	649	11.1

Values are medians unless stated otherwise. NDNS 65+, National Diet and Nutrition Survey of people aged 65 years old and over; EPIC, European Prospective Investigation into Cancer and Nutrition; DNFCS, Dutch National Food Consumption Survey; InCHIANTI, Ageing in Chianti; Carb, Carbohydrates; B12, Vitamin B12; D, Vitamin D; Ca, Calcium.

[1] Values are means.

[2] Does not include alcohol.

[3] Non-starch polysaccharides.

[4] Only folic acid.

[5] Dietary folate equivalents (1 µg DFE = 1 µg food folate = 0.5 µg folic acid supplement on empty stomach = 0.6 µg folic acid from fortified food or as a supplement taken with meals).

1.6. Nutritional deficiencies - folate and vitamin B12

In the UK, over 10% of older adults (aged 65 and over) and 18% of those aged 85 and over are at medium or high risk of malnutrition [54]. Public expenditure on disease-related malnutrition is estimated to exceed £13 billion per year and over half is expended on older adults [55]. The very old are at increased risk of nutritional deficiencies due to inherent biological ageing changes but also due to higher prevalence of chronic diseases and its treatments, polymedication, increased hospitalization and financial restrains, reduced mobility, social isolation and increased dependence on others [56]. These health and social factors are coupled with loss of lean mass, relative increase in fat mass, loss of bone density, fluid and electrolyte regulation, decline in taste sensitivity and malabsorption [57]. Further, age *per se* and some widely used drugs in this age group, have adverse effects on the sense of taste [58,59], on appetite [60] or on nutrient use by the body [61]. For instance, poor appetite [62] may lead to inadequate protein intake, which is a risk factor for the development of sarcopenia (loss of muscle mass and function) and physical frailty [63]. Although micronutrient malabsorption is not an inherent consequence of ageing, the absorption of pH-dependent vitamins and minerals, such as folate, vitamin B12, calcium, iron and β-carotene might be partially compromised [64,65]. For example, about 10-30% of older adults have atrophic gastritis (caused by *Helicobacter pylori* infection, long-term use of proton pump inhibitors, H_2 receptor antagonists and biguanides) which leads to hypochlorhydria [64]. This has a detrimental effect on acid-pepsin digestion and favours small bowel bacterial growth resulting in impaired vitamin B12 absorption [66]. In addition, those with autoimmune atrophic gastritis produce antibodies against intrinsic factor which can lead to pernicious anaemia [66]. Very old adults are also at higher risk of vitamin D deficiency due to reduced skin stores of 7-dehydrocholesterol (provitamin D) (coupled with reduced sun exposure), renal impairment and reduced renal conversion of its biologically inert to active form (i.e. 25-hydroxyvitamin D to calcitriol), immobility, malnutrition and environmental factors [reviewed in Hill *et al.* [67]]. Micronutrient deficiencies contribute to disability, frailty and impaired physical function in very old adults [68].

Energy requirements are largely dependent on energy expenditure so that the decrease in energy needs in later life mirrors the age-dependent fall in physical activity, through frailty, disability or disease. While energy requirements generally decrease with age there is currently no convincing evidence that vitamin and mineral requirements decrease as

well. In 2011, the Scientific Advisory Committee on Nutrition (SACN) released new energy dietary reference values (DRVs) for the UK. Energy DRVs were set at 11.5 MJ for men and 9.1 MJ for women aged 25-34 and; 9.6 MJ for men and 7.7 MJ for women aged 75 and over (decrease of ~2MJ) [34]. However, folate and vitamin B12 DRVs remain constant throughout adulthood according to the Committee on Medical Aspects of Food Policy (COMA) 1991 report [69]. This difference in requirements between energy and micronutrients adds to the potential risk for folate and vitamin B12 deficiencies in this age group.

A review of micronutrient deficiencies in community-dwelling older adults (aged 65 and over) living in western countries reported that 29% and 16% of men and, 30% and 19% of women had intakes below the Nordic Nutrition Recommendations (NRR) estimated average requirement (EAR) for folate (200 µg/d) and vitamin B12 (1.4 µg/d), respectively [70]. Another review found out that 17-34% of older men and 18-46% of older women living in Europe had folate intakes below the EAR and 0-20% of older men and 0-21% of older women had vitamin B12 intakes below the EAR [71].

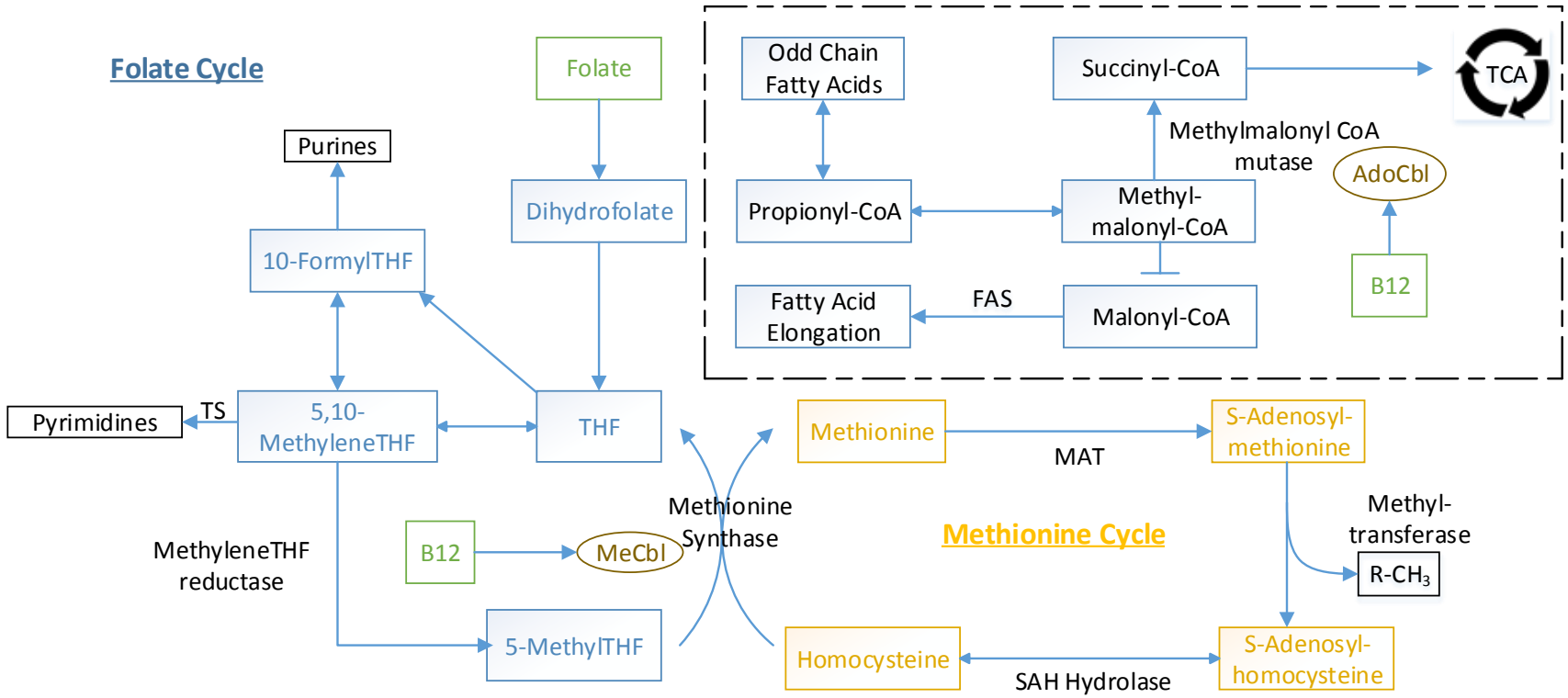
Seventeen percent of free-living older adults participating in the National Diet and Nutrition Survey (NDNS) of people aged 65 and over were below the UK EAR for folate of 150 µg/day [69,72] and 2% had vitamin B12 intakes below the EAR of 1.25 µg/day [69,72]. Intake of most micronutrients was approximately 10% lower in those aged 85 and over than those aged 65-74 years old [49]. The NDNS of people aged 65 and over concluded that the great majority of vitamin and mineral deficiencies increased with age and fall with socioeconomic status [72,73]. The current NDNS rolling programme estimated that 1% of older adults (aged 65 and over) were below the UK lower reference nutrient intake (LRNI) for folate (100 µg/d) and vitamin B12 (1.0 µg/d) and that 7.3% of men and 10.8% of women had red blood cell folate (RBC) concentrations below 340 nmol/L and 5.9% of men and women had serum vitamin B12 concentrations below 150 pmol/L [74].

1.7. The role of folate and vitamin B12 in one-carbon metabolism and fatty acid synthesis

The involvement of folate and vitamin B12 on 1-C metabolism and fatty acid synthesis is shown in **Figure 1.1** Methionine synthase requires a form of vitamin B12, methylcobalamin (MeCbl) to convert 5-methylTHF to tetrahydrofolate (THF) (folate cycle) and homocysteine to methionine (methionine cycle). Methionine is further converted to S-

adenosylmethionine (SAM), the main methyl donor in the brain. Another form of vitamin B12, adenosylcobalamin acts as a co-factor for methylmalonyl-CoA mutase and without it methylmalonyl-CoA would accumulate and not be converted to succinyl-CoA. For example, a build-up of methylmalonyl-CoA would have negative effects on the membranes of the neuronal tissue by two possible mechanisms: methylmalonyl-CoA would inhibit malonyl-CoA and result in decreased fatty acid synthesis and; be converted back to proprionyl-CoA (can substitute acetyl-CoA in fatty acid synthesis), resulting in the incorporation of odd chain fatty acids in the myelin sheath. Within the folate cycle, 10-formylTHF provides 1-C units for purine synthesis (guanine and adenine) and 5,10-methyleneTHF acts as a cofactor in the reaction leading to thymidylate synthase that will eventually synthesize pyrimidines (thymidine). Another intermediate in the 1-C metabolism, 5-methylTHF, acts as a co-substrate in the conversion of homocysteine to methionine by methionine synthase. Vitamin B12 and folate deficiency also lead to hyperhomocysteinemia, reduced synthesis of neurotransmitters (dopamine, epinephrine and serotonin) and decreased synthesis of purines/pyrimidines.

Figure 1.1. Folate and vitamin B12 in one-carbon metabolism and fatty acid synthesis.



TCA, tricarboxylic acid cycle; THF, tetrahydrofolate; AdoCbl, adenosylcobalamin; FAS, fatty acid synthase; TS, thymidylate synthase; MeCbl, methylcobalamin; MAT, methionine adenyltransferase; SAH, S-adenosyl homocysteine.

1.8. Folate

Folate is essential in 1-C transfer reactions as the 1-C units are used for *de novo* biosynthesis of purines and pyrimidines, remethylation of homocysteine to methionine and interconversions of serine and glycine. Therefore it is no surprise that, besides the classic symptom of over folate deficiency, megaloblastic anaemia [75], folate deficiency/ insufficiency has been associated with neural tube defects [76], stroke [77,78], bone health [79,80], cognitive impairment [81-83], certain types of cancer [84-86] and all-cause mortality [87].

Folate or vitamin B9 is a general term to describe water-soluble tetrahydrofolate (THF) derivatives belonging to the group of B vitamins. Folate is composed of a mixture between monoglutamates and polyglutamates and is naturally present in foods such as spinach, asparagus, kale, brussel sprouts, black-eyed peas and liver [88]. Folic acid is the synthetic fully oxidized monoglutamate used in fortified foods and dietary supplements and it is more stable than folate [89] (**Figure 1.2.**). Naturally occurring folate is unstable and a variable degree of losses occur when light or oxygen or high temperatures are present [89]. The UK has set the dietary reference value (DRV) for folate at 100, 150 and 200 µg/day for lower reference nutrient intake (LRNI), estimated average requirement (EAR) and reference nutrient intake (RNI), respectively for adults aged 50 and over [69].

Intestinal and tissue absorption

Ingested polyglutamated folate is hydrolyzed to a monoglutamated form and absorbed in the jejunum by an active and pH-dependent mechanism with the aid of the proton-coupled folate transporter (PCFT1) [90]. Folates that are not initially absorbed are later absorbed in the ileum by passive diffusion [91,92]. Once the folate monoglutamates have gone through the jejunal brush border membrane, these are transported to the liver via portal circulation where they are stored and released to peripheral tissues when needed. Tissue absorption is cell-specific but generally also occurs through active transport with the presence of folate transporters (folate receptors, reduced folate carrier and PCFT1).

Bioavailability

Folate bioavailability is dependent on the food matrix, stability of labile folates, presence of vitamin C and folate-binding proteins and folate pool sizes [89,93,94]. Nonetheless, there is a consensus that folic acid is better absorbed than dietary folate [95]. The US Institute

of Medicine estimated that the absorption efficiency of folic acid from supplements (if taken with food) or from fortified food was 85% and 100% from supplements taken on an empty stomach [89] whilst dietary folate absorption efficiency was 50% [89,94]. In an attempt to overcome the different bioavailabilities of folate and folic acid in different conditions, dietary folate equivalents (DFE) were introduced where 1 µg DFE equals 1 µg of dietary folate or 0.6 µg folic acid from fortified foods/ supplements on a full stomach or 0.5 µg folic acid consumed while fasting [89].

Biomarkers of status

Folate status can be assessed by measuring plasma/ serum folate or by measuring folate in erythrocytes with red blood cell (RBC) folate. Plasma folate concentrations can be analysed by high-performance liquid chromatography (HPLC), by liquid chromatography–mass spectrometry (LC-MS), by liquid chromatography-tandem mass spectrometry (LC/MS-MS), or, far more frequently, by microbiological assay or folate-binding protein assays (such as chemiluminescence assays, radioassays and ion-capture assays) [96]. The chromatography methods have the added advantage that they are able to measure different folate species while the microbiological assay and protein-binding assays only measure total folate [97]. However, the chromatography methods are often costly, need time-consuming extraction procedures or large volumes of plasma [97]. Folate concentrations obtained from the protein-binding assays are lower than those from microbiological assays and show a wide variation between different kits [98]. The Microbiological assay [using a strain of *Lactobacillus rhamnosus (formerly Lactobacillus casei), Streptococcus faecium or Pediococcus cerevisiae*] is generally considered to be the "gold standard" [99].

Folate measured in plasma/ serum reflects short-term folate intake and folate measured in erythrocytes is a sensitive indicator of long-term status (preceding 120 days) and reflects tissue folate stores [33,100]. A serum folate concentration <10 nmol/L or a RBC folate concentration <340 nmol/L is broadly considered as deficient [89].

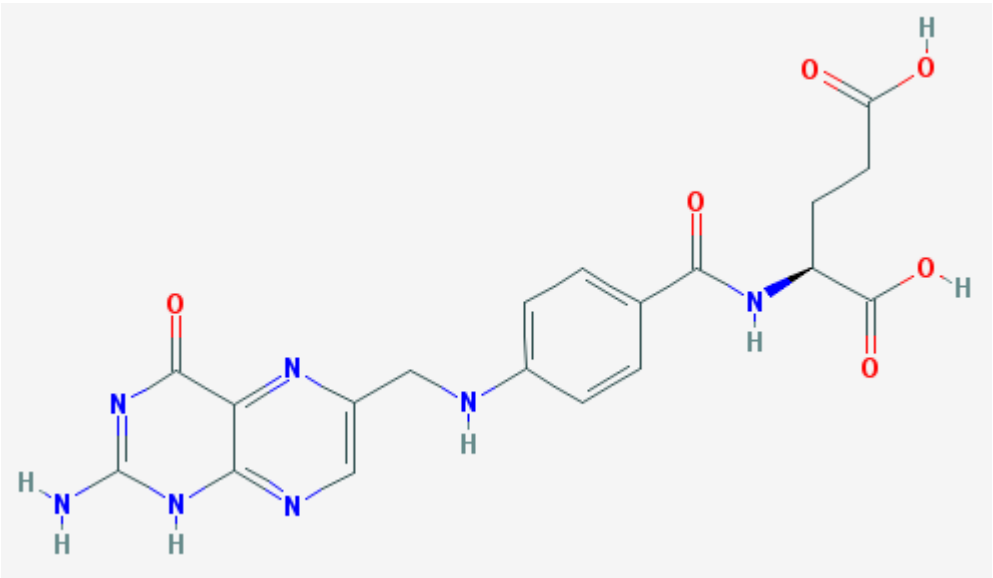
Genetic variation in folate status

Several polymorphisms of genes coding for enzymes and transport proteins involved in 1-C metabolism have been identified and reported to have a detrimental effect on folate status. The C677T *methylenetetrahydrofolate reductase* (*MTHFR*) single nucleotide polymorphism (SNP) (rs1801133) is the most widely studied. The *MTHFR* gene encodes an

enzyme of the same name that is responsible for the reduction between 5,10-methylene-tetrahydrofolate and 5-methyltetrahydrofolate (the major form of folate in plasma) providing 1-C unit in the process. Homozygosity for the T allele equates to a reduction of ~70% in the MTHFR enzyme activity which is translated to 20-25% lower serum folate concentrations than in those with the CC genotype. The reduction in MTHFR enzyme activity is less dramatic for the A1298C mutation (rs1801131) in the same gene. However, a synergistic effect may exist between the two SNPs where individuals with both mutations have the highest tHcy and lowest RBC folate concentrations [101]. The 19-bp deletion of *dihydrofolate reductase* (*DHFR*) was associated with a modest increase in folate status in women but not in men [102] and not in all studies [103]. A SNP in the *solute carrier family 19 member 1* (which encodes the reduced folate carrier 1 enzyme) from the G allele to the A allele (rs1051266) was associated with lower RBC folate concentrations [104].

Other SNPs such as *methionine synthase reductase* A66G (rs1801394), *glutamate carboxypeptidase II* C1561T (rs61886492), *MTR* A2756G (rs1805087) and *MTRR* A66G (rs1801394) may also influence folate status [89].

Figure 1.2. Chemical structure of folic acid ($C_{19}H_{19}N_7O_6$).



C, carbon; H, hydrogen; N, nitrogen O, oxygen.

1.9. Vitamin B12

Vitamin B12 acts as a coenzyme for methionine synthase (MS) and methylmalonil-CoA mutase. Vitamin B12 is closely interlinked with folate through the MS co-enzyme,

having been associated to many of the same diseases, i.e. megaloblastic anaemia [105], neuropathy [106], cognitive impairment [107], CVD [108] and bone health [109].

Vitamin B12 or cobalamin is a water-soluble vitamin belonging to the B vitamins group. All compounds with vitamin B12 activity are generally called cobalamins due to the cobalt-containing chemical structure (**Figure 1.3**) but only two act as co-enzymes in human metabolism; methylcobalamin and 5-deoxyadenosylcobalamin [110]. Hydroxocobalamin is considered an intermediate and cyanocobalamin is a synthetic compound used in dietary supplements and food fortification [110]. Humans, animals, plants and fungi cannot synthesize vitamin B12, only bacteria and archaea are able to do so. However, due to bacterial symbiosis, some foods are natural sources of B12 such as dairy products (milk, yogurt, and cheese), organ meat (liver, kidneys, giblets, and pate), meat (beef, pork), fish and seafood (clams, oysters, mackerel and herring). Vitamin B12 is not known to be destroyed by heat but can be destroyed by light. Strict vegetarians and vegans may have to resort to fortified foods such as soy milk and breakfast cereals to achieve the recommended daily intake of vitamin B12 [88,111]. DRVs for vitamin B12 vary depending on the authority setting them but they have been set for adults older than 50 years at 1.0, 1.25 and 1.5 μg/day for the lower reference nutrient intake (LRNI), estimated average requirement (EAR) and reference nutrient intake (RNI) in the UK, respectively [69].

Intestinal and tissue absorption

Vitamin B12 has to undergo a complex process in order to be absorbed in the distal ileum. Bound to protein in food, vitamin B12 has to be released by pepsin and hydrochloric acid in the stomach (vitamin B12 in fortified foods does not have to go through these steps as it is already in the free form). The ensuing free form of vitamin B12 binds to haptocorrin, forming a B12-haptocorrin complex. This complex is later broken down in the duodenum by pancreatic proteases which enable vitamin B12 to bind to the glycoprotein intrinsic factor (IF), be recognized (by cubilin) and absorbed by endocytosis in the enterocytes of the distal ileum [66,112].

Once absorbed, most of the vitamin B12 binds to haptocorrin (70-90%) while the remaining 10-30% bind to transcobalamin II and III forming holotranscobalamin [66,113]. Only holotranscobalamin can be taken up by tissue cells by an endocytosis mechanism through specific calcium-dependent transcobalamin receptors [114]. The haptocorrin-bound vitamin B12 acts as a circulating storage but most of the vitamin B12 is stored in the liver (around 5

mg or 50% of total body stores). Adenosylcobalamin is the major form of vitamin B12 present in the liver but hydroxocobalamin and methylcobalamin are present as well. Other organs such as the kidney, brain and spleen can also store a relatively high amount of vitamin B12. Around 1.4 µg per day of vitamin B12 is believed to be secreted by the liver into the bile but because of the enterohepatic circulation, once the bile enters the small intestine, vitamin B12 will once again bind to IF and be reabsorbed in the ileum (up to 90%). Even so, the highest loss of vitamin B12 occurs through faeces and whenever circulating vitamin B12 exceeds blood's capacity, the excess is also secreted in urine [115]. Since intake of vitamin B12 is small relative to its body stores, vitamin B12 deficiency can take years to manifest [116].

Bioavailability

Ileal receptors saturate with intakes of 1.5 and 2.5 µg of vitamin B12 per meal. It is estimated that only 50% and 5% of vitamin B12 is absorbed with intakes of ~1 and 25 µg, respectively, and only ~1% is be absorbed by passive diffusion if IF is not present. The bioavailability of vitamin B12 in any form or dose is estimated to be roughly 40% in healthy adults with intact IF secretion [112].

Biomarkers of status and function

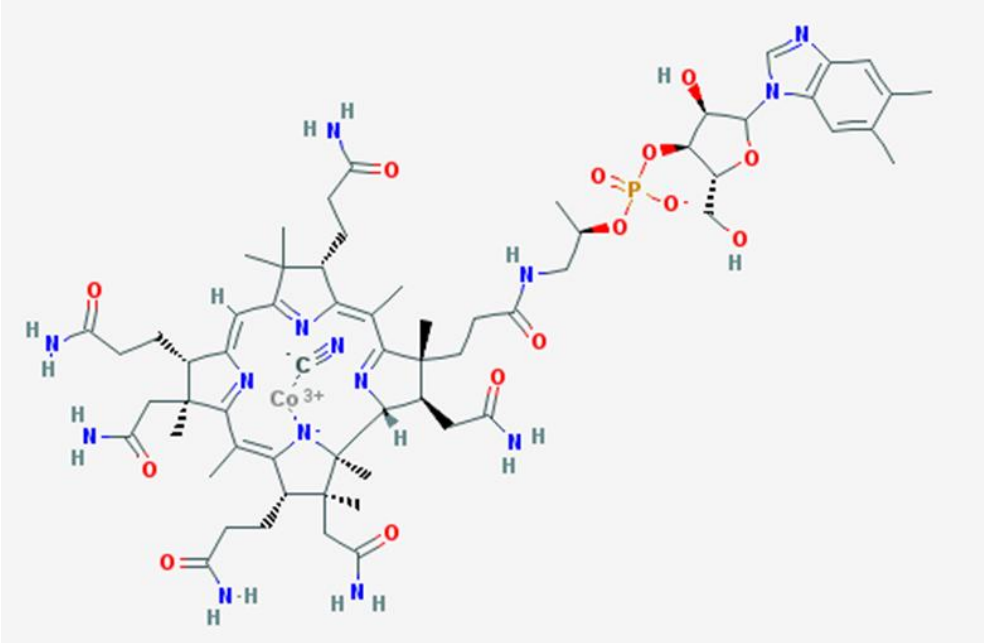
Vitamin B12 status can be assessed by vitamin B12 measured in plasma/ serum, holotranscobalamin or by methylmalonic acid (MMA) which is a functional biomarker. While plasma/ serum vitamin B12 measures all forms of vitamin B12, holotranscobalamin only measures the metabolically active fraction of vitamin B12. MMA concentrations are elevated with deficient vitamin B12 levels because the conversion of methylmalonyl-CoA to succinyl-CoA is a vitamin B12-dependent process. Deficiency of vitamin B12 leads to accumulation of methylmalonyl-CoA which is further metabolised to MMA [33,100]. The European Food Safety Authority (EFSA) and other authorities have broadly classified plasma vitamin B12 deficiency in adults as a concentration <148 pmol/L [112].

Genetic variation in vitamin B12 status

FUT2 encodes galactoside 2-alpha-L-fucosyltransferase 2 which is involved in the regulation of the H antigen [117]. *FUT2* GG genotype was associated with higher vitamin B12 status, especially in women [118]. Transcobalamin 1 and transcobalamin 2 are circulating

vitamin B-12 binding proteins and *rs526934* and *rs757874*, respectively were associated with lower vitamin B-12 status [117]. Several other genes polymorphisms affect vitamin B12 status, such as *CBS (rs2124459), CD320 (rs2336573), CUBN (rs11254363), DNMT2 (rs2295809) and PON1 (rs3917577). CBS encodes* cystathionine β-synthase which converts homocysteine to cystathionine, the CD320 receptor binds to holotranscobalamin and internalises it by endocytosis, CUBN encodes cubilin which is the receptor for the vitamin B12-IF complex, DNMT21 is a RNA methyltransferase and PON1 is an esterase that can remove detrimental oxidised-lipids [117,119]. Zinck *et al.* reported that individuals with SNPs in *CBS (rs2124459), CD320 (rs2336573), CUBN (rs11254363), DNMT2 (rs2295809), and PON1 (rs391757)* were less likely to have inadequate plasma vitamin B12 concentrations (<220 pmol/L) than the reference major allele [117].

Figure 1.3. Chemical structure of vitamin B12 (cyanocobalamin) ($C_{63}H_{88}CoN_{14}O_{14}P$).



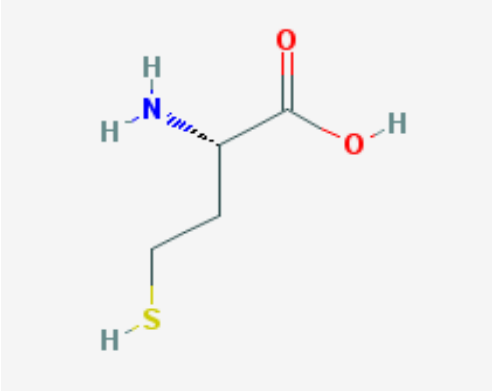
C, carbon; Co, cobalt; H, hydrogen; N, nitrogen O, oxygen; P, phosphorus.

1.10. Homocysteine

Elevated homocysteine concentrations have been associated with cardiovascular diseases[120,121], fracture risk [122] and impaired cognition [33,107,123]. Although the meaning of elevated homocysteine and whether it is a cause, an intermediate or a consequence of disease severity is still debatable, reduction of hyperhomocysteinemia is frequently pointed out as a potential explanation for the association between B vitamins and health outcomes.

Homocysteine is a potentially cytotoxic non-protein sulphur-containing α-aminoacid derived from methionine catabolism (**Figure 1.4.**). Homocysteine is central to two pathways: the methionine or remethylation cycle and the transsulfuration pathway. In the methionine cycle, homocysteine is remethylated to methionine by methionine synthase, this is further converted to S-adenosylmethionine (SAM) which donates methyl groups by being demethylated to S-adenosylhomocysteine which is then hydrolysed back to homocysteine (**Figure 1.1**). In liver and kidneys, homocysteine is converted to cystathionine and further hydrolysed to cysteine in the transsulfuration pathway. Because of the involvement of B vitamins but especially folate and vitamin B12 in the remethylation of homocysteine, dietary intakes of these vitamins are inversely associated with total homocysteine concentrations. In fact, supplementation with folic acid typically lowers homocysteine concentrations by 13-25% with a further reduction of 7% with the addition of vitamin B12 [124], making it a functional marker of folate and vitamin B12. Homocysteine can be assessed by measuring the concentration of plasma total homocysteine (tHcy). Hyperhomocysteinemia is frequently defined as a tHcy concentration >15 µmol/L [125].

Figure 1.4. Chemical structure of homocysteine ($C_4H_9NO_2S$).



C, carbon; H, hydrogen; O, oxygen; S, sulfur.

1.11. Relationship between folate and vitamin B12 intake, and status

The complexity of the dose-response relationships between intake and status are influenced by limitations in dietary assessment, food composition data, choice of biomarkers, genotypic variation, bioavailability and complex metabolic pathways. In addition, several single nucleotide polymorphisms (SNP) modulate folate and vitamin B12 status. For example, homozygosity of the T allele (measured in the forward orientation)

(rs1801133) of the *MTHFR* gene (which encodes methylenetetrahydrofolate reductase) is associated with low folate status [117].

There is conflicting evidence about relationships between folate and vitamin B12 intake and, folate and vitamin B12 status, respectively, in older adults. Some studies report a significant association between folate and vitamin B12 intake and status in older adults [83,126-130] while others do not [131-133]. Differences in folate and vitamin B12 bioavailability from total diets and specific food sources may provide a partial explanation for the observed discrepancies. Folate bioavailability from foods is substantially lower than that from supplements or from foods fortified with folic acid with estimated bioavailability of 50 and 85%, respectively [89]. If intrinsic factor (IF) secretion is intact, approximately 40% of vitamin B12 is absorbed [112].

1.12. One-carbon metabolism and cognition

There are now 46.8 million people with dementia worldwide and that number is predicted to reach 74.7 million by 2030 [134]. The Cognitive Function and Ageing Study (CFAS) II estimated that there were 670,000 older adults with dementia in 2011 in England alone [135]. Despite some evidence of reduction in the prevalence and incidence of dementia [135,136], it is predicted that cases of dementia will increase primarily for the following reasons: (a) dementia incidence doubles every 6.3 years after 60 years old [134]; (b) the population is ageing worldwide due predominantly to the rise in the numbers of the very old (aged 85 years and over), the fastest growing age segment in the United Kingdom and most western societies [7]. Dementia is one of the most important predictors of disability and poses a major societal challenge [137]. Since cognitive decline and dementia impact quality of life detrimentally and are currently not treatable, research priorities have focused on preventing or delaying the onset of dementia through modifiable risk factors, such as nutrition [138].

Folate and vitamin B12 are central to one-carbon (1-C) metabolism. Inadequate or aberrant 1-C metabolism may be associated with cognitive function through mechanisms such as hyperhomocysteinemia [139], reduced synthesis of neurotransmitters, phosphatidylcholine and pyrimidines [140], altered DNA methylation patterns [141], reduced fatty acid synthesis and incorporation of odd chain fatty acids into the myelin sheath [142]. Although the biological rationale for associations between folate, vitamin B12 and

homocysteine with cognitive function seem plausible, conflicting results have been reported. Folate, vitamin B12 and homocysteine have been associated with cognitive decline in some longitudinal studies [82,123,143] but not all [144-146], in some randomized controlled trials (RCTs) [83,147], for one vitamin but not for the other or only for certain cognitive domains.

1.13. One-carbon metabolism and mortality

Folate provides 1-C units for *de novo* biosynthesis of purines and pyrimidines, transmethylation of homocysteine to methionine and interconversions of serine and glycine [148]. Vitamin B12 acts as a coenzyme for methionine synthase (MS), responsible for the transmethylation of homocysteine to methionine and consequent production of S-adenosylmethionine (SAM) and methylmalonyl-CoA mutase, which converts methylamalonyl-CoA to succinyl-CoA in the fatty acid synthesis pathway [148]. SAM is the universal methyl donor for methylation of all cellular macromolecules, including DNA and histones, and therefore plays a critical role in regulation of gene expression [148]. Given the close involvement in 1-C metabolism, it is no surprise that folate and vitamin B12 have been associated with CVD, cancer and all-cause mortality. A recent meta-analysis pooling data from 30 randomized controlled trials (RCTs) reported that folic acid supplementation reduced CVD risk by 4% and the risk of stroke by 10% [149]. Systematic reviews and meta-analyses have shown that raised homocysteine concentration and low folate status are associated with increased cancer risk whilst [150] higher folate intake or status is associated with lower risk at some cancer sites [85,86]. However, randomised controlled intervention studies show no effect of supplemental folic acid on cancer risk [151]. The picture is similar for vitamin B12. Some have found that high concentrations of vitamin B12 are associated with all-cause mortality [152,153] and cancer (particularly haematological and smoking and alcohol related cancers) [154,155] while others did not [156-159]. It is hypothesized that “very high” vitamin B12 concentrations may be due to i) increased release of vitamin B12 from storage ii) upregulation of haptocorrin and transcobalamin synthesis, iii) decreased clearance of vitamin B12 from plasma and/or iv) diminished binding affinity of vitamin B12 for transporter proteins (all of which may be associated with disease development) but no clear mechanism has been determined [114,160].

1.14. Objectives

There is a dearth of data exploring the dietary intake and nutritional status of very old adults and its association with health outcomes. Therefore, the overall objective of this PhD thesis was to provide an accurate snapshot of the dietary habits of the very old and examine health trajectories with respect to 1-C metabolism biomarkers in a unique cohort such as the Newcastle 85+ Study. More specifically, this PhD aimed to:

- CHAPTER 3 and 4

    1) Describe the daily intake of energy, macronutrients, non-starch polysaccharides, vitamins and minerals in the very old at baseline;

    2) Determine the principal food sources of macro and micronutrients;

    3) Investigate how dietary intakes compare against the current UK DRVs;

    4) Explore socioeconomic and lifestyle influences on dietary intake.

- CHAPTER 5

    1) Determine the prevalence of "inadequate" folate and vitamin B12 intake and status;

    2) Examine the associations between the top contributing dietary sources of folate and vitamin B12, and status;

    3) Investigate whether high dietary intakes of folate and vitamin B12 are associated with reduced risk of "inadequate" status.

- CHAPTER 6

    1) Investigate the association between red blood cell folate (RBC folate) concentrations and cognitive impairment and the rate of cognitive decline in global and attention-specific cognition over 5 years;

    2) Examine the association between plasma vitamin B12 concentrations and cognitive impairment and the rate of cognitive decline in global and attention-specific cognition over 5 years;

3) Explore the association between total homocysteine (tHcy) concentrations and cognitive impairment and the rate of cognitive decline in global and attention-specific cognition over 5 years.

- CHAPTER 7

1) Determine the association between RBC folate concentration and all-cause and cardiovascular specific mortality over a 9-year period;

2) Explore the association between plasma vitamin B12 concentration and all-cause and cardiovascular specific mortality over a 9-year period;

3) Investigate the association between tHcy concentrations and all-cause and cardiovascular specific mortality over a 9-year period.

1.15. Summary

The very old are now the fastest growing age group of western societies. However, very little is known about the dietary habits in this age group. Many studies arbitrarily exclude very old people for no reason other than age [161], whilst others only include a small number, resulting in a lack of statistical power. Further, healthy life expectancy has not seen such a rapid rise as life expectancy, which raises the need for new strategies to postpone the age-related morbidity. Modulation of modifiable risk factors, such as nutrition and 1-C metabolism biomarkers is of special interest. Given the close involvement in 1-C metabolism, it is no surprise that folate, vitamin B12 and homocysteine have been associated with cognition, cardiovascular disease (CVD) and all-cause mortality. However, the current evidence is inconclusive and most is based on studies in younger populations.

Chapter 2 will describe general methods that are common to two or more experimental chapters throughout this thesis. Chapter 2 will describe the Newcastle 85+ Study database which was used for all analyses, the study's recruitment and cohort retention profile and general strengths and weaknesses; the dietary assessment and food group allocation; blood collection, 1-C metabolism biomarkers and genotyping; and general statistical analysis.

CHAPTER TWO

2. General Methods

2.1. The Newcastle 85+ Study

The Newcastle 85+ Study is a longitudinal population-based study of health trajectories and outcomes in the very old. All people turning 85 in 2006 (born in 1921) who were permanently registered with the 64 general practices within Newcastle upon Tyne or North Tyneside primary care trusts (North East England) were approached. The study's initial aim was to provide a comprehensive snapshot of the very old and investigate their health trajectories and outcomes at different phases of follow-up in the hope of better understanding the different aspects of the ageing process.

Recruitment profile

Most (83%) of the 64 general practices agreed to participate (n=53). Participating general practitioners were asked to exclude people with an end-stage terminal illness and individuals who were deemed unsafe for a research nurse to visit alone [37]. Cognitively impaired or institutionalized participants were not excluded. All eighty-five year olds remaining (who were not excluded) were sent a letter of invitation. In total, 1459 people were invited to participate, 1042 were recruited and 845 (319 men and 526 women) had multidimensional health assessment and GP record review data. A detailed flowchart with the recruitment profile and the sample available for the most important exposures and outcomes of interest used in this thesis is shown in **Figure 2.1**.

The multidimensional health assessments were carried out by one of the eleven trained research nurses at the participant's usual residence, including home or institution. Three interviews lasting ≈90 min, including questionnaires (socioeconomic, health, diet

intake and lifestyle variables), measurements and functional tests (anthropometry, cognition, physical function, pulmonary and cardiovascular) were completed at each phase (for study questionnaires visit http://research.ncl.ac.uk/85plus). A fourth visit was done to collect a fasting blood sample (nutrition, health and ageing biomarkers). The mean duration of the multidimensional health assessment (excluding blood collection and body weight measurement) was 206 minutes over 25 days [37]. An overview of the variables collected in the Newcastle 85+ Study from baseline to phase 4 is shown in **Appendix E**. All computerized and paper GP records, including hospital correspondence and the results of investigations, were reviewed by a research nurse for diseases, medication use and recent use of general practice services (last 12 months). Inter-rater agreement of GP record data extraction was examined for 24 randomly selected records. Intra-class correlations for a core set of diseases (angina, myocardial infarction, heart failure, hypertension, and stroke) ranged from 0.45 to 0.79 which indicated a moderate or good agreement between research nurses [37]. Only total number of prescribed medications showed some disagreement between the research nurses but not type of medication and number of consultations with a GP/ practice nurse and in which setting [37].

Assessment and retention profile

Data were collected at baseline (phase 1, 2006/2007), 18 (phase 2, 2007-2009) (GP records were not reviewed on this phase), 36 (phase 3, 2009/2010) and 60 months later (phase 4, 2011/2012). The final analytical sample with complete baseline (phase 1) multidimensional health assessment and GP record review (minus 2 participants who withdrew from the study) was 845 (81% of the original 1042 that were recruited). The recruited cohort was socio-demographically representative of the general UK population [37]. From phase 1 to phase 2, 25% (n=215) of the participants withdrew or died, from phase 2 to phase 3 23% (n=147) and from phase 3 to phase 4 29% (n=139) (**Figure 2.1**).

This study was conducted according to the guidelines laid down by the Declaration of Helsinki and all procedures involving human subjects were approved by the Newcastle and North Tyneside local research ethics committee (06/Q0905/2). Written informed consent was obtained from all participants, and when unable to do so, consent was obtained from a carer or a relative according to the UK Mental Capacity Act 2005.

General health characterization at baseline

At baseline, most participants were community-dwelling while 10% lived in care homes [37]. Hypertension, atherosclerotic disease, osteoarthritis, and cataracts were very prevalent in the participants of the Newcastle 85+ Study, with each close to 50% [37]. No participant was completely free of chronic diseases and almost 90% had at least three diagnosed diseases [37]. The median number of chronic diseases was 5 (interquartile range: 3-6). Women had a higher disease count than men. Sixty percent reported to have hearing impairment, 38% to be visually impaired, 38% had had a fall in the last year and 21% had severe or profound urinary incontinence [37]. Despite the relatively high number of diseases and conditions, almost 80% rated their health as good, very good or excellent compared to others of the same age [37]. Further details of the study have been reported elsewhere [35,37,161].

Strengths and weaknesses of the Newcastle 85+ Study

The Newcastle 85+ Study is a unique cohort owing to the age group, the large number of participants and the extensive multidimensional health data. The high response rate achieved in recruitment and retention is also an important strength, especially considering the age group and extensive assessment involved [161]. A single-aged cohort offers several advantages such as minimizing the effect of age variability but it limits generalizations. In 1921 life expectancy was 56.2 years for men and 60.0 years for women and only 18% of men and 33% of women were expected to reach 85 years old. Cohort life expectancy (which take into account mortality rates in later years) set it at 61.3 for men and 68.0 for women [162]. It is impossible to fully exclude any survival or length bias that arose due to selective survivability as all participants were 85 or turned 85 at recruitment (~20 years beyond cohort life expectancy), an age where mortality rates are high [163].

The Newcastle 85+ Study cohort was socio-demographically representative of the UK (comparison between sociodemographic status of the Newcastle 85+ Study and the 2001 national census) and included institutionalised and cognitively impaired very old (two commonly excluded characteristics in other cohorts). The participants were all from Newcastle-upon-Tyne and North Tyneside (mainly urban areas) and of a predominantly white background. Generalisations to other geographical locations and to populations with

different ethnic makeup should be undertaken with caution. The rapid processing of blood samples after venipuncture is another strength of this study.

2.2. Dietary intake assessment and food group allocation

A pilot study conducted between 2003 and 2004 in 171 eighty five year old Newcastle-upon-Tyne residents informed the choice of the dietary assessment method to be used (Food Frequency Questionnaire (FFQ) or repeated Multiple Pass 24h Recall (24hr-MPR)) in the Newcastle 85+ Study [164]. Four criteria were used: i) Energy intake (EI) relative to estimated basal metabolic rate (BMR), ii) EI and daily intake of selected nutrients vs. the 1994-95 UK National Diet and Nutrition Survey (NDNS) dietary intake data for community-dwelling 85 and older adults [49], iii) time burden for research nurses/ nutrition researchers and iv) utility and acceptability of each method by the participants and research nurses. Against these four criteria, the repeated 24hr-MPR was superior to the FFQ quantitatively and qualitatively [164]. The 24hr-MPR provides detailed retrospective information on food eaten while only requiring short-term memory. Participant burden is also minimal (especially against weighed or unweighed food diaries) [165], it is completed by an interviewer and not the responder [166], and the conversational nature of the process reduces the risk of overlooking details. However, the 24hr-MPR also presents disadvantages such as still relying on memory, having to estimate portion size, requiring trained interviewers and tiresome data preparation/coding. From the available options at the time of dietary assessment in the Newcastle 85+ Study, the 24hr-MPR was an acceptable, and potentially preferred method, of dietary assessment in this age group [164].

Dietary intake was assessed by 24 hour Multiple Pass Recall (24hr-MPR) on two non-consecutive occasions (one week apart and on different days of the week) by trained research nurses and portion sizes estimated using the “Photographic Atlas of Food Portion Sizes” [167]. The repeated 24hr-MPR assesses dietary intake in the previous 24 hours and includes 3 passes to ensure that every detail is recorded. Pass 1/ quick list: the participant was asked to recall the food and drink they had consumed in the previous 24 hours, while thinking of what they had done. An initial prompt was given for commonly omitted foods, such as snacks and sweets, and the whole intake was recorded without interruption. Pass 2/ detailed record: the interviewee was asked to provide more detail, including the time and occasion of everything recorded in the quick list. Portion sizes were also estimated during

this pass. Pass 3/ review: all the recorded food and drink, portion sizes, time of meals and method of confection (if appropriate) were reviewed by the interviewer and participant. Mean time to complete a single 24h-MPR was 22 minutes. Dietary assessment of participants in residential care and those requiring proxy respondents were included successfully [168]. Eighty five percent and 90% of the participants believed that the 24hr-MPRs reflected their usual food and drink intake, respectively. All dietary intake data were independently double entered. Any discrepancies were identified, checked against original records and corrected prior to data analysis. Individual foods were coded and allocated to 15 first level food groups [cereals and cereal products (grains), milk and milk products, eggs and egg dishes, oils and fat spreads, meat and meat products, fish and fish dishes, vegetables, potatoes, savoury snacks, nuts and seeds, fruit, sugar, preserves and confectionery, non-alcoholic beverages, alcoholic beverages and miscellaneous (soups, sauces and remaining foods that did not belong in other food groups)], 48 second level and 132 third level food groups with increasing specificity (**Appendix A**). Use of the 24-MPR was consented by 805 participants and complete dietary intake data (without protocol violation and 2x24hr-MPRs) were available for 793 participants.

2.3. Blood collection, one-carbon metabolism biomarkers and genotyping

Blood samples were taken after an 8h overnight fast at baseline. Forty mL of blood was drawn from the antecubital vein between 7:00 a.m. and 10:30 a.m., placed on ethylenediamine tetraacetic acid (EDTA) tubes and taken immediately to the laboratory (95% of the samples reached the laboratory within 1 h) [43]. 778 participants had blood taken and 95% provided fasting samples [37]. As part of the full blood count, red blood cell count, white blood cell count and platelets were analysed and haematocrit calculated. RBC folate concentration analysis required an extra manual pre-treatment where blood samples were treated with a folate lysis reagent (ascorbic acid) and incubated for 90 min. Red blood cell folate (RBC folate) and plasma vitamin B12 concentrations were determined by a two-step assay using a chemiluminescence microparticle immunoassay on an Abbott ARCHITECT analyser and data were available for 752 and 753 participants, respectively. RBC folate was further adjusted for haematocrit. tHcy concentration was measured by an Abbot IMx immunoassay at baseline and available for 766 participants [169]. All biochemical analyses

were done by the department of clinical biochemistry at Newcastle Royal Victoria Infirmary (Newcastle-upon-Tyne, UK) [170].

Whole blood DNA was extracted by means of QiaGEN Amp Maxi DNA Purification Kit. As part of the EU Longevity Genetics Consortium, genome-wide association studies (GWAS) were performed on 765 participants from the Newcastle 85+ Study using Illumina Omni genotyping arrays. Data were obtained from 710 individuals and after quality control, 642 individuals were retained for the final analysis [171].

2.4. Other socioeconomic, health and lifestyle variables

Socioeconomic

Multidimensional health questionnaires recorded sex (male/ female), housing type [standard, sheltered (self-contained housing with communal areas such as a lounge, laundry or garden and on-call support) or institutional], living arrangements (living alone, with spouse or with others), years of full time education, social class according to the National Statistics Socio-Economic Classification (NS-SEC) three class scheme [Higher managerial, administrative and professional occupations (Class 1); intermediate occupations (Class 2); and routine and manual occupations (Class 3) [172]] based on past main occupation [37]. Meal provision included meals provided by the social services, voluntary services or other private help in the previous 4 weeks, and luncheon club attendance was defined as at least one visit in the previous 4 weeks.

Health

Medical records held by the general practitioner were reviewed by research nurses for diagnosed dementia/Alzheimer's disease, diabetes type 1 and 2, hypertension and cardiac disease (heart failure, angina, myocardial infarction, coronary artery bypass graft, coronary angioplasty/stent, pacemaker, atrial flutter/fibrillation), cerebrovascular disease (stroke, transient ischaemic attack, carotid endarterectomy), peripheral vascular disease, arthritis, respiratory disease, and cancer diagnosed within the previous 5 years excluding non-melanoma skin cancer. A disease count was created by scoring these diseases as either present or absent [173]. Glomerular filtration rate (GFR) was estimated by the chronic kidney disease epidemiology collaboration (CKD-EPI) guidelines using sex, ethnicity, serum creatinine and age; and a GFR < 30 ml/minute/1.73m^2 was defined as renally impaired [174].

Depression was assessed by the 15-item geriatric depression scale [no depression (0-5), mild or moderate (6-7) and severe depression (≥8 points)]. Self-rated health compared to others of the same age was categorised into excellent or very good, good, fair or poor. Being able to cook a hot meal and being able to go shopping for groceries was categorized into no difficulty, able to but with help or an aid, and unable to do this by him/herself. High sensitivity C-reactive protein (hs-CRP) was measured with a Dade Behring Cardiophase hsCRP immunoassay. Alanine transaminase (ALT), alkaline phosphatase and bilirubin were measured as part of the liver panel.

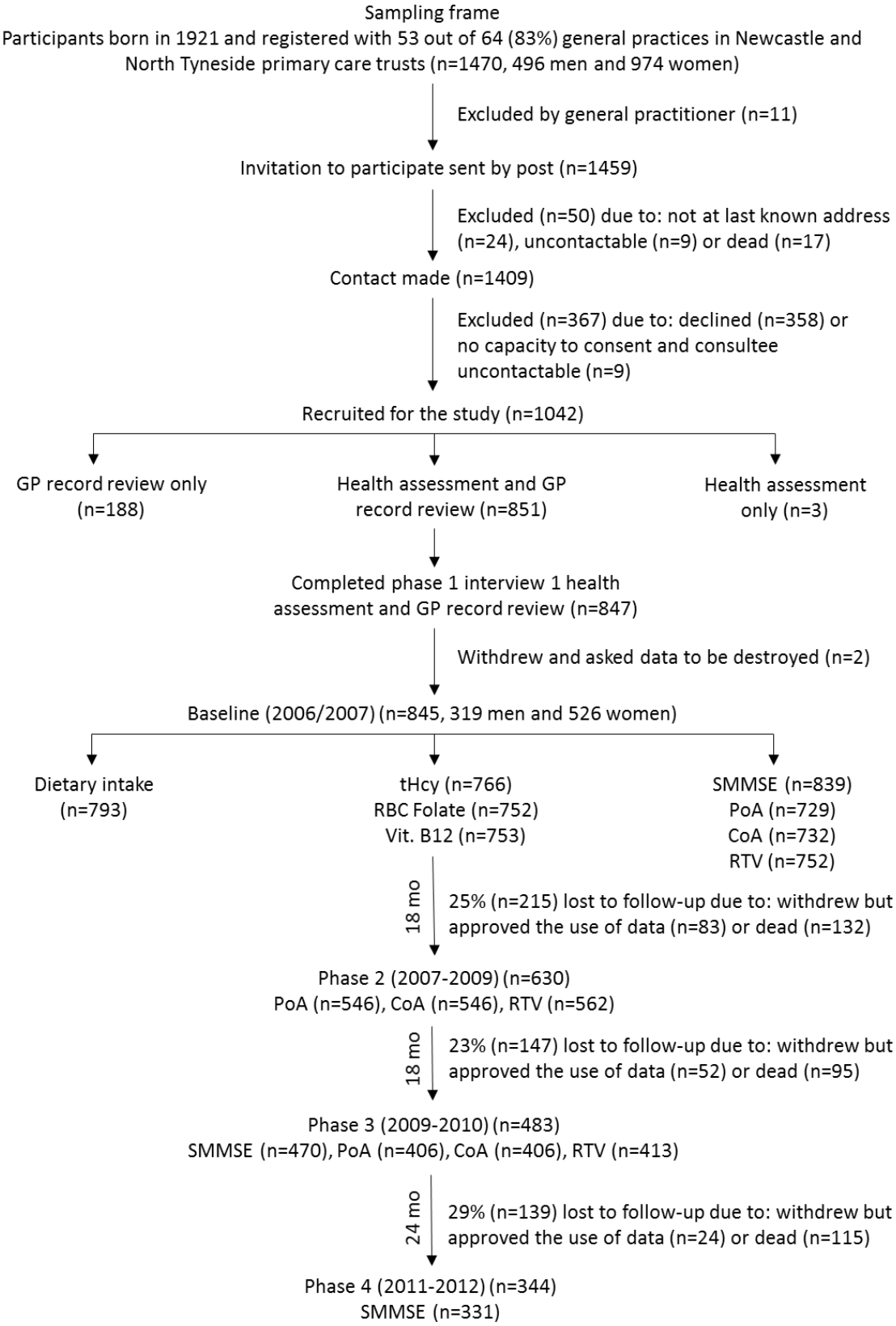
Lifestyle

Weight and height were measured and used to calculate body mass index (BMI). Participants were categorised into those with low (scores 0-1), medium (scores 2-6) and high (scores 7-18) physical activity based on a validated and purpose designed physical activity questionnaire [175]. Supplement use was divided into three categories viz. no supplements, one supplement and, two or more supplements. Information on supplement use was limited to type and brand, therefore micronutrient-containing supplements were assumed to be taken according to manufacturer's specifications.

2.5. General statistical analysis

Statistical analysis was conducted using the IBM statistical tool SPSS v22.0 (IBM, New York, USA) unless otherwise mentioned. The Shapiro-Wilk test, quantile-quantile plots and histograms were used to assess normality. Normally distributed continuous data are presented as means and standard deviations (SD), and non-Gaussian distributed variables as medians and interquartile ranges (IQR). Categorical data are presented as percentages (with corresponding sample size). Sex differences (or other differences between two groups) were assessed with the Chi-squared test (χ^2) for categorical variables and, by independent t-test and Mann-Whitney U test for parametric and non-parametric continuous data, respectively. Differences between 3 or more groups (e.g. quartiles) were assessed by the Chi-squared test (χ^2) for categorical variables and, ANOVA and Kruskal-Wallis test for parametric and non-parametric continuous data, respectively. Values of $p<0.05$ were considered broadly statistically significant. Specific statistical methods will be reported in each experimental chapter.

Figure 2.1 Flowchart of recruitment and cohort retention profiles of the Newcastle 85+ Study.



CoA, continuity of attention; GP, general practitioner; mo, months; PoA, power of attention; tHcy, RBC folate, red blood cell folate; RTV, reaction time variability; SMMSE, standardised mini-mental state examination; total homocysteine; vit., vitamin.

2.6. Summary

The Newcastle 85+ Study is a longitudinal cohort study of health trajectories and outcomes in the very old (all 85 years at baseline). Data were collected at baseline, 18, 36 and 60 months later. GP records review and multidimensional health assessment were performed at each collection phase by research nurses and the final analytical sample was of 845 participants at baseline. Dietary intake was assessed by 24hr-MPR on two non-consecutive occasions and was available for 793 participants. Blood samples were taken after an 8h overnight fast at baseline and taken to the laboratory within 1h. RBC folate and plasma vitamin B12 concentrations were determined by a chemiluminescence microparticle immunoassay on an Abbott ARCHITECT analyser and tHcy concentration was measured by an Abbot IMx immunoassay.

Food and nutrient intake data are scarce in very old adults – the fastest growing age segments of most western societies, including the UK. Chapter 3 is the first experimental chapter of this thesis and will cover the macronutrient intake and food sources in the very old. Besides determining dietary intake of energy, carbohydrate, fat, protein and non-starch polysaccharide (NSP), and corresponding food sources according to 15 major food groups, it will assess nutrient “adequacy” and investigate associated factors.

CHAPTER THREE

3. Macronutrient intake and food sources in the very old

[Mendonça N *et al.* (2016) Macronutrient intake and food sources in the very old: Analysis of the Newcastle 85+ Study. *Br J Nutr*. **115**(12):2170-80.]

Key words: dietary intake, 'aged, 80 and over', very old, Newcastle 85+

3.1. Abstract

Food and nutrient intake data are scarce in very old adults (85 years and older) – one of the fastest growing age segments of Western societies, including the UK. Our primary objective was to assess energy and macronutrient intakes and respective food sources in 793 85-year-olds (302 men and 491 women) living in North-East England and participating in the Newcastle 85+ cohort Study. Dietary information was collected using a repeated multiple-pass recall (2 × 24 h recalls). Energy, macronutrient and NSP intakes were estimated, and the contribution (%) of food groups to nutrient intake was calculated. The median energy intake was 6·65 (interquartile ranges (IQR) 5.49–8.16) MJ/d – 46.8% was from carbohydrates, 36·8% from fats and 15.7% from proteins. NSP intake was 10·2 g/d (IQR 7.3–13.7). NSP intake was higher in non-institutionalised, more educated, from higher social class and more physically active 85-year-olds. Cereals and cereal products (grains) were the top contributors to intakes of energy and most macronutrients (carbohydrates, non-milk extrinsic sugars, NSP and fat), followed by meat and meat products. The median intakes of energy and NSP were much lower than the estimated average requirement for energy (9.6 MJ/d for men and 7.7 MJ/d for women) and the dietary reference value (DRV) for NSP (≥18 g/d). The median SFA intake was higher than the DRV (≤11% of dietary energy). This study highlights the paucity of data on dietary intake and the uncertainties about DRV for this age group.

3.2. Introduction

Although the population is ageing and very old adults are at increased risk of nutritional deficiencies, very little is known about the dietary habits in this age group. Many studies arbitrarily exclude very old people for no reason other than age [161], whilst others only include a small number, resulting in a lack of statistical power. For example, a Europe-wide multi-centre study of food intake in older adults (SENECA: Survey in Europe on Nutrition and the Elderly: a Concerted Action) had an upper age limit of 79 years [176]. Out of the current (years 1-4) 4156 participants (sample size was weighted for unequal selection and non-response) in the UK's National Diet and Nutrition Survey (NDNS) rolling programme, only 15 men and 23 women were aged 85 and over [177]. Twenty years ago, the 1994-95 NDNS of people aged ≥65 years was the first representative dietary survey in the UK to include significant numbers of adults aged 85 and over (172 men and 287 women) but this

survey has not been repeated [49]. This survey reported that dietary intakes for most nutrients in the very old did not meet the Dietary Reference Values (DRV) [49]. The UK's DRVs for "older people" add further to the evidence of how frequently very old people are overlooked. Apart from the Estimated Average Requirement (EAR) for energy intake and the Reference Nutrient Intake (RNI) for protein which sets a DRV for individuals aged 75 and over and 50 and over, respectively, all other DRVs include everyone aged ≥18 in the same category [34,69]. In summary, there is a need for more reports of dietary intake data for those aged ≥85.

The chapter aimed to describe the intake of energy, macronutrients and non-starch polysaccharides (NSP) by participants in the Newcastle 85+ Study, and to determine their principal food sources. Further, intakes are compared against the current UK DRVs and, socioeconomic and lifestyle influences on dietary intake are explored.

3.3. Methods

Participants

This chapter uses data from the Newcastle 85+ Study and details were reported in *Chapter 2, General Methods - 2.1 The Newcastle 85+ Study*.

Estimation of energy and macronutrients intake and, food group contributions

Dietary intake assessment and food allocation to tertiary food groups was described on *Chapter 2, General Methods - 2.2 Dietary assessment and food group allocation.* Energy and macronutrient [alcohol, total carbohydrate, non-milk extrinsic sugars (NMES), non-starch polysaccharides (NSP), fat, total, saturated (SFA), polyunsaturated (PUFA) and monounsaturated (MUFA), and protein] intakes were estimated using McCance and Widdowson's sixth edition food composition tables [178] and a purpose-designed Microsoft Office Access database. Food group contribution to total energy and macronutrient intake was calculated and sources of at least 85% of each nutrient intake are reported. In addition, consumed foods were also disaggregated into five groups (bread, rice, potatoes, pasta and other starchy foods; milk and dairy foods; food and drinks high in fat

and/or sugar; meat, fish, eggs, beans and other non-dairy sources of protein and; fruit and vegetables) and compared with the Eatwell Plate (Balance of Good Health) [179].

Estimation of misreporting

Dietary misreporting is an acknowledged limitation of all dietary assessment methods. Goldberg *et al.* tried to address this problem by introducing cut-off values to identify misreporters [180]. The cut-offs are derived from estimations of energy intake (EI) divided by the estimated basal metabolic rate (BMR_{est}) ($EI:BMR_{est}$) [181]. Fredrix equations have been shown to be the most accurate in older subjects [182] and were used to calculate each participant's BMR_{est}. Under-reporters and over-reporters were defined as having an $EI:BMR_{est}$ below 1.05 and over 2.0, respectively. A 17% within subject variation for energy intake, 15% between-subject variation for physical activity level (PAL) [181] and 3.5% within-subject variation for BMR measurements [183] were assumed. The individual limits were calculated assuming a PAL value of 1.55, the World Health Organization (WHO) value for "light activity" and a 95% confidence interval [181]. As a sensitivity analysis, nutrient intakes with the cut-offs applied were calculated and compared to the nutrient intakes of all participants (without the cut-offs).

Socioeconomic, health and lifestyle factors

Details of variables used were described in *Chapter 2, General Methods - 2.4 Other socioeconomic, health and lifestyle variables*.

Statistical analysis

Case selection and simple descriptive analysis were used to identify the baseline characteristics, dietary intake and population below or above the DRVs. Energy and nutrient intakes were compared across housing, living arrangements (with whom participants live), years of full-time education, past occupation (NS-SEC) and physical activity groups by multinomial logistic regression. All models were adjusted for sex and NSP was also adjusted for energy intake. General details are presented in *Chapter 2, General Methods - 2.5 General statistical analysis.*

3.4. Results

Characteristics of the Newcastle 85+ Study population

Dietary intake data were available for 793 participants of the Newcastle 85+ Study (302 men and 491 women; female:male ratio of 1.6), who were all born in 1921 (aged 85.5±0.4 years at the time of data collection). Health and sociodemographic characteristics of these 793 participants by sex are included in **Table 3.1**. The majority of participants lived in standard housing (78%), alone (61%) and had nine years or less of full time education (64%). Approximately half (51%) had had a routine or manual occupation (NS-SEC class 3) and 44% had a medium physical activity level. Using WHO adult body mass index (BMI) cut-offs[184], 7% of participants were underweight while 10% were obese, suggesting the existence of a double burden of malnutrition. Participants with 2x24hr-MPR data and those without (n=40) or with only 1x24hr-MPR (n=12) did not differ with respect to sex, living arrangements (who participants live with), education, social class and BMI. However, people without complete dietary data were more likely to live in institutional housing, to be physically inactive, to be unable to cook a hot meal and unable to do grocery shopping independently compared with those with complete dietary data.

Dietary intake

Intakes of energy, EI:BMR_{est}, macronutrients and NSP are reported in **Table 3.3**. Median energy intake was 6.65 (IQR: 5.49-8.16) MJ per day of which 46.8% was derived from carbohydrate, 36.8% from total fat and 15.7% from protein. EI:BMR_{est} was 1.33 (IQR: 1.08-1.60) for both men and women. As expected, men had significantly higher intakes of energy, macronutrients and NSP than women. However, when expressed as relative contribution to energy or per 1 MJ, only percent of energy from protein was significantly higher for men (p=0.010). Conversely, the percentage of energy from SFA was lower in men (p=0.012) whereas the PUFA: SFA ratio was higher in men than in women (p=0.017).

Sensitivity analysis

Sixty-two participants (13 men and 49 women) did not have records of weight and/or height and so were excluded from the analysis of effects of misreporting which was conducted for the remaining 731 participants. Using 1.05-2.0 EI:BMR_{est} as a cut-off, 26.3% (n=192) of participants were identified as potential misreporters (30.4% men and 23.5%

women). Of the 731 very old, 21.6% (n=158) were defined as under-reporters (25.6% men and 19.0% women) and 4.7% (n=34) as over-reporters (4.8% men and 4.5% women). Cognitive impairment at baseline was associated with misreporting (OR: 1.61, 95% CI, 1.11-2.33, p=0.012). Dietary intake of non-misreporters (n=607) and the differences between total reporters (n=731) are presented in **Supplemental Table 3.2**. Since there were more under-reporters than over-reporters, daily energy intake increased by 0.36 MJ or 86 Kcal when cut-offs were applied, marginally increasing intakes of all macronutrients and NSP. Data from all participants (n=793) were used in our primary analyses because of the uncertainty regarding the identification of misreporters.

Contribution of food groups to dietary intake

Cereals and cereal products (CCP) and non-alcoholic beverages were the only food groups consumed by all participants (**Table 3.2**). Since CCP includes macronutrient-rich foods such as bread, buns and breakfast cereals, CCP were frequent top contributors for macronutrients (**Figure 3.1**). One third of the 34.2% of energy intake that came from CCP came from bread (32.7%). Similarly, more than a third of the CCP contribution to carbohydrate intake (48.3%) was also from bread (38.7%). Non-alcoholic beverages contributed to 18.4% of NMES intake of which 60% was from fruit juice and the remaining 40% from soft drinks. Added sugar was coded separately from tea/coffee if it was added. Therefore, tea and coffee contributed to 0% of NMES intake, even though these were ubiquitously consumed in this population. More than half of the 42.3% of NSP intake attributable to CCP came from bread (50.8%). Nearly half of the contribution of vegetables to NSP intake (22%) came from peas and cruciferous vegetables (49.7%). The biggest contributors to fat intake were CCP with 23.1% (38.5% of which was provided by buns, cakes, pastries and fruit pies), followed by meat and meat products (20.8%), and oils and fat spreads (19.9%). Meat and meat products were greater contributors to fat intake in men than in women (23.8% vs. 18.5%) while the opposite was true for oils and fat spreads (18.0% vs. 21.4%). There were similar sex differences in contributions to SFA intake. The large majority of SFA intake attributable to oils and fat spreads consumption (21.6%) came from butter (81.9%). Similarly, most of PUFA that came from oils and fat spreads (31.9%) came from fat spreads (87.4%). Meat and meat products were the main sources of protein (34.6%), followed by CCP (24.2%) and, milk and milk products (11.5%). Most sex differences

occurred when meat and meat products were a top contributor to macronutrient intake but the male: female ratio did not exceed 1.3.

Comparison of food intake with the Eatwell Plate

As a public health tool, the Eatwell Plate (Balance of Good Health) is intended to illustrate the recommended intake of five food groups (%). Foods and drinks high in fat and/or sugar (FS) accounted for 18% of the "Newcastle 85+ plate", much higher than the 8% recommended by the Eatwell Plate (not the recently updated version), leading to lower than recommended proportion of fruit and vegetables, bread, rice, potatoes, pasta and other starchy foods and, albeit to a less extent, of meat, fish, eggs, beans and other non-dairy sources of protein in the Newcastle 85+ Study (**Supplemental Figure 3.1**).

Nutrient adequacy

Compliance of the Newcastle 85+ Study cohort with the UK DRVs is shown in **Supplemental Table 3.1**, while **Figure 3.2** shows the distribution of energy, NMES, NSP and SFA intake compared with the corresponding DRVs. The median energy intakes were below the recently established EAR for dietary energy in the UK [(34)] and only 20% of the cohort met the 9.6 MJ and 7.7 MJ for men and women, respectively. Fifty per cent of men and 24% of women reported drinking alcohol and most of those were below the 32g and 24g advisable maximum limits of alcohol intake per day for men (77.5%) and women (90.8%), respectively[(185)]. Because alcohol consumption was relatively low, the percentage of energy inadequacy decreased by only 5% in men (from 80.1% to 75.1%) and 3% in women (from 80.2% to 77.2%) when alcohol was included in energy intake estimations. Median carbohydrate intake was also below the DRV for men and women and carbohydrate contributed ≥50% of food energy intake in one third (33%) of the population. Median NMES intake did not reach 11% energy from NMES per day but more than 40% of the group derived more energy from NMES than the dietary guidelines. In contrast, neither men nor women met the NSP intake DRV of 18g per day and only 9% of the cohort had higher intakes. Moreover, median NSP intake was also below 12g per day (66% of the population had lower intakes), the estimated lower end of the reference range[(69)]. Median total fat and SFA contribution to energy intake was higher than 35% and 11%, respectively. Nearly 60% of the group exceeded the recommended contribution of fat to energy intake while this percentage rose to 72.1% for SFA. However, median protein intake was higher than the RNI of 0.75g/Kg

[69], reflecting that 78.1% and 67.4% of men and women, respectively, had higher protein intakes than the RNI.

Dietary intake by housing, socioeconomic status and physical activity

Table 3.4 reports daily energy, macronutrient and NSP intake stratified by housing, living arrangements, years of full time education, social class (NS-SEC) and physical activity. When adjusted for sex, participants in institutional housing were more likely to have higher intakes of energy, carbohydrate and higher percentage of energy from NMES than those who lived in standard housing. However, institutionalised participants were more likely to have lower intakes of NSP (also adjusted for energy intake) and percentages of energy from MUFA and PUFA. Participants living in sheltered housing were more likely to have lower NSP intakes than those living in standard housing. There were no statistically significant differences between those living with their spouse or with others compared to those living alone except for protein intake. Participants who lived with others were more likely to have a lower protein intake than those who lived alone ($p=0.032$). Adjusted for sex and energy, participants who experienced 12 years or more of full time education had higher NSP intakes than those with ≤ nine years of full time education ($p=0.008$). Similarly, those with previous higher managerial, administrative or professional (Class 1) and intermediate (Class 2) occupations (NS-SEC) had higher NSP intakes than those with routine or manual occupations (Class 3) ($p=0.001$ and $p=0.018$, respectively). Participants with high physical activity had higher intakes of NSP and, percentage of energy from MUFA, PUFA and protein than those with low physical activity. The same was true for NSP and percentage of energy from PUFA in those with medium physical activity.

Table 3.1. Health and sociodemographic characteristics of the Newcastle 85+ Study participants with complete dietary data by sex.

	All	Men	Women	p-value[1]
Sex	- (793)	38 (302)	62 (491)	-
Age (mean±sd)	85.5±0.4	85.5±0.46	85.5±0.43	0.472[2]
Housing				0.001
Standard	78 (620)	85 (256)	74 (364)	
Sheltered	17 (137)	12 (37)	21 (100)	
Institutional	4 (34)	3 (8)	5 (26)	
Living Arrangements[3]				<0.001
Alone	61 (437)	42 (119)	74 (318)	
With Spouse only	28 (204)	51 (145)	14 (59)	
With Others	11 (79)	8 (23)	13 (56)	
Year of full-time education				0.608
≤9 years	64 (501)	61 (184)	66 (317)	
10-11 years	23 (183)	25 (75)	23 (108)	
12-20 years	12 (97)	13 (39)	12 (58)	
Past Occupation (NS-SEC)				<0.001
Higher Managerial/ Administrative/ Professional (Class 1)	34 (259)	40 (118)	31 (141)	
Intermediate (Class 2)	15 (109)	8 (23)	19 (86)	
Routine and manual (Class 3)	51 (385)	52 (155)	50 (230)	
Body Mass Index (Kg/m^2)				0.125
Underweight (<18.5)	7 (48)	5 (13)	8 (35)	
Eutrophic (18.5-24.9)	51 (374)	51 (146)	51 (228)	
Overweight (25.0-29.9)	32 (236)	36 (105)	30 (131)	
Obese (≥30.0)	10 (72)	9 (25)	11 (45)	
Physical Activity[4]				<0.001
Low	22 (176)	20 (60)	24 (116)	
Medium	44 (343)	33 (99)	50 (244)	
High	34 (270)	47 (142)	26 (128)	
Diet Change in past year				0.082
Yes	7 (53)	5 (15)	8 (38)	
No	93 (718)	95 (279)	92 (439)	
Food provision (Social Services + Private + Voluntary)				0.610
No visit	94 (671)	94 (268)	93 (403)	
At least once in 4 weeks	6 (47)	6 (17)	7 (30)	
Luncheon Club				0.010
Attended	7 (55)	4 (12)	9 (43)	
Not Attended	93 (734)	96 (289)	91 (445)	
Cook a Hot Meal independently				0.051
No Difficulty	79 (624)	83 (251)	76 (373)	
Some Difficulty	7 (48)	5 (16)	7 (32)	
Unable	15 (118)	11 (34)	17 (84)	
Shopping for Groceries independently				<0.001
No Difficulty	48 (378)	64 (191)	38 (187)	
Some Difficulty	13 (100)	12 (37)	13 (63)	
Unable	40 (313)	24 (73)	49 (240)	

Values are percentages (numbers) unless stated otherwise. [1] Chi-squared test ($\chi 2$) for no sex difference [2] Independent t-test for no sex difference [3] Excludes participants in institutional care. [4] Purpose designed physical activity questionnaire [(186)].

Table 3.2. Percentage (%) of consumers and consumption (g/d) of major food groups in the Newcastle 85+ Study participants by sex.

Food Groups	All			Men			Women			p-value[1]
	%	Median (g/d)	IQR	%	Median (g/d)	IQR	%	Median (g/d)	IQR	
Cereals and Cereal Products	100	211.0	141.6-307.0	100	265.6	190-382.8	100	186.6	125.6-263.6	<0.001
Non-Alcoholic Beverages	100	1210	918-1528	100	1210	899-1549	100	1210	950-1518	0.876
Meat and Meat Products	94	112.5	65.8-167.8	97	135	88.6-197.6	92	98	57.6-148.0	<0.001
Oils and Fat Spreads	93	16.0	10.0-28.0	92	18	10.0-29.8	93	16.0	10.0-26.0	0.213
Vegetables	91	103.8	60.5-159.5	91	114.6	70.0-169.0	91	96.6	54.0-147.6	0.006
Milk and Milk Products	90	141.5	69.0-225.0	89	138.6	54.2-225	90	143.6	75.0-225.0	0.412
Potatoes	82	99.0	66.0-153.5	83	131	78.6-178.2	81	90.6	49.8-140.0	<0.001
Sugar, Preserves and Confectionery	80	20.0	10.8-36.0	83	25	13.0-43.4	78	17.6	10.0-32.0	<0.001
Fruit	74	143.0	79.5-231.3	74	151	86.0-243.6	75	130.8	75.8-221.8	0.221
Miscellaneous	73	55.8	22.8-142.0	75	55	22.0-145.4	73	57.6	25.0-140.8	0.962
Eggs and Egg Dishes	39	30.0	25.0-60.0	40	50	25.0-60.0	38	30.0	25.0-60.0	0.102
Fish and Fish Dishes	36	56.0	32.0-85.5	33	60	37.6-77.6	38	52.2	28.0-90.0	0.259
Alcoholic Beverages	34	125.5	50.0-300.9	50	250	100.0-568.0	24	75.0	40.0-142.0	<0.001
Savoury Snacks	11	14.0	7.0-14.5	11	14	7.0-20.2	10	14.0	7.0-14.0	0.730
Nuts and Seeds	7	15.0	6.4-20.8	9	20	13.2-23.8	7	9.0	5.0-19.4	0.015

%, Percentage of consumers throughout the 2x24hr-MPR; IQR, Interquartile Range.

[1] Mann-Whitney U test for no sex differences between consumption (g/d) of each food group (only consumers).

Table 3.3. Daily energy, EI:BMRest, macronutrient and NSP intakes in the Newcastle 85+ Study by sex.

Macronutrients	All		Men		Women		p-value[1]
	Median	IQR	Median	IQR	Median	IQR	
Energy (MJ)[2]	6.65	5.49-8.16	7.73	6.36-9.20	6.15	5.09-7.25	<0.001
Energy (Kcal)	1588	1018-1949	1848	1519-2201	1471	1217-1733	<0.001
EI:BMR_{est}	1.33	1.08-1.60	1.33	1.04-1.57	1.33	1.11-1.61	0.287
Alcohol (g)[4]	13.2	6.8-23.2	17.3	9.6-30.7	8.7	4.8-14.3	<0.001
Carbohydrate (g) (% en)	193.9 (46.8)	156.9-238.1	228.3 (46.8)	180.9-271.9	177.3 (46.8)	147.2-218.6	<0.001 (0.760[3])
NMES (g) (% en)	42.7 (10.1)	25.4-63.8	50.2 (10.5)	32.5-78.2	38.0 (9.8)	23.2-56.7	<0.001 (0.055)
NSP (g)	10.2	7.3-13.7	11.3	8.8-15.5	9.3	6.8-12.2	<0.001
NSP (g) per 1 MJ	1.51	1.17-2.01	1.52	1.16-1.95	1.50	1.18-2.04	0.485
Total Fat (g) (% en)	65.8 (36.8)	50.1-84.2	74.7 (36.4)	57.7-95.0	60.4 (37.2)	47.1-77.1	<0.001 (0.093[3])
SFA (g) (% en)	24.3 (13.6)	17.3-32.4	27.0 (12.9)	18.8-35.5	22.8 (13.7)	16.4-30.9	<0.001 (0.012)
MUFA (g) (% en)	15.5 (8.8)	11.1-21.3	18.2 (8.6)	12.6-23.9	14.2 (8.9)	10.6-19.7	<0.001 (0.761)
PUFA (g) (% en)	6.3 (3.4)	3.9-9.9	7.3 (3.6)	4.7-11.4	5.7 (3.4)	3.5-8.5	<0.001 (0.237)
P:S ratio	0.25	0.15-0.42	0.28	0.17-0.43	0.23	0.14-0.41	0.017
Protein (g) (% en)	61.3 (15.7)	48.9-75.7	73.0 (15.9)	57.9-90.1	54.5 (15.5)	45.1-67.2	<0.001 (0.010)
Protein (g/Kg)	0.99	0.77-1.24	1.04	0.81-1.32	0.96	0.75-1.17	<0.001

EI:BMR_{est}, intake energy intake as a multiple for estimated basal metabolic rate [(182)]; NSP, non-starch polysaccharides; IQR, interquartile range; % en, percentage of energy; NMES, non-milk extrinsic sugars; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; P:S ratio, PUFA/SFA ratio.

[1] Mann-Whitney U test for no sex difference unless stated otherwise. [2] Does not include alcohol.

[3] Independent t-test for no sex difference. [4] Alcohol consumers only.

Table 3.4. Daily energy, macronutrient and NSP intake of the Newcastle 85+ Study participants by demographic, socioeconomic and lifestyle characteristics.

Macronutrients	Housing			Living Arrangements[3]			Education (years)			Past-Occupation (NS-SEC)			Physical Activity		
	Standard (n=620)	Sheltered (n=137)	Institutional (n=34)	Alone (n=437)	Spouse (n=204)	Others (n=79)	≤9 (n=501)	10-11 (n=183)	≥12 (n=97)	Class 1 (n=385)	Class 2 (n=109)	Class 3 (n=259)	Low (n=176)	Medium (n=343)	High (n=270)
Energy (MJ)	6.62	6.78	7.65*	6.36	7.28	6.64	6.57	6.69	6.89	6.76	6.63	6.64	6.77	6.37	6.92
Energy (Kcal)	1581	1619	1828	1520	1739	1587	1571	1599	1646	1617	1586	1588	1617	1522	1653
Alcohol (g)[1]	13.3	10.1	-[2]	11.5	16.1	9.7	10.6	15.0	13.6	12.8	10.2	13.4	12.6	10.2	15.0
Carbohydrate (g)	191.6	198.4	222.0**	184.2	220.4	186.2	192.4	190.1	211.4	200.9	195.8	192.7	197.2	185.8	203.1
% Energy	46.8	46.9	47.6	46.5	47.2	47.8	46.9	46.1	48.1	47.1	47.5	46.6	46.7	47.3	46.2
NMES (g)	42.3	41.5	63.6**	38.8	49.5	40.2	41.5	42.6	45.9	44.5	45.7	41.4	46.6	41.5	43.9
% Energy	10.0	10.2	12.9*	9.8	10.6	9.5	10.0	10.3	9.9	10.1	10.3	9.8	10.1	10.1	10.1
NSP (g)	10.4	9.4**	7.1***	9.9	11.0	10.1	9.9	10.1	11.7**	10.8**	10.4*	9.9	9.1	10.0**	11.3***
Total Fat (g)	65.5	64.7	74.4	63.9	68.1	60.8	65.5	66.6	65.0	66.0	63.9	67.1	66.8	62.3	69.7
% Energy	36.8	37.3	37.7	36.9	36.4	37.3	36.9	37.6	35.6	35.7	36.6	37.2	37.3	36.7	36.7
SFA (g)	24.0	24.9	25.6	23.5	25.3	22.3	23.5	25.5	24.4	24.4	24.9	24.1	25.3	23.3	25.4
% Energy	13.6	13.7	12.9	13.6	13.2	13.5	13.5	14.4	13.1	13.4	13.4	13.7	14.1	13.5	13.4
MUFA (g)	15.8	14.9	14.5	15.3	16.3	15.4	15.5	15.7	16.1	15.2	16.5	16.0	14.7	14.8	16.7*
% Energy	9.0	8.2	7.8**	9.0	8.6	9.3	8.8	9.0	8.4	8.6	9.1	8.9	8.3	8.7	9.2*
PUFA (g)	6.5	5.9	4.1*	6.5	6.4	6.4	6.6	6.3	5.9	6.4	6.5	6.3	5.2	6.2*	6.9***
% Energy	3.6	3.3	2.3**	3.7	3.3	3.8	3.6	3.4	3.0	3.4	3.7	3.4	2.8	3.5*	3.8**
Protein (g)	61.2	61.1	61.9	58.3	67.5	55.0*	59.4	62.0	65.7	63.5	59.5	61.1	59.6	58.0	66.2*
% Energy	15.7	15.3	14.0	15.7	15.9	14.3	15.7	15.5	15.7	15.7	15.8	15.5	15.3	15.4	16.1*

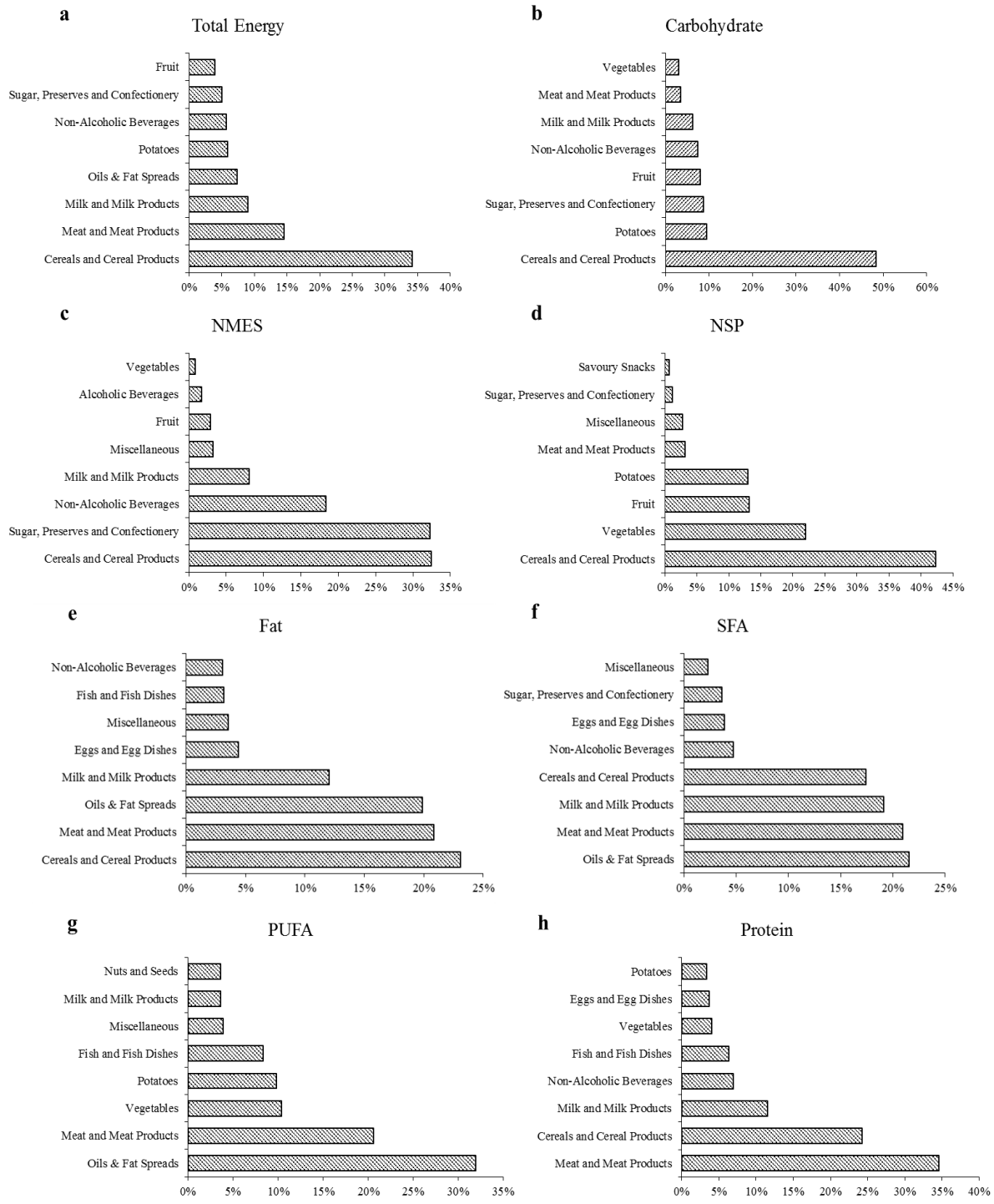
NSP, non-starch polysaccharides; NS-SEC, National Statistics Socioeconomic Classification. Class 1: Higher managerial, administrative and professional occupations; Class 2: Intermediate occupations; Class 3: Routine or manual occupations. NMES, non-milk extrinsic sugars; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. All models were adjusted for sex except NSP which was adjusted for sex and energy intake. Standard housing, living alone, ≤9 years of full time education, class 3 of past occupation and low physical activity were the reference categories.

* p<0.05 ** p<0.01*** p<0.001.

[1] Only alcohol drinkers.

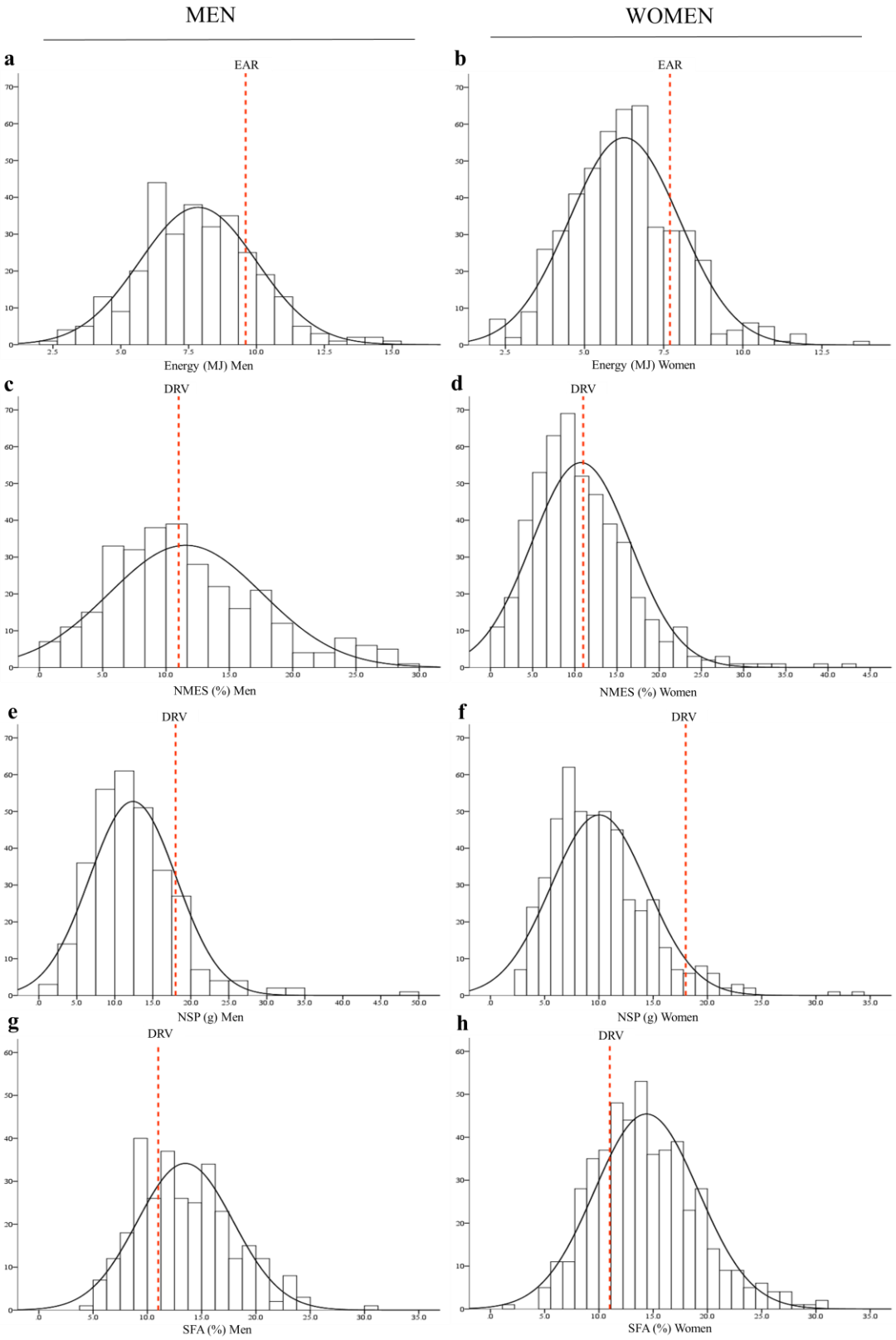
[2] Not reported due to low participant number.[3] Excludes people in institutional care.

Figure 3.1. Contribution (%) of 15 food groups to average energy and macronutrient intake in the Newcastle 85+ Study.



Contribution to **a.** energy **b** carbohydrate **c** NMES **d** NSP **e** fat **f** SFA **g** PUFA and **h** protein intake. NMES, non-milk extrinsic sugars; NSP, non-starch polysaccharides; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.

Figure 3.2. Distribution and adequacy of energy and macronutrient intake in the Newcastle 85+ Study.



Distribution and adequacy of food energy intake (MJ) in **a**, Men and **b**, Women; of NMES intake (%) in **c**, Men and **d**, Women; of NSP intake (g) in **e**, Men and **f**, Women and; of SFA intake (%) in **g**, Men and **h**, Women. Vertical dashed lines represent the DRVs in the UK for adults[69] and for adults aged 75 and over for energy[34]. EAR, Estimated Average Intake; DRV; Dietary Reference Value; NMES, non-milk extrinsic sugars; NSP, non-starch polysaccharides; SFA, saturated fatty acids.

3.5. Supplemental material

Supplemental Table 3.1. United Kingdom dietary reference values and group compliance (%) in the Newcastle 85+ Study by sex.

Macronutrients	All	Men		Women	
	Group Compliance (%)	DRV	Group Compliance (%)	DRV	Group Compliance (%)
Energy	19.8	9.6 MJ[1]	19.9	7.7 MJ[1]	19.8
Alcohol[2]	83.3	32 g[3]	77.5	24 g[2]	90.8
Carbohydrate	33.0	50 % en	34.4	50 % en	32.2
NMES	56.5	11 % en	52.6	11 % en	58.9
NSP	9.0	18 g	14.2	18 g	5.7
Total Fat	41.1	35 % en	44.0	35 % en	39.3
SFA	27.9	11 % en	32.5	11 % en	25.1
Protein	71.5	0.75 g/Kg	78.1	0.75 g/Kg	67.4

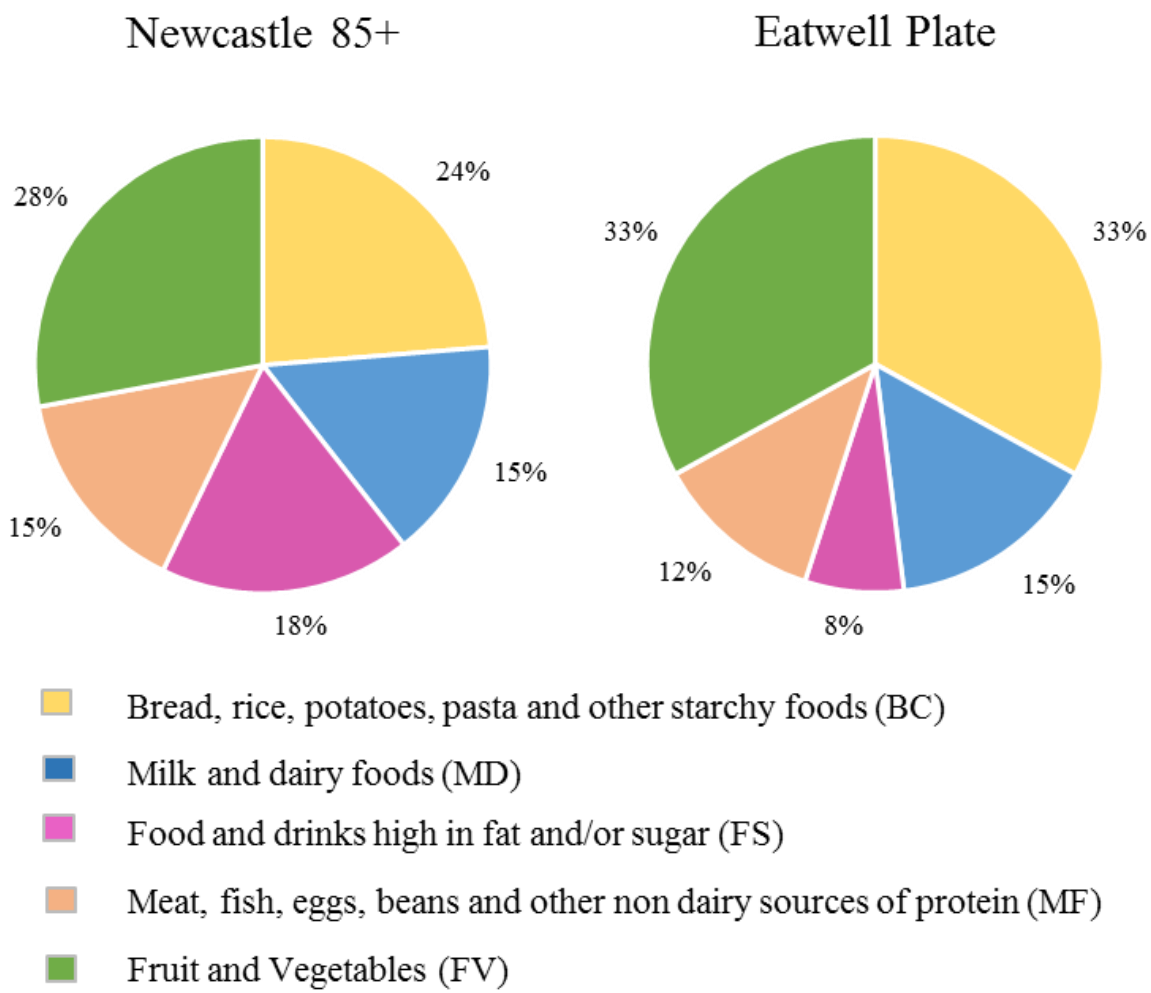
DRV, Dietary Reference Value; NMES, non-milk extrinsic sugars; NSP, non-starch polysaccharides; SFA, saturated fatty acids; % en, percentage of energy.
DRVs were taken from the Committee on Medical Aspects of Food Policy report [69] unless stated otherwise. Group compliance is the percentage above or below the DRVs as appropriate.
[1] Scientific Advisory Committee on Nutrition [34].
[2] Only alcohol drinkers.
[3] Sensible-Drinking limits[185].

Supplemental Table 3.2. Daily energy, EI:BMRest, macronutrient and NSP intakes of 539 non-misreporters and difference between 731 total reporters by sex in the Newcastle 85+ Study[1].

Macronutrients	All			Men			Women		
	Median	IQR	Dif	Median	IQR	Dif	Median	IQR	Dif
Energy (MJ)	7.00	6.13-8.34	-0.36	8.47	7.39-9.40	-0.75	6.39	5.66-7.25	-0.24
Energy (Kcal)	1674	1466-1993	-86	2024	1766-2248	-178	1527	1353-1734	-57
EI:BMR_{est}	1.41	1.24-1.62	-0.08	1.43	1.27-1.61	-0.1	1.40	1.23-1.63	-0.07
Carbohydrate (g) (% en)	207.6 (46.5)	172.3-242.4	-14.1	242.2 (46.6)	213.9-280.8	-14.2	185.9 (46.3)	162.5-219.5	-8.9
NMES (g) (% en)	47.7 (10.4)	30.2-66.2	-5.1	58.2 (10.8)	39.3-83.4	-8.1	41.9 (10.2)	26.7-59.6	-3.9
NSP (g)	10.8	8.3-14.4	-0.6	12.5	9.8-16.1	-1.2	10.2	7.6-13.2	-0.7
Total Fat (g) (% en)	70.6 (37.1)	57.2-87.2	-5.1	81.1 (36.4)	66.1-99.9	-6.6	63.9 (37.4)	53.5-77.3	-3.6
SFA (g) (% en)	26.3 (13.7)	19.9-34.2	-2.2	29.5 (13.0)	22.4-37.5	-2.6	24.2 (14.0)	18.6-31.4	-1.5
MUFA (g) (% en)	16.8 (8.9)	12.5-22.3	-1.1	19.7 (8.7)	14.8-25.0	-1.5	15.6 (9.0)	11.7-20.1	-1.3
PUFA (g) (% en)	6.9 (3.6)	4.5-10.5	-0.4	8.2 (3.6)	5.5-11.8	-0.8	6.3 (3.5)	3.9-9.5	-0.4
P:S ratio	0.26	0.15-0.41	0.00	0.29	0.17-0.41	0.00	0.24	0.14-0.41	0.01
Protein (g) (% en)	65.3 (15.6)	52.5-79.0	-3.8	77.7 (15.8)	65.6-93.3	-4.4	57.9 (15.4)	49.3-68.9	-3.5

EI:BMR_{est}, energy intake by estimated basal metabolic rate[(182)]; NSP, non-starch polysaccharides; IQR, interquartile range; IQR, interquartile range; Dif, Difference between without and with cut-offs (accurate reporters); % en, percentage of energy; NMES, non-milk extrinsic sugars; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; P:S ratio, PUFA/SFA ratio.
Cut-offs were defined as an EI:BMR_{est} at 1.05-2.0. Sixty-two participants did not have weight and/or height; therefore, cut-offs could not be applied to the entire cohort and only to 731 participants.
[1] 26.3% were misreporters (21.6% underreporters and 4.7% overreporters).

Supplemental Figure 3.1. Comparison between the Newcastle 85+ Study and old Eatwell Plate (Balance of Good Health).



3.6. Summary of Chapter 3

Food and nutrient intake data are scarce in very old adults (85 years and older) – one of the fastest growing age segments of western societies, including the UK. The primary objective of this chapter was to assess energy and macronutrients intake and, respective food sources in 793 eighty-five-year-olds (302 men and 491 women) living in North-East England and participating in the Newcastle 85+ cohort Study. Dietary information was collected using a repeated multiple pass recall (2x24hr recalls). Energy, macronutrient and non-starch polysaccharide (NSP) intakes were estimated and the contribution (%) of food groups to nutrient intake was calculated. Median energy intake was 6.65 (IQR: 5.49-8.16) MJ/ day, 46.8% was from carbohydrate, 36.8% from fat and 15.7% from protein. NSP intake was 10.2 g/ day (IQR: 7.3-13.7). NSP intake was higher in non-institutionalised, more educated, from higher social class and more physically active 85 year olds. Cereals and cereal products were the top contributors to intakes of energy and most macronutrients (carbohydrate, non-milk extrinsic sugars, NSP and fat), followed by meat and meat products. Median intakes of energy and NSP were much lower than the estimated average requirement (EAR) for energy (9.6 MJ for men and 7.7 MJ for women per day) and the dietary reference value (DRV) for NSP (≥ 18g/ day). Median saturated fatty acids intake was higher than the DRV (≤ 11% of dietary energy). This chapter provides much needed information on dietary intake and DRVs for this age group.

A number of socioeconomic, biological and lifestyle characteristics change with advancing age and place very old adults at increased risk of micronutrient deficiencies. Chapter 4 will continue to investigate the dietary intake of the very old, focusing on micronutrients, such as iron, folate and vitamin B12, the food sources and associated factors, as well as determining micronutrient intake “inadequacy” against UK DRVs.

CHAPTER FOUR

4. Micronutrient intake and food sources in the very old

[Mendonça N *et al.* (2016) Micronutrient intake and food sources in the very old: Analysis of the Newcastle 85+ Study. *Br J Nutr*. **116**(4):751-61.]

Key words: dietary intake, vitamins, minerals, 'aged, 80 and over', Newcastle 85+

4.1. Abstract

A number of socio-economic, biological and lifestyle characteristics change with advancing age and place very old adults at increased risk of micronutrient deficiencies. The aim of this study was to assess vitamin and mineral intakes and respective food sources in 793 85-year-olds (302 men and 491 women) in the North-East of England, participating in the Newcastle 85+ Study. Micronutrient intakes were estimated using a multiple-pass recall tool (2 × 24 h recalls). Determinants of micronutrient intake were assessed with multinomial logistic regression. Median vitamin D, Ca and Mg intakes were 2.0 (interquartile range (IQR) 1.2–6.5) μg/d, 731 (IQR 554–916) mg/d and 215 (IQR 166–266) mg/d, respectively. Fe intake was 8.7 (IQR 6.7–11.6) mg/d, and Se intake was 39.0 (IQR 27.3–55.5) μg/d. Cereals and cereal products were the top contributors to intakes of folate (31.5 %), Fe (49.2%) and Se (46.7%) and the second highest contributors to intakes of vitamin D (23.8 %), Ca (27.5%) and K (15.8 %). More than 95% (n=756) of the participants had vitamin D intakes below the UK's Reference Nutrient Intake (10 μg/d). In all, >20% of the participants were below the Lower Reference Nutrient Intake for Mg (n=175), K (n=238) and Se (n=418) (comparisons with dietary reference values (DRV) do not include supplements). As most DRV are not age specific and have been extrapolated from younger populations, results should be interpreted with caution. Participants with higher education, from higher social class and who were more physically active had more nutrient-dense diets. More studies are needed to inform the development of age-specific DRV for micronutrients for the very old.

4.2. Introduction

The scarcity of dietary data on very old adults, and lack of evidence for relationships with risk factors and health outcomes, have resulted in DRVs based on extrapolations from younger populations [187]. For example, In the UK, apart from the Reference Nutrient Intake (RNI) for vitamin D which sets a Dietary Reference Intake (DRV) for people aged 65 and over, all other DRVs for vitamins or minerals apply equally to everyone aged ≥50 [69].

The 1994-95 National Diet and Nutrition Survey (NDNS) of people aged 65 and over identified a significant number of older adults with inadequate micronutrient intakes, namely vitamin D, magnesium and potassium [72]. A review of micronutrient intakes across Europe revealed that inadequacy (assessed against the Nordic Nutrition Recommendations, estimated average intake) was present in more than 20% of older adults (≥65 years) for

vitamin D, folate, calcium and selenium [71]. Similarly, a review of non-institutionalised older adults living in western countries concluded that at least 30% were below the Estimated Average Requirement (EAR) for vitamin D, vitamin B12, calcium, magnesium and selenium [70].

The aim of this chapter was to assess daily energy, vitamin and mineral intakes of 85 year olds participating in the Newcastle 85+ Study; determine its food sources; compare intakes with the current UK DRVs; and explore socioeconomic and lifestyle determinants of micronutrient intake.

4.3. Methods

Participants

This chapter uses data from the Newcastle 85+ Study and details were reported in *Chapter 2, General Methods - 2.1 The Newcastle 85+ Study*.

Micronutrient estimation, food group contribution and supplement use

Dietary intake assessment and food allocation to tertiary food groups was described on *Chapter 2, General Methods - 2.2 Dietary assessment and food group allocation*. Energy, vitamin and mineral intakes were estimated using the McCance and Widdowson's sixth edition food composition tables (used as published) [178] together with a purpose-designed in house Microsoft Office Access database on the nutrient composition of commonly consumed foods [16]. Intakes of energy, vitamin A, β-carotene, vitamin B2, vitamin B6, folate, vitamin B12, vitamin E, vitamin C, vitamin D, calcium, iron, magnesium, potassium, sodium, selenium and zinc are reported here (excluding supplements). Vitamin and mineral density per 1 MJ of energy was also calculated. The average contribution of food groups to vitamin and mineral intakes was reported so that ≥90% of intakes were explained.

Supplement use details were described in *Chapter 2, General Methods - 2.4 Other socioeconomic, health and lifestyle variables*. Supplement users were characterised by supplement type: those taking fish and omega-3 oil preparations, single mineral/vitamin preparations, multivitamin and/or multimineral preparations and, other supplements. Micronutrient intakes from all sources (including supplements) and the difference (%) between micronutrient intakes from dietary sources only (excluding supplements) were determined but supplements were not included in the main analysis.

Statistical analysis

Baseline characteristics, micronutrient intake and percentage of participants below the Lower Reference Nutrient Intake (LRNI), EAR, RNI and UL were calculated using descriptive statistics. If available, LRNI was the preferred DRV to be reported. The LRNI is only supposed to meet the needs of 2.5% of a given population and intakes below this are likely to be "inadequate". Most micronutrient intake data were continuous and non-normally distributed therefore, sex differences were determined by the Mann-Whitney U test. Vitamin and mineral intakes were stratified by housing, living arrangements (with whom participants lived), years of full time education, social class [coded to the National Statistics Socio-economic Classification (NS-SEC) 3 class system [(172)]] and physical activity groups, and compared by multinomial logistic regression. Apart from energy, which was adjusted for sex only, all vitamins and minerals were adjusted for sex and energy. General statistical methods are presented in *Chapter 2, General Methods - 2.5 General statistical analysis.*

4.4. Results

Vitamin intakes

Men had higher vitamin intakes than women except for vitamin C (**Table 4.2**). However, the overall higher vitamin intake by men disappeared when the results were expressed per 1 MJ. Specifically, women's vitamin A intake was 12 µg-RE/MJ or 13% higher (p=0.008) and vitamin C intake was 20 mg/MJ or 28% higher (p=0.001) than men's intake. Despite 43% of participants (n=335) consuming one or more supplements on a regular basis (**Table 4.1**), on a population level, vitamin intakes changed only marginally when supplements were included except for vitamin A and D which increased by 19.2% (from 620 to 752 µg-RE) and by 22.5% (from 2.0 to 2.5 µg), respectively (**Supplemental Table 4.1**). Due to the modest differences to micronutrient intake when including supplements, and limitations in supplement frequency data, micronutrient consumption from supplements was not included in the main analysis.

Vitamin food sources

Figure 4.1 shows the percent contribution of food groups to vitamin intake for all participants. Meat and meat products contributed to 40% of vitamin A intake - the majority coming from liver and liver products and dishes (94.4%). Vegetables were the second biggest contributor (22.4%) to vitamin A intake, of which most came from carrots (71.1%). Cereals and cereal products (CCP) were the biggest contributors (31.5%) to folate intake, 86.9% of which came from bread and breakfast cereals. Vegetables were the second biggest contributor (15.8%) to folate intake with 42.4% coming from cruciferous vegetables. Half (49.6%) of the vitamin B12 intake from meat and meat products (52.3%) came from liver and liver products and dishes. One third (33.8%) of vitamin D intake came from fish and fish dishes (98.9% of which was from oily fish), and 23.8% from CCP (45.2% of which was from breakfast cereals and 43.3% from buns, cakes, pastries and fruit pies).

Mineral intakes

Similar to vitamin intake, men had an overall higher mineral intake than women (24% higher on average) (**Table 4.2**). When expressed per 1 MJ of energy, men still had higher intakes of iron ($p=0.005$), selenium ($p=0.028$) and zinc ($p<0.001$) compared to women but lower calcium intakes ($p=0.008$). On a population level, supplement contribution to mineral intakes was almost negligible (**Supplemental Table 4.1**). The highest difference between dietary intake with and without supplements was only 2.7% for zinc (from 7.1 to 7.3 mg).

Mineral food sources

Figure 4.1 shows the percent contribution of food groups to vitamin intakes for all participants. Milk and milk products were the biggest contributors (31.3%) to calcium intake while CCP was second with 27.5% (36.6% of which came from bread). Non-alcoholic beverages contributed 18.9% to calcium intake mainly because tea and coffee (with added milk) were included in this group (95.4% came from tea, coffee and water). Non-alcoholic beverages accounted for 19% of potassium intake (81.5% of which was from tea, coffee and water). CCP (15.8%) and potatoes (14.6%) were the second and third, respectively, biggest contributors to potassium intake. CCP explained 46.7% of selenium intake, and 93.2% of this came from bread. Meat and meat products made a higher contribution to intakes of iron

(19.3% vs. 14.2%), vitamin D (20.3% vs. 13.4%) and vitamin B12 (59.2% vs. 47.8%) for men than for women.

Micronutrient adequacy

The failure of both men and women in the Newcastle 85+ Study to meet several micronutrients' DRVs was widespread (**Figure 4.2** and **Supplemental Table 4.2**). Twenty percent of the participants had intakes below the LRNI for magnesium, potassium and selenium. The proportion of participants below the LRNI for vitamin A, vitamin B12 and zinc was around 10%. However, 4.6% (n=36) of the participants had vitamin A intakes above the UL. The widest disparity between intake and recommendations was seen for vitamin D intake, with more than 95% (n=756) of participants having intakes below the RNI for vitamin D of 10 µg per day (EAR or LRNI for vitamin D have not been defined for the UK) [(69)] and 52.7% (n=418) of participants were below the LRNI for selenium. In contrast, 82.2% (n=652) of participants were above the RNI for sodium of 1600 mg per day [(69)]. The 95th percentile of sodium intake was 4663 mg per day and within those that were above the RNI, median intake was 2594 mg. Fewer men had intakes below the DRV for vitamin B12, iron, potassium and folate than women. The widest difference between men and women not meeting the LRNI was for vitamin B12 (5.0% vs. 12.4%, $p<0.001$) and iron (2.3% vs. 7.8%, $p<0.001$). Meat and meat products were top contributors for both micronutrients.

Micronutrient intake by housing, socioeconomic status and physical activity

Table 4.3 reports the energy, vitamin and mineral intakes in the Newcastle 85+ Study stratified by housing, living arrangements, years of full time education, social class (past occupation according to NS-SEC) and physical activity. All micronutrient models were adjusted for sex and food energy intake. Energy and vitamin D intake were higher in participants who lived in institutional care (nursing or residential) than in standard housing. Conversely, vitamin E, magnesium and potassium intakes were lower in institutional than in standard housing. Participants who lived with their spouses had higher potassium and selenium intake than those who lived alone. Those with 12 or more years of full time education had higher intakes of vitamin C, vitamin D, calcium, magnesium and potassium than those with ≤ nine years of full time education. Social class also associated with the intake of several vitamins and minerals. Participants with previous higher managerial, administrative and professional occupations (class 1) had higher intakes of vitamin B2,

folate, calcium, iron, magnesium, potassium and zinc than those who had routine and manual occupations (class 3). Those with high physical activity had a more nutrient-dense diet in vitamin B6, folate, vitamin E, vitamin C, iron, magnesium, potassium and zinc than those with lower physical activity.

Table 4.1. Energy intake and supplement use by sex.

	All	Men	Women	p-value[1]
Sex	793	38 (302)	62 (491)	-
Energy (MJ)	6.65 (5.49-8.16)	7.73 (6.36-9.20)	6.15 (5.09-7.25)	<0.001[2]
Carbohydrate (% en)	46.8 (42.6-51.5)	46.8 (42.7-52.0)	46.8 (42.5-51.4)	0.760[3]
Fat (% en)	36.8 (32.0-41.8)	36.4 (31.6-41.1)	37.2 (32.2-42.2)	0.093[3]
Protein (% en)	15.7 (13.5-18.3)	15.9 (13.8-18.9)	15.5 (13.6-17.9)	0.006[3]
Dietary Supplement Use				0.252
None	58 (456)	62 (185)	55 (271)	
1	29 (227)	27 (81)	30 (146)	
2+	14 (108)	12 (35)	15 (73)	
Dietary Supplement Type				0.590
Fish and Omega-3 Oil	48 (162)	48 (56)	48 (106)	
Mineral/ Vitamin Preparations	10 (32)	8 (9)	11 (23)	
Multivitamin and/or Multimineral	12 (39)	10 (12)	12 (27)	
Other	31 (102)	34 (39)	29 (63)	

Values are percentages (numbers). Energy and % en from macronutrients are presented as medians (interquartile range). % en, percentage of energy; NS-SEC, National Statistics Socioeconomic Classification.
[1] Chi-squared test ($\chi 2$) for no sex difference unless otherwise stated.
[2] Mann-Whitney U test for no sex difference.
[3] Independent t-test for no sex difference.

Table 4.2. Daily energy, vitamin and mineral intakes of the Newcastle 85+ Study participants by sex and per 1 MJ of energy[1].

Micronutrients	All		Men			Women			
	Median	IQR	Median	IQR	Median/ 1 MJ	Median	IQR	Median/ 1 MJ	p-value[3]
Energy (MJ)[2]	6.65	5.49-8.16	7.73	6.36-9.20	-	6.15	5.09-7.25	-	<0.001
Vitamins									
Vitamin A (μg RE)	620	398-910	674	414-988	86.5	593	390-851	98.5	0.008
β-Carotene (μg)	1516	517-2883	1769	606 -3167	212.5	1335	488-2666	215.0	0.577
Vitamin B2 (mg)	1.5	1.2-1.9	1.7	1.3-2.1	0.22	1.4	1.1-1.8	0.23	0.138
Vitamin B6 (mg)	1.7	1.2-2.1	2.0	1.5-2.5	0.25	1.5	1.1-1.9	0.25	0.217
Folate (μg)	208	157-264	245	183-295	30.9	189	146-243	31.7	0.564
Vitamin B12 (μg)	2.9	1.9-4.4	3.4	2.2-5.2	0.46	2.6	1.6-3.9	0.42	0.047
Vitamin E (mg)	4.7	3.2-7.5	5.0	2.4-8.3	0.65	4.5	2.9-6.9	0.69	0.128
Vitamin C (mg)	56.5	30.5-99.1	55.5	32.4-98.4	7.10	57.2	30.0-99.4	9.27	0.001
Vitamin D (μg)	2.0	1.2-6.5	2.3	1.4-3.7	0.33	1.8	1.0-2.9	0.30	0.200
Minerals									
Calcium (mg)	731	554-916	829	634-1007	103.7	683	537-862	111.2	0.008
Iron (mg)	8.7	6.7-11.6	10.5	8.4-13.5	1.35	7.8	6.1-9.9	1.28	0.005
Magnesium (mg)	215	166-266	251	196-309	32.6	196	156-239	32.4	0.316
Potassium (mg)	2477	1890-3023	2798	2230-3448	356.6	2262	1804-2797	373.4	0.100
Sodium (mg)[4]	2388	1829-3188	2987	2216-3743	372.1	2162	1691-2707	361.6	0.101
Selenium (μg)	39.0	27.3-55.5	48.3	33.9-65.1	6.19	35.2	25.3-48.4	5.83	0.028
Zinc (mg)	7.1	5.5-9.6	8.6	6.8-11.1	1.12	6.3	5.1-8.2	1.05	<0.001

IQR, Interquartile Range; RE, Retinol Equivalents.

[1] Does not include supplements. [2] Does not include energy from alcohol.

[3] Mann-Whitney U test for no sex difference (Median/ 1 MJ of energy). [4] Does not include table salt and salt used for cooking.

Table 4.3. Daily energy, vitamin and mineral intakes according to demographic, socioeconomic and lifestyle characteristics[1].

Micronutrients	Housing			Live With			Education (years)			Past-Occupation (NS-SEC)			Physical Activity		
	Stand (n=620)	Sheltered (n=137)	Institut (n=34)	Alone (n=437)	Spouse (n=204)	Others (n=79)	≤9 (n=501)	10-11 (n=183)	≥12 (n=97)	Class 1 (n=385)	Class 2 (n=109)	Class 3 (n=259)	Low (n=176)	Medium (n=343)	High (n=270)
Energy (MJ) [2]	6.62	6.78	7.65*	6.36	7.28	6.64	6.57	6.69	6.89	6.76	6.63	6.64	6.77	6.37	6.92
Vitamins															
Vitamin A (µg RE)	606	623	709	600	642	582	602	625	667	639	636*	600	627	599	648
β-Carotene (µg)	1589	1093	1546	1381	1792	1365	1492	1493	1470	1575	1576	1339	1382	1339	1730
Vitamin B2 (mg)	1.5	1.5	1.8	1.4	1.6	1.4	1.5	1.6	1.7	1.6**	1.5*	1.5	1.6	1.4	1.6
Vitamin B6 (mg)	1.7	1.6	1.7	1.6	1.9	1.6	1.6	1.7	1.8	1.7	1.7	1.6	1.5	1.6*	1.9***
Folate (µg)	208	195	231	195	231	191	201	209	234	214*	208	203	185	201	232**
Vitamin B12 (µg)	2.9	2.7	3.8	2.7	3.1	2.2	2.8	3.1	3.0	3.0	2.8*	2.8	3.0	2.5	3.2
Vitamin E (mg)	4.7	4.7	3.9*	4.7	4.8	4.6	4.7	4.7	5.1	4.7	5.2	4.5	4.5	4.4	5.2*
Vitamin C (mg)	59.0	49.6	62.1	55.2	56.7	62.3	54.8	55.5	80.0**	61.7	64.5	52.1	46.6	56.4	66.6*
Vitamin D (µg)	1.9	1.9	3.5**	1.8	2.1	1.9	1.9	2.1*	2.1*	2.0	1.9	1.9	2.6	1.8*	2.1
Minerals															
Calcium (mg)	730	731	736	713	799	638*	710	738	778*	753*	730	722	735	702	771
Iron (mg)	8.9	8.0***	9.0	8.3	9.8	7.9	8.3	9.6	9.9	9.3**	8.7	8.6	8.6	8.4*	9.5**
Magnesium (mg)	220	205**	195***	209	236	196	211	216.	235**	226***	223***	209	197	208***	235***
Potassium (mg)	2504	2445*	2363**	2348	2738*	2276	2397	2495	2904**	2656***	2440	2402	2278	2381**	2725***
Sodium (mg)[3]	2357	2482*	2678	2363	2532	2077*	2351	2464	2390	2381	2363	2392	2401	2285*	2573
Selenium (µg)	39.1	36.2	41.5	37.9	40.8*	34.0	38.1	40.0	39.0	38.1	39.7*	39.3	37.8	38.1	41.1
Zinc (mg)	7.2	7.0	7.4	6.9	7.9	6.2	7.0	7.3	7.6	7.4**	7.2*	7.0	7.0	6.7	8.0*

NS-SEC, National Statistics Socioeconomic Classification; Stand, Standard; Institut, Institutional Housing; Class 1: Higher managerial, administrative and professional occupations; Class 2: Intermediate occupations; Class 3: Routine or manual occupations.

All models were adjusted for sex and energy intake except for energy intake which was only adjusted for sex. Standard housing, living alone, ≤9 years of full time education, class 3 of past occupation and low physical activity were the reference categories.

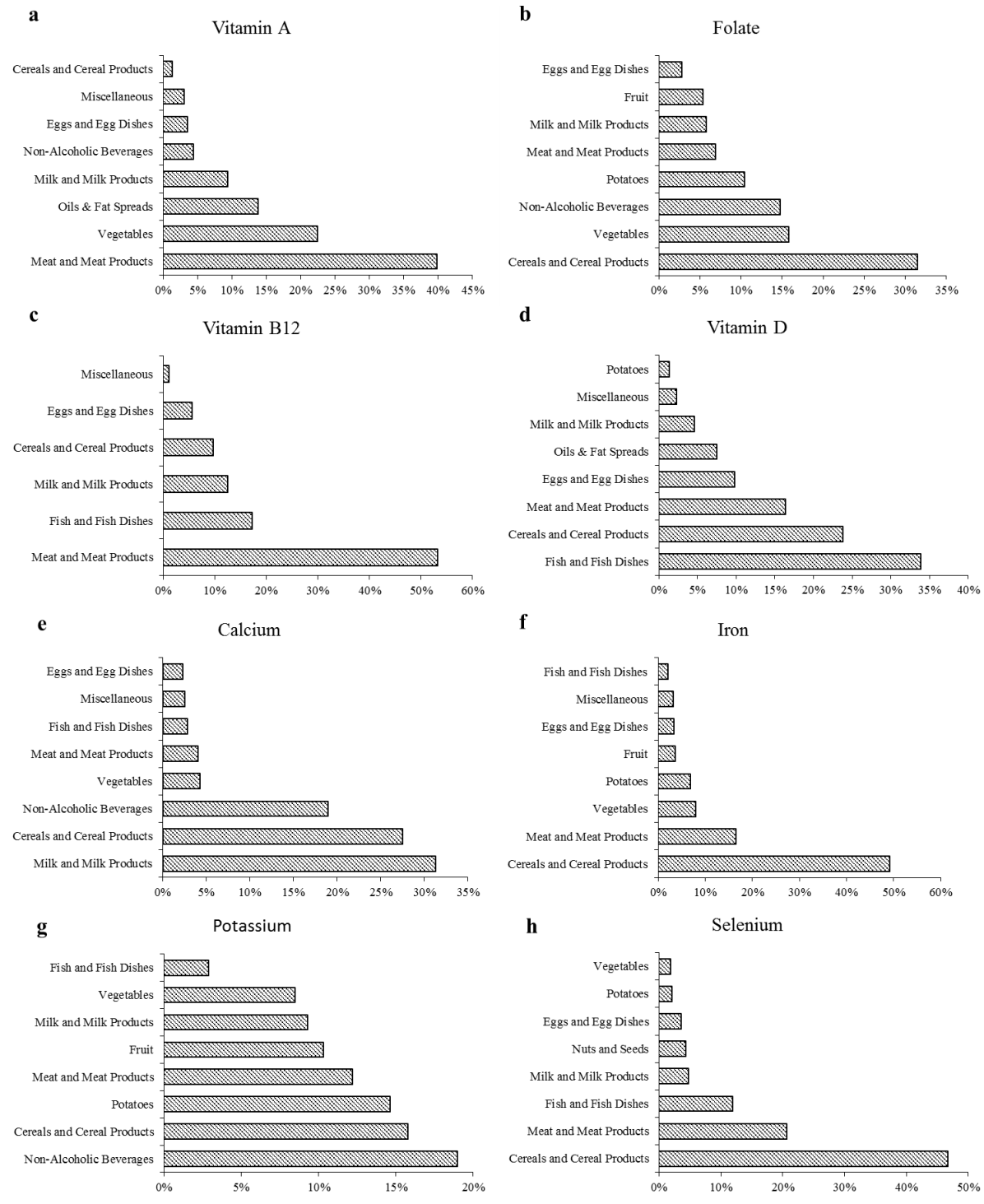
* p<0.05 ** p<0.01*** p<0.001.

[1] Does not include supplements.

[2] Does not include energy from alcohol.

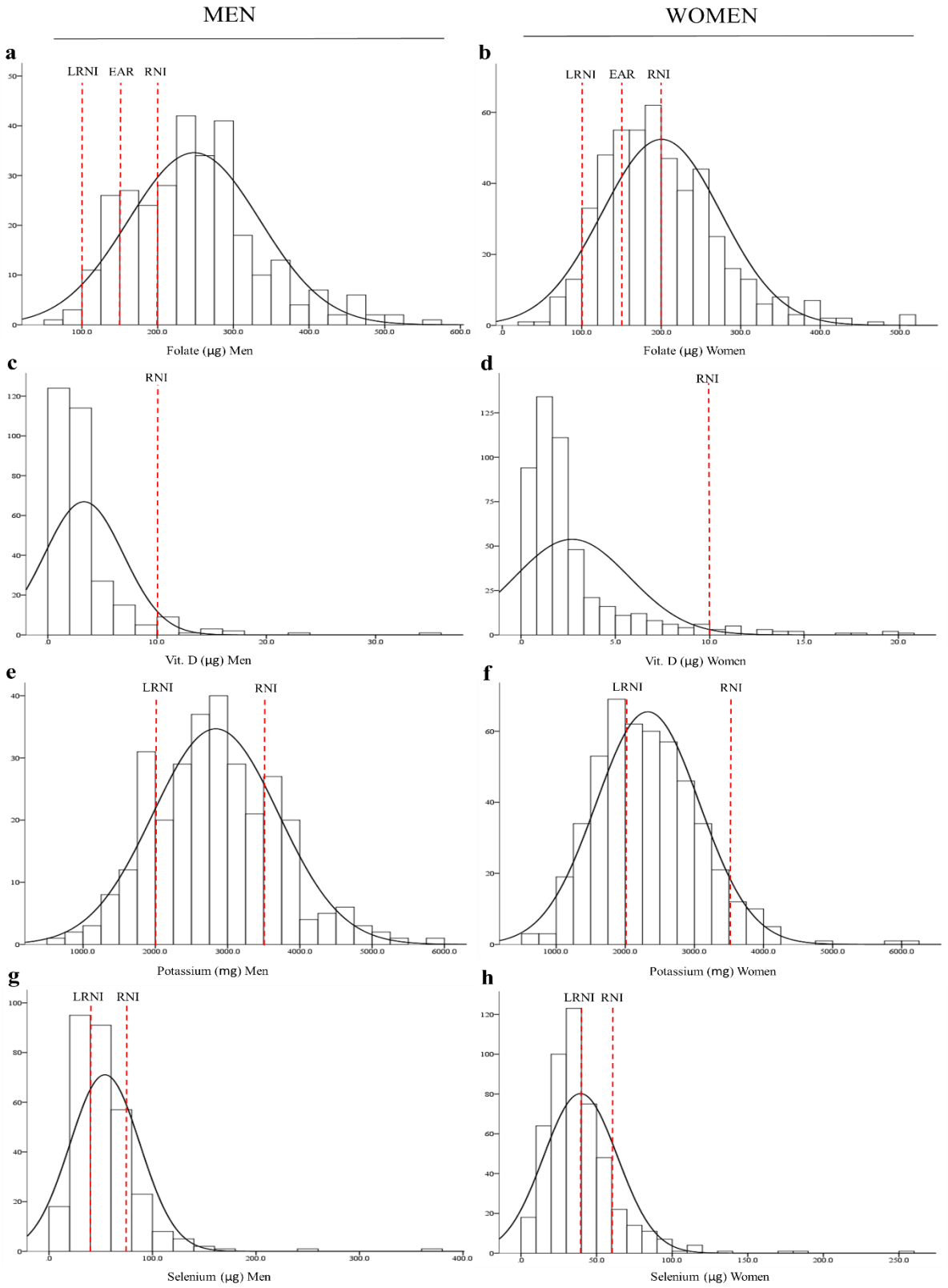
[3] Does not include table salt and salt used for cooking.

Figure 4.1. Contribution (%) of 15 food groups to average micronutrient intake in the Newcastle 85+ Study.



Contribution to **a**, Vitamin A; **b**, Folate; **c**, Vitamin B12; **d**, Vitamin D; **e**, Potassium; **f**, Calcium; **g**, Iron; and **h**, Selenium intake.

Figure 4.2. Intake distribution and inadequacy of selected micronutrients by sex.



Intake of folate (µg) in **a**, Men and **b**, Women; of vitamin D (µg) in **c**, Men and **d**, Women; of potassium (µg) in **e**, Men and **f**, Women; of selenium (µg) in **g**, Men and **h**, Women. Horizontal dashed lines represent the LRNI, EAR and RNI for people aged 50 and over, except for vitamin D which is set for ≥65 years[6]. Nutrient intakes do not include supplements. RNI, Reference Nutrient Intake; EAR, Estimated Average Intake; LRNI, Lower Reference Nutrient Intake.

4.5. Supplemental material

Supplemental Table 4.1. Vitamin and mineral intakes from all sources (including supplements) and, the difference (%) between including and not including supplement estimation by sex.

Micronutrients	All			Men				Women			
	Median	IQR	Dif (%)	Median	IQR	Median/ 1MJ	Dif (%)	Median	IQR	Median/ 1 MJ	Dif (%)
Vitamins											
Vitamin A (µg RE)	752	462-1255	19.2	801	479-1281	104	17.2	711	450-1243	116	18.1
Vitamin B2 (mg)	1.6	1.2-2.0	6.5	1.7	1.3-2.2	0.2	0.0	1.4	1.1-1.9	0.2	0.0
Vitamin B6 (mg)	1.7	1.3-2.2	0.0	2.0	1.5-2.5	0.3	0.0	1.6	1.2-2.0	0.3	6.5
Folate (µg)	212	158-276	1.9	247	186-300	32	0.8	193	147-253	31	2.1
Vitamin B12 (µg)	3.0	1.9-4.6	3.4	3.5	2.2-5.4	0.5	2.9	2.6	1.6-4.1	0.4	0.0
Vitamin E (mg)	5.0	3.3-8.2	6.2	5.3	3.5-8.6	5.8	5.8	4.9	3.1-7.9	0.8	8.5
Vitamin C (mg)	60.5	31.7-110.3	6.8	57.7	35.1-108.8	0.7	3.9	62.4	31.2-112.2	10.2	8.7
Vitamin D (µg)	2.5	1.3-6.2	22.2	2.7	1.6-6.3	0.4	16.0	2.3	1.2-6.2	0.4	24.4
Minerals											
Calcium (mg)	735	555-922	0.5	833	640-1008	104	0.5	691	538-868	112	1.0
Iron (mg)	8.9	6.8-11.8	2.3	10.6	8.3-13.7	1.4	1.0	7.9	6.2-10.2	1.3	1.3
Magnesium (mg)	218	169-269	1.1	254	200-312	33	1.3	198	157-247	33	0.6
Selenium (µg)	39.4	27.6-57.5	1.0	49.2	34.4-67.9	6.4	1.9	35.7	25.4-50.1	5.9	1.4
Zinc (mg)	7.3	5.7-9.9	2.7	8.7	7.0-11.7	1.1	1.2	6.6	5.3-8.7	1.1	4.7

IQR, Interquartile Range; RE, Retinol Equivalents; Dif, difference between median vitamin and mineral intakes from all sources (including supplements) and dietary sources only. There is no β-carotene and sodium supplementation use.

Supplemental Table 4.2. Percentage (%) of the Newcastle 85+ Study participants below the RNI, EAR and LRNI for the UK by sex[1].

Micronutrients	All			Men			Women			p-value[2]
	<LRNI	<EAR	<RNI	<LRNI	<EAR	<RNI	<LRNI	<EAR	<RNI	
Vitamins										
Vitamin A (µg RE)	10.5	28.1	51.7	13.1	31.9	52.3	8.8	25.8	51.3	0.786
Vitamin B2 (mg)	6.8	10.9	26.0	3.6	11.4	23.5	8.8	9.9	27.5	0.214
Vitamin B6 (mg)	-	-	27.1	-	-	20.9	-	-	31.0	0.002
Folate (µg)	3.4	22.1	46.4	1.3	13.5	30.3	4.7	27.4	56.4	<0.001
Vitamin B12 (µg)	9.6	13.6	17.5	5.0	8.0	9.9	12.4	17.1	22.2	<0.001
Vitamin C (mg)	4.2	19.0	34.1	2.6	17.4	30.5	5.1	20.0	36.3	0.095
Vitamin D (µg)	-	-	95.3	-	-	94.4	-	-	95.9	0.313
Minerals										
Calcium (mg)	5.7	19.4	44.6	3.3	14.8	31.9	5.7	22.3	52.6	<0.001
Iron (mg)	5.7	25.0	49.6	2.3	4.4	29.6	7.8	33.3	62.0	<0.001
Magnesium (mg)	22.1	51.3	81.3	22.2	50.0	71.2	22.0	52.1	87.6	<0.001
Potassium (mg)	30.0	-	87.5	18.9	-	77.2	36.9	-	93.9	<0.001
Sodium (mg)[3]	0.0	-	17.8	0.0	-	10.9	0.0	-	22.0	<0.001
Selenium (µg)	52.7	-	85.9	37.5	-	83.6	62.2	-	87.3	0.145
Zinc (mg)	10.2	32.0	60.3	11.2	31.6	60.9	9.6	32.3	59.2	0.625

RNI, Reference Nutrient Intake; EAR, Estimated Average Intake; LRNI, Lower Reference Nutrient Intake; RE, Retinol Equivalents.

RNI, EAR and LRNI were taken from the UK dietary reference values for people aged 50 and over, except for vitamin D which is set for older adults [(6)].

[1] Does not include supplements.

[2] Chi-squared test ($\chi 2$) for no sex difference in percentage below RNI.

[3] Does not include table salt and salt used for cooking.

4.6. Summary

A number of socioeconomic, biological and lifestyle characteristics change with advancing age and place very old adults at increased risk of micronutrient deficiencies. The aim of this chapter was to assess vitamin and mineral intake and respective food sources in 793 eighty-five-year-olds (302 men and 491 women) in the North-East of England, participating in the Newcastle 85+ Study. Micronutrient intakes were estimated using a multiple pass recall tool (2x24hr recalls). Determinants of micronutrient intake were assessed with multinomial logistic regression. Median vitamin D, calcium and magnesium intakes were 2.0 (IQR: 1.2-6.5) μg/day, 731 (IQR: 554-916) mg/day and 215 (IQR: 166-266) mg/day, respectively. Iron intake was 8.7 (IQR: 6.7-11.6) mg/day and selenium intake was 39.0 (IQR: 27.3-55.5) μg/day. Cereals and cereal products were the top contributors to intakes of folate (31.5%), iron (49.2%) and selenium (46.7%) and the second biggest contributors to intakes of vitamin D (23.8%), calcium (27.5%) and potassium (15.8%). More than 95% (n=756) of the participants had vitamin D intakes below the UK's Reference Nutrient Intake (10 μg/d). Twenty percent or more of the participants were below the Lower Reference Nutrient Intake for magnesium (n=175), potassium (n=238) and selenium (n=418) (comparisons to dietary reference values (DRVs) do not include supplements). Since most DRVs are not age-specific and have been extrapolated from younger populations, results should be interpreted with caution. Participants with higher education, from higher social class and more physically active had more nutrient-dense diets. More studies are needed to inform the development of age-specific DRVs for micronutrients for the very old.

Very old adults are at increased risk of folate and vitamin B12 deficiencies due to reduced food intake and gastrointestinal absorption. Chapter 5 will explore folate and vitamin B12 status, the associations between the top contributing dietary sources of folate and vitamin B12 and status, and whether high dietary intakes of both vitamins are associated with reduced risk of "inadequate" status.

CHAPTER FIVE

5. Intakes of folate and vitamin B12 and biomarkers of status in the very old

[Mendonça N *et al.* (2016) Intakes of folate and vitamin B12 and biomarkers of status in the very old: The Newcastle 85+ Study. *Nutrients*. 8(10), 604.]

Key words: 'aged 80 and over'; Newcastle 85+ Study; red blood cell folate; vitamin B12; FUT2; MTHFR; food groups.

5.1. Abstract

Very old adults are at increased risk of folate and vitamin B12 deficiencies due to reduced food intake and gastrointestinal absorption. The main aim was to determine the association between folate and vitamin B12 intake from total diets and food groups, and status. Folate or vitamin B12 intakes (2 x 24 h multiple pass recalls) and red blood cell (RBC) folate or plasma vitamin B12 (chemiluminescence immunoassays) concentrations were available at baseline for 731 participants aged 85 from the Newcastle 85+ Study (North-East England). Generalized additive and binary logistic models estimated the associations between folate and vitamin B12 intakes from total diets and food groups, and RBC folate and plasma B12. Folate intake from total diets and cereal and cereal products (grains) was strongly associated with RBC folate ($p < 0.001$). Total vitamin B12 intake was weakly associated with plasma vitamin B12 ($p = 0.054$) but those with higher intakes from total diets or meat and meat products were less likely to have deficient status. Women homozygous for the *FUT2* G allele had higher concentrations of plasma vitamin B12. Cereals and cereal products are a very important source of folate in the very old. Higher intakes of folate and vitamin B12 lower the risk of "inadequate" status.

5.2. Introduction

There is conflicting evidence about relationships between folate and vitamin B12 intake and, folate and vitamin B12 status, respectively, in older adults. Some studies report a significant association between folate and vitamin B12 intake and status in older adults [83,126-130] while others do not [131-133]. In light of concerns about dietary inadequacy, it is imperative to assess folate and vitamin B12 status in older people, particularly the very old (85 years and older). The aims were to determine i) the prevalence of "inadequate" folate and vitamin B12 intake and status in the Newcastle 85+ Study, ii) the associations between the top contributing dietary sources of folate and vitamin B12, and status, and iii) whether high dietary intakes of both vitamins are associated with reduced risk of "inadequate" status.

5.3. Methods

Participants

This chapter uses data from the Newcastle 85+ Study and details were reported in *Chapter 2, General Methods - 2.1 The Newcastle 85+ Study*.

Dietary assessment and food groups

Dietary intake assessment and food allocation to tertiary food groups was described on *Chapter 2, General Methods - 2.2 Dietary assessment and food group allocation*. The top three food group contributors to folate or vitamin B12 intakes (accounted for > 50% of total intake) were included in the analysis. These food groups were also widely consumed by this population and, therefore a possible target for public health policies/ fortification. Information on supplement use was limited to type and brand and, therefore, this was only used as a dichotomous covariate (yes/no) [188].

Nutritional biomarkers and single nucleotide polymorphisms

Blood samples collection, RBC folate and plasma vitamin B12 analyses, and genotyping were described in *Chapter 2, General Methods - 2.3 Blood collection, one-carbon metabolism biomarkers and genotyping.* Complete dietary intake data, and RBC folate and plasma vitamin B12 concentrations were available for 731 and 732 participants, respectively. The single nucleotide polymorphisms (SNP) in the *MTHFR* (rs1801133, chromosome 1, position 11796321), *FUT2* (rs492602, chromosome 19, position 48703160), *MTR* (rs1805087, chromosome 1, position 236885200) and *TCN1* (rs526934, chromosome 11, position 59866020) genes were chosen as candidate modifiers of RBC folate and plasma vitamin B12 concentrations. All SNPs were assessed for deviation from the Hardy-Weinberg equilibrium.

Statistical analysis

Linearity and homoscedasticity assumptions were tested with residuals versus predicted values plots. Differences between RBC folate and plasma vitamin B12 concentrations according to *MTHFR* (rs1801133), *MTR* (rs1805087), *TCN1* (rs526934) and *FUT2* (rs492602) genotype were assessed using Kruskal Wallis tests followed by Dunn-Bonferoni tests if the null hypothesis was rejected.

The semi-parametric generalized additive models (gam) were investigated in R version 3.0.1 (R foundation for statistical computing) using the package "gam" and used to plot the relationship between vitamin intakes (thin plate regression splines for smoothing) and corresponding biomarkers. The generated reference value of zero in the y-axis corresponds to the RBC folate/ plasma vitamin B12 concentrations for the mean intake of folate and vitamin B12, respectively. Odds ratio (OR) (and 95% confidence interval (CI)) of RBC folate concentrations <600 nmol/L and plasma B12 <148 pmol/L according to quartiles of folate and vitamin B12 intake from total diets and top contributing food groups were computed using binary logistic regression. The commonly used cut-off to define folate deficiency of RBC folate <340 nmol/L [89] could not be used for these models due to the low percentage of deficiency among study participants. Gam and binary logistic regression models were adjusted for sex, energy intake, folic acid or vitamin B12 containing supplement use, folate/ vitamin B12 intake from other food groups*, MTHFR* or *FUT2* genotype. The vitamin B12 models were additionally adjusted for H_2 receptor antagonists, biguanides and proton pump inhibitor (PPI) use. General statistical methods are presented in *Chapter 2, General Methods - 2.5 General statistical analysis.*

5.4. Results

Folate and vitamin B12 intake and status "inadequacies"

Although 43% of participants (n=335) consumed one or more supplements on a regular basis [188], only 4.8% were users of folic acid and vitamin B12 as part of multivitamin supplements (**Table 5.1**). A low percentage of participants (3.1%) had folate intakes below the UK LRNI (100 µg/d) [69] or had RBC folate concentrations below the classic cut-off for deficiency of 340 nmol/L (3.6%) [89]. Folate intake and status were "inadequate" in only five participants. Cereals and cereal products, vegetables and fruit and fruit juice were the top food group contributors to folate intake, explaining almost 60% of total folate intake. Vitamin B12 intakes were below the UK LRNI (1 µg/d) [69] in 9.2% (n=67) of the population while 17.1% (n=125) were below 148 pmol/L of plasma vitamin B12 [112] (110 of these 125 had also total homocysteine concentrations > 15 µmol/L). In addition, 17 participants had "inadequate" intakes as well as deficient plasma concentrations of vitamin B12. There were twice as many women with vitamin B12 intakes below the UK LRNI than men (5.0% vs. 12.4%, p<0.001) but not with plasma vitamin B12 concentrations <148 pmol/L (17.4% vs.

16.9%, p=0.238). Eighty-six percent (n=628) of the participants had plasma vitamin B12 concentration <400 pmol/L, a concentration that has been associated with high total homocysteine and methylmalonic acid concentrations [189,190]. Intake of the top three food groups (meat, fish and dairy) explained more than 80% of total vitamin B12 intake.

Folate, vitamin B12 status and genotype

RBC folate and plasma vitamin B12 concentrations according to *FUT2, MTHFR, MTR and TCN1* and genotypes are shown in **Table 5.2**. Individuals with the *MTHFR* (rs1801133) AG or GA genotype [minor allele frequency (MAF) = 0.33 in the Newcastle 85+ Study vs. 0.32 for the A allele from the 1000 Genomes Project British population phase 3 [191]] had higher RBC folate concentrations than those homozygous for G (p=0.024). Participants with the *FUT2* (rs492602) GG genotype had higher concentrations of plasma vitamin B12 than other *FUT2* genotypes (p<0.001) [MAF=0.45 in the Newcastle 85+ Study vs. 0.48 for the G allele in residents of England and Scotland [191]]. The association between *FUT2* genotype and plasma vitamin B12 concentrations was significant in women (p<0.001) but not in men (p=0.140).

Association between folate intake and status

The associations between folate intake from all food sources, from cereals and cereal products, from fruit and fruit juice and from vegetables, and RBC folate concentrations are shown in **Figure 5.1** (gam model adjusted for sex, energy intake, *MTHFR* genotype, folate intake from the two other food groups and folic acid supplement use). Total folate intakes were associated with RBC folate (p<0.001). The steepest part of the dose-response curve appeared to be for folate intakes of 50-200 µg per day but RBC folate concentrations continued to increase with increasing folate intake up to ≈500 µg per day. Folate intake from cereals and cereal products, and from fruit and fruit juice were also associated with RBC folate concentrations (p<0.001 and p=0.014, respectively) (**Figure 5.1**)

Risk of low folate status by folate intake

Table 5.3 shows the odds ratios (and 95% CI) of low RBC folate (<600 nmol/L) according to total, cereals and cereal products, vegetables and, fruit and fruit juice folate intake quartiles. Individuals in the highest quartile of total folate intake (>264 µg/d) were less likely to have RBC folate concentrations <600 nmol/L than those in the lowest quartile (<157 µg/d) in the unadjusted model (OR: 0.58, 95% CI: 0.36, 0.94) and adjusted model (OR: 0.43, 95% CI:

0.23, 0.82). Individuals in higher quartiles of folate intake from cereals and cereal products and from vegetables were also less likely to have low RBC folate concentrations (<600 nmol/L) than those in quartile 1. The same was not true for folate intake from fruit and fruit juice in any model.

Association between vitamin B12 intake and status

Total vitamin B12 intake was weakly associated with plasma vitamin B12 concentrations while adjusting for sex, energy intake, vitamin B12 intake from the other two food groups, *FUT2* genotype, vitamin B12 supplement use and H_2 antagonists, biguanides or PPI use (p=0.054) (**Figure 5.2**). Plasma vitamin B12 concentration appeared to decrease when vitamin B12 intake exceeded 10 µg/d but the CI were very wide thereafter. Vitamin B12 intake from meat and meat products, milk and milk products and fish and fish dishes were not associated with plasma vitamin B12 concentration.

Risk of deficient vitamin B12 status by vitamin B12 intake

Participants with total vitamin B12 intake above the median (2.88 µg/d) were half as likely to be deficient for plasma B12 as those with the lowest intake (<1.87 µg/d) in the adjusted model (**Table 5.4**). Individuals in quartile 4 of vitamin B12 intake from meat and meat products (>2.10 µg/d) were also half as likely to be deficient for plasma vitamin B12 as those in quartile 1 in the unadjusted (OR: 0.55, 95% CI: 0.31-0.98) and adjusted models (OR: 0.41, 95% CI: 0.20-0.81). The same trend was present for milk and milk products but this did not reach statistical significance (p=0.054).

Table 5.1. Population characteristics, folate and vitamin B12 intakes and biomarkers of one carbon metabolism in the Newcastle 85+ Study.

	All	Men	Women	p-value[1]
Sex (%) (n)	732	39 (287)	61 (445)	-
BMI (kg/m^2) (mean±SD)	24.4±4.3	24.7±3.9	24.3±4.6	0.244[2]
Smokers (%) (n)	5.6 (41)	4.2 (12)	6.5 (29)	0.183
Alcohol Drinkers (%) (n)	72 (364)	84 (192)	62 (172)	<0.001
Total Energy Intakes (MJ/d)	6.78 (5.62-8.31)	8.01 (6.65-9.59)	6.26 (5.17-7.38)	<0.001
Folate and vitamin B12 supplement use (%) (n)	4.8 (35)	3.8 (11)	5.4 (24)	0.334
H_2 antagonists, PPI and biguanides use (%) (n)	26.8 (196)	27.2 (78)	26.5 (118)	0.844
Total Homocysteine (µmol/L)	16.7 (13.5-21.4)	18.0 (14.5-21.9)	16.1 (13.1-21.0)	0.001
>15 µmol/L (%) (n)	63.1 (471)	70.3 (206)	58.5 (265)	0.001
Folate				
Intake (µg/d)	209 (157-265)	246 (185-296)	189 (144-242)	<0.001
< 100 µg/d (%) (n)	3.1 (23)	0.7 (2)	4.7 (21)	0.002
Top food group contributors	Cereals (32%), Vegetables (16%), Fruit (9%)	Cereals (32%), Vegetables (15%), Fruit (8%)	Cereals (31%), Vegetables (17%), Fruit (10%)	-
Red Blood Cell Folate (nmol/L)	863 (451-1287)	868 (596-1282)	854 (614-1287)	0.728
<340 nmol/L (%) (n)	3.6 (26)	2.1 (6)	4.5 (20)	0.103
Vitamin B12				
Intake (µg/d)	2.9 (1.9-4.4)	3.5 (2.2-5.2)	2.5 (1.6-3.9)	<0.001
< 1.0 µg/d (%) (n)	9.2 (67)	4.5 (13)	12.1 (54)	<0.001
Top food group contributors	Meat (53%), Fish (17%), Milk (13%)	Meat (59%), Fish (16%), Milk (10%)	Meat (48%), Fish (19%), Milk (15%)	-
Plasma Vitamin B12 (pmol/L)	232 (170-324)	228 (166-309)	238 (174-337)	0.238
<148 pmol/L (%) (n)	17.1 (125)	17.4 (50)	16.9 (75)	0.841

BMI, body mass index; Cereals, Cereals and cereal products; Fruit, Fruit and fruit juice; Meat, Meat and meat products; Fish, Fish and fish dishes; Milk, Milk and milk products; PPI, proton pump inhibitors. Values are medians and IQR unless otherwise stated.

[1] No sex difference by Chi-squared test ($\chi 2$) for categorical or Mann-Whitney test for non-parametric continuous variables.

[2] Independent t-test.

Table 5.2. Plasma vitamin B12 and red blood cell folate concentrations by FUT2, MTHFR, MTR, and TCN1 genotypes in the Newcastle 85+ Study.

	RBC folate (nmol/L)	p-value[1]	Plasma vitamin B12 (pmol/L)	p-value[1]
FUT2 (rs492602)		0.531		<0.001
AA (n=128)	894 (629-1349		216 (146-281)	Ref.
A/G (n=308)	917 (603-1322)		221 (163-309)	0.413
GG (n=187)	835 (595-1206)		277 (209-381)	<0.001
MTHFR (rs1801133)		0.028		0.244
GG (n=276)	871 (614-1275)	Ref.	234 (168-331)	
A/G (n=279)	845 (584-1263)	1.000	230 (164-312)	
AA (n=67)	1010 (693-1626)	0.060	249 (193-339)	
MTR (rs1805087)		0.547		0.277
AA (n=419)	881 (613-1278)		240 (173-337)	
A/G (n=178)	845 (596-1332)		226 (162-297)	
GG (n=26)	1053 (580-1593)		247 (162-310)	
TCN1 (rs526934)		0.065		0.298
AA (n=331)	877 (606-1317)		237 (178-336)	
A/G (n=247)	845 (595-1223)		231 (160-325)	
GG (n=45)	1074 (630-1439)		222 (182-273)	

RBC folate, Red blood cell folate; *FUT2*, Fucosyltrasnferase 2; *MTHFR*, Methylenetetrahydrofolate reductase; *MTR*, Methionine synthase; *TCN1*, Transcobalamin 1. Ref., Reference used for post hoc comparisons.

[1] Kruskal Wallis test followed by Dunn-Bonferroni post-hoc test if the null hypothesis was rejected.

Table 5.3. Odds ratio (95% CI) of low RBC folate concentration according to quartiles of total folate intake and intakes from cereals and cereal products, from vegetables and from fruit and fruit juice in the Newcastle 85+ Study.

Folate intake	Model 1 (unadjusted)		Model 2 (adjusted)	
Total (μg/d)	<600 nmol/L (n=170)	p	<600 nmol/L (n=170)	p
<157	1.00 (ref)	-	1.00 (ref)	-
157-208	0.64 (0.40, 1.04)	0.071	0.65 (0.38, 1.09)	0.103
209-264	0.72 (0.45, 1.15)	0.173	0.58 (0.34, 1.02)	0.057
>264	0.58 (0.36, 0.94)	0.028	0.43 (0.23, 0.82)	0.010
Cereals and Cereal products (μg/d)	<600 nmol/L (n=170)	p	<600 nmol/L (n=170)	p
<36	1.00 (ref)	-	1.00 (ref)	-
36-59	0.96 (0.61, 1.49)	0.840	0.84 (0.51, 1.38)	0.493
59-92	0.40 (0.24, 0.66)	<0.001	0.32 (0.18, 0.57)	<0.001
>92	0.41 (0.25, 0.68)	0.001	0.33 (0.18, 0.61)	<0.001
Vegetables (μg/d)	<600 nmol/L (n=154)	p	<600 nmol/L (n=154)	p
<15	1.00 (ref)	-	1.00 (ref)	-
15-30	0.72 (0.43, 1.21)	0.212	0.49 (0.25, 0.95)	0.035
30-51	0.86 (0.52, 1.41)	0.550	0.59 (0.32, 1.08)	0.089
>51	0.79 (0.48, 1.30)	0.357	0.52 (0.28, 0.99)	0.045
Fruit and Fruit Juice (μg/d)	<600 nmol/L (n=127)	p	<600 nmol/L (n=127)	p
<7.3	1.00 (ref)	-	1.00 (ref)	-
7.3-16	0.90 (0.53, 1.52)	0.682	1.01 (0.56, 1.83)	0.979
16-34	0.61 (0.35, 1.07)	0.086	0.67 (0.36, 1.25)	0.213
>34	0.76 (0.44, 1.31)	0.329	0.79 (0.43, 1.44)	0.437

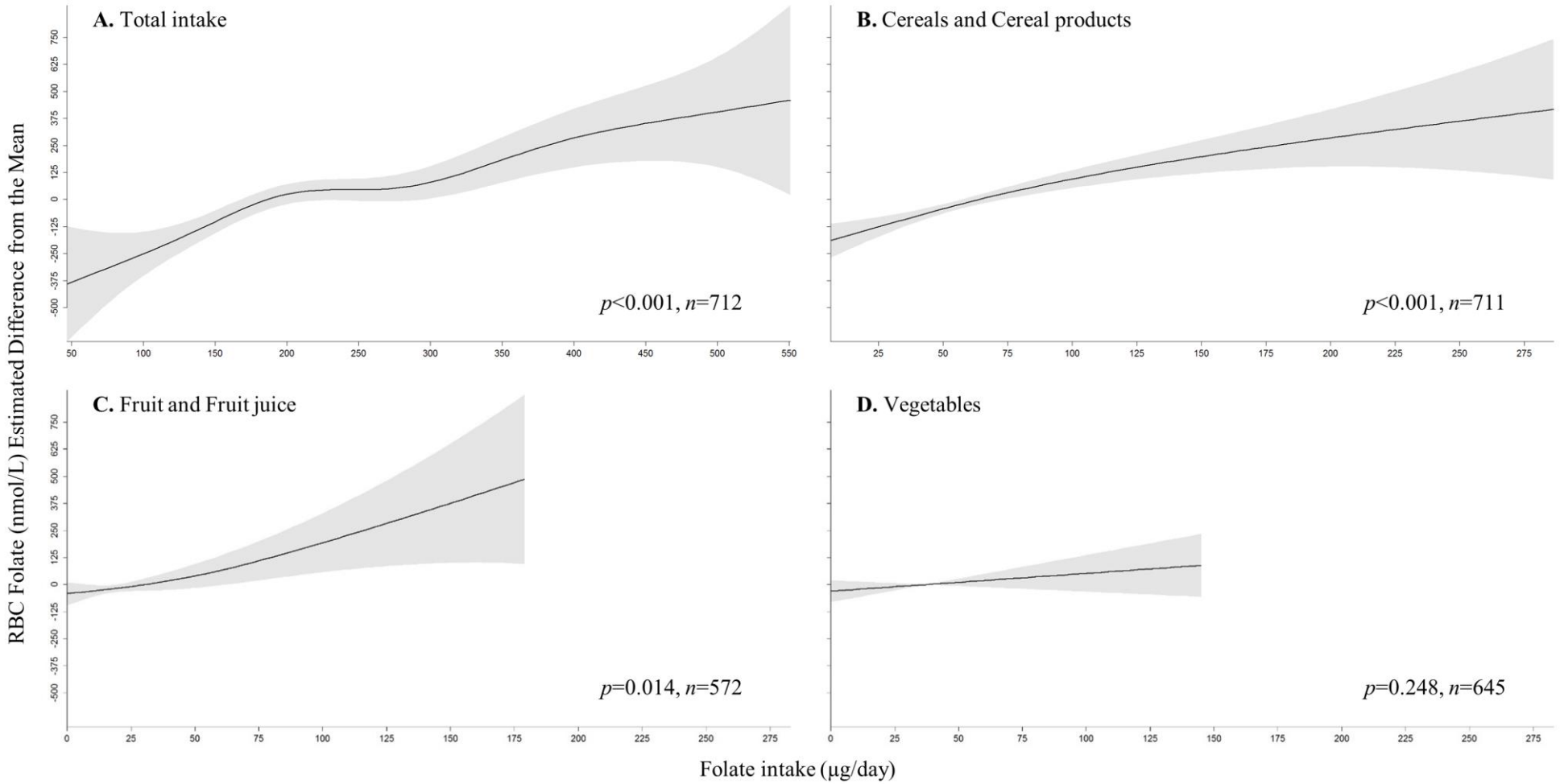
RBC folate, Red blood cell folate; p, p-value. Low folate status was defined as RBC folate concentration <600 nmol/L. Binary logistic regression model. Model 1 is unadjusted and Model 2 is adjusted for sex, energy intake, folate intake from the other two food sources (except for total folate), *MTHFR* genotype and folic acid-containing supplement use.

Table 5.4. Odds ratio (95% CI) of plasma vitamin B12 deficiency according to quartiles of intake of total vitamin B12 and intakes from meat and meat products, from fish and fish products, and from milk and milk products in the Newcastle 85+ Study.

Vitamin B12 intake	Model 1 (unadjusted)		Model 2 (adjusted)	
Total (µg/d)	<148 pmol/L (n=125)	p	<148 pmol/L (n=125)	p
<1.87	1.00 (ref)	-	1.00 (ref)	-
1.87-2.88	0.70 (0.42, 1.18)	0.180	0.57 (0.32, 1.01)	0.056
2.88-4.40	0.60 (0.35, 1.02)	0.057	0.50 (0.28, 0.92)	0.026
>4.40	0.53 (0.31, 0.92)	0.024	0.40 (0.21, 0.76)	0.005
Meat and Meat products (µg/d)	<148 pmol/L (n=118)	p	<148 pmol/L (n=118)	p
<0.35	1.00 (ref)	-	1.00 (ref)	-
0.35-1.03	0.72 (0.42, 1.24)	0.236	0.69 (0.38, 1.25)	0.220
1.03-2.10	0.84 (0.50, 1.44)	0.533	0.78 (0.43, 1.42)	0.422
>2.10	0.55 (0.31, 0.98)	0.043	0.41 (0.20, 0.81)	0.010
Fish and Fish products (µg/d)	<148 pmol/L (n=43)	p	<148 pmol/L (n=43)	p
<0.46	1.00 (ref)	-	1.00 (ref)	-
0.46-1.06	0.61 (0.23, 1.65)	0.331	0.66 (0.23, 1.91)	0.444
1.06-2.45	0.86 (0.34, 2.15)	0.743	0.66 (0.23, 1.86)	0.427
>2.45	1.00 (0.41, 2.42)	0.992	0.70 (0.25, 1.97)	0.503
Milk and Milk products (µg/d)	<148 pmol/L (n=102)	p	<148 pmol/L (n=102)	p
<0.27	1.00 (ref)	-	1.00 (ref)	-
0.27-0.53	0.84 (0.47, 1.52)	0.562	0.88 (0.46, 1.71)	0.711
0.53-0.88	1.12 (0.64, 1.96)	0.698	1.28 (0.70, 2.37)	0.425
>0.88	0.58 (0.31, 1.08)	0.086	0.49 (0.24, 1.01)	0.054

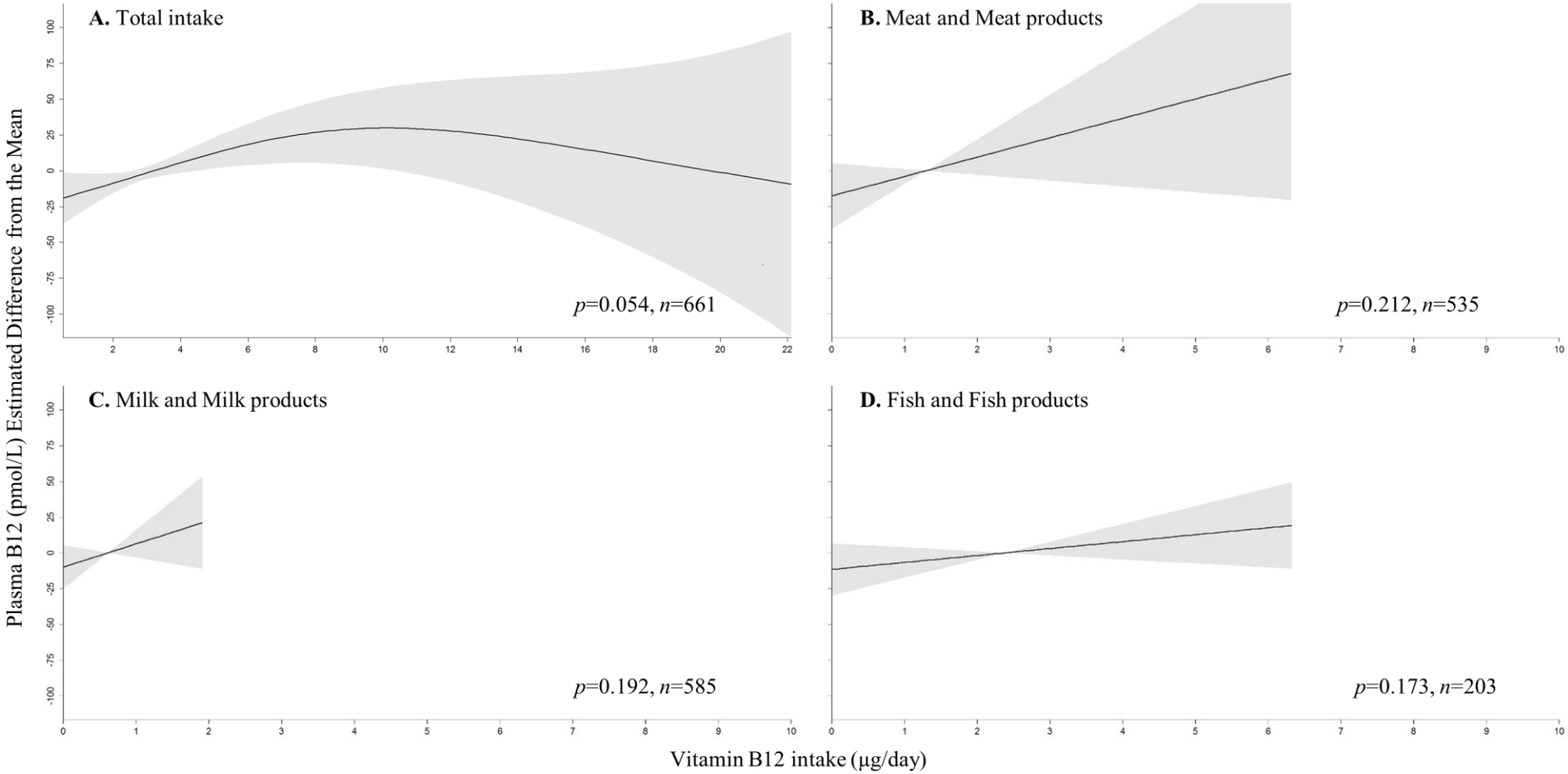
P, P-value. Binary logistic regression model. Deficient plasma vitamin B12 concentration was defined as <148 pmol/L. Model 1 is unadjusted and Model 2 is adjusted for sex, energy intake, *FUT2* genotype, vitamin B12 intake from the other two food sources (except total vitamin B12 intake) (e.g. the meat and meat products model is adjusted for fish and fish dishes, and milk and milk products), vitamin B12 containing supplement use, H_2 antagonists, biguanides and proton pump inhibitors use.

Figure 5.1. Estimated difference from the mean (and 95% CI) of RBC folate concentration according to folate intake from all dietary sources, from cereals and cereal products, from fruit and fruit juice and from vegetables.



Generalized additive model (gam) adjusted for sex, energy intake, MTHFR genotype, folic acid supplement use and folate intake from the two other food sources (e.g. the cereals and cereal products model is adjusted for fruit and fruit juice, and vegetables). The highest 2.5th percentiles of RBC folate concentrations are not included. Three participants with a folate intake above 150 µg only from vegetables were not included. One participant with a folate intake of 327 µg only from fruit and fruit juice was excluded. P values are from the corresponding gam model.

Figure 5.2. Estimated difference from the mean (and 95% CI) of plasma B12 concentrations according to vitamin B12 intake from all dietary sources, from meat and meat products, from milk and milk products and from fish and fish products.



Generalized additive model (gam) adjusted for sex, energy intake, *FUT2* genotype, H_2 antagonists, proton pump inhibitors or biguanides use, vitamin B12 supplement use and vitamin B12 intakes from the other two food sources (e.g. the meat and meat products model is adjusted for fish and fish dishes, and milk and milk products). The lowest and highest 2.5th percentiles of vitamin B12 intakes and plasma vitamin B12 concentrations are not included except for meat and meat products where the highest 5th percentile was excluded. P values are from the corresponding gam model.

5.5. Summary

Very old adults are at increased risk of folate and vitamin B12 deficiencies due to reduced food intake and gastrointestinal absorption. The main aim was to determine the association between folate and vitamin B12 intake from total diets and food groups, and status. Folate or vitamin B12 intakes (2x24h multiple pass recalls) and red blood cell (RBC) folate or plasma vitamin B12 (chemiluminescence immunoassays) concentrations were available at baseline for 731 participants aged 85 from the Newcastle 85+ Study (North-East England). Generalized additive and binary logistic models estimated the associations between folate and vitamin B12 intakes from total diets and food groups, and RBC folate and plasma B12. Folate intake from total diets and cereal and cereal products was strongly associated with RBC folate ($p<0.001$). Total vitamin B12 intake was weakly associated with plasma vitamin B12 ($p=0.054$) but those with higher intakes from total diets or meat and meat products were less likely to have deficient status. Women homozygous for the *FUT2* G allele had higher concentrations of plasma vitamin B12. Cereals and cereal products are a very important source of folate in the very old. Higher intakes of folate and vitamin B12 lower the risk of "inadequate" status.

Chapter 6 will determine the associations between RBC folate, plasma vitamin B12 and tHcy concentrations at baseline, and cognitive impairment and the rate of cognitive decline in global and attention-specific cognition over 5 years in the very old.

CHAPTER SIX

6. One-carbon metabolism biomarkers and cognitive decline in the very old

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Key words: 'aged, 80 and over', Newcastle 85+, cognition, folate, B12, homocysteine

6.1. Abstract

Although the biological rationale for the association between folate, vitamin B12, and homocysteine with cognitive function seems plausible, conflicting results have been reported. This study aimed to determine the associations between 1-carbon (1-C) metabolism biomarkers (folate, vitamin B12, and homocysteine), and cognitive impairment at baseline and the rate of cognitive decline over 5 years in the very old. The Newcastle 85+ Study was a prospective longitudinal study of people 85 years old and followed over 5 years in North-east England. The analytical sample included 765 community-dwelling and institutionalized very old participants with 1-C metabolism biomarkers and cognitive measures. Global cognition was measured by the Standardized Mini-Mental State Examination (SMMSE) at baseline, and at 3 and 5 years of follow-up and, attention-specific cognition with the Cognitive Drug Research (CDR) System at baseline, and at 1.5 and 3.0 years of follow-up. Baseline red blood cell folate (RBC folate), plasma vitamin B12, and total homocysteine (tHcy) concentrations were determined by immunoassay. Linear mixed models were used to estimate the associations between quartiles of 1-C metabolism biomarkers and cognition over 3 (CDR) and 5 years (SMMSE). Compared with participants in the lowest quartile of RBC folate concentrations (<612 nmol/L), those in the highest quartile of RBC folate concentrations (>1280 nmol/L) had 1 more point on the SMMSE (β=+1.02, SE=0.43, P=0.02). Those in quartile 4 of tHcy (>21.4 mmol/L) had 1 point less in the SMMSE at baseline than those in the lowest quartile (<13.5 mmol/L) (β=-1.05, SE=0.46, P=0.02). Plasma vitamin B12 was not predictive of global or attention-specific cognition at baseline and at follow-up. None of the 1-C metabolism biomarkers except tHcy was associated with the rate of decline in attention scores over 3 years. RBC folate and tHcy, but not plasma vitamin B12, were associated with better global cognition in the very old at baseline but were not predictive of rate of decline over 5 years.

6.2. Introduction

Although the biological rationale for associations between folate, vitamin B12 and homocysteine with cognitive function seem plausible, conflicting results have been reported. Folate, vitamin B12 and homocysteine have been associated with cognitive decline in some longitudinal studies [82,123,143] but not all [144-146], in some randomized controlled trials (RCTs)

[83,147], for one vitamin but not the other or only for certain cognitive domains. Insufficient follow-up time, small sample size, participants' age at recruitment and different cognitive tests used are frequently reported reasons for the conflicting results. Furthermore, studies targeting the very old are lacking. We hypothesized that higher red blood cell (RBC) folate and plasma vitamin B12 concentrations would be associated with better cognitive performance and slower rate of cognitive decline, primarily through homocysteine-lowering effects. This study aimed to determine the associations between RBC folate, plasma vitamin B12 and total homocysteine (tHcy) concentrations at baseline, and cognitive impairment and the rate of cognitive decline in global and attention-specific cognition over 5 years in a large population of the very old who participated in the Newcastle 85+ Study.

6.3. Methods

Participants

This chapter uses data from the Newcastle 85+ Study and details were reported in *Chapter 2, General Methods - 2.1 The Newcastle 85+ Study*.

Biomarkers of 1-C metabolism

Blood samples collection, RBC folate, plasma vitamin B12 and tHcy analyses, and genotyping were described in *Chapter 2, General Methods - 2.3 Blood collection, one-carbon metabolism biomarkers and genotyping.*

Cognitive assessment

The Standardized Mini-mental State Examination (SMMSE) was used to assess global cognitive status at baseline, 3- (36 months) and 5-year (60 months) follow-up. The SMMSE is a short-standardised screening test for cognitive impairment in older adults that ranks global cognitive function from 0 to 30. Individuals with SMMSE scores ≥26 were considered as normal and <26 as cognitively impaired [192]. Three automated tests of attention from the Cognitive Drug Research (CDR) System were used to assess cognition at baseline and again after 1.5- (18 months) and 3-years (36 months). The tests were simple reaction time (SRT), which assesses focussed attention and concentration; choice reaction time (CRT) which assessed similar abilities in addition to information processing and decision making; and the digit vigilance task (DVT) that assesses the ability to sustain attention [193]. Using the

measures of speed and accuracy from the tasks, three validated composite measures were derived: power of attention (PoA), the sum of three speed scores which reflects the ability to focus attention and the intensity of concentration; reaction time variability (RTV) which is a sum of the coefficients of variance of the reaction time scores and reflects variations in attention during the tasks; and continuity of attention (CoA) which combines the accuracy scores from CRT and DVT and reflects the ability to sustain attention [30,194]. All three of these composite scores have been validated previously [195,196]. Higher scores in the SMMSE and the CoA, and lower scores for the PoA and RTV measures reflect superior performance (**Appendix F**).

Other confounders

Details on genotyping were described in *Chapter 2, General Methods - 2.3 Blood collection, one-carbon metabolism biomarkers and genotyping.* The gene encoding apolipoprotein E (*APOE*) was genotyped for common polymorphisms at rs429358 and rs7412 (to provide information on zygosity at ε2, ε3 and ε4 alleles). Details on the variables recorded by the multidimensional health questionnaires and the GP medical records review were also described in *Chapter 2, General Methods - 2.4 Other socioeconomic, health and lifestyle variables*.

Statistical analysis

General statistical methods are presented in the *Chapter 2, General Methods - 2.5 General statistical analysis.* Linearity assumptions were tested with residuals versus predicted values plots. Multicollinearity of confounders was assessed with variance inflation factor, tolerance and eigenvalues. To account for within-person variability and missing values, the longitudinal effects of RBC folate, plasma vitamin B12 and tHcy on cognitive function were assessed by linear mixed models. Random effects terms included both intercept and slopes of SMMSE and attention scores with the time in the study coded as 0 (baseline), 1 (1.5 or 3 years) or 2 (3 or 5 years), respectively. Parameters (β coefficients) were estimated by the maximum likelihood method and the model followed an autoregressive heterogeneous covariance structure. RTV was logarithmically (ln) transformed to correct a positive skew and aid convergence. Other attention scores (PoA and CoA) were not transformed because the distribution of the residuals of each respective linear mixed model was roughly normal and did not interfere with convergence. An interaction between the biomarker and time was added to assess the rate of cognitive decline.

Binary logistic regression models were fitted to predict cognitive impairment (SMMSE≤25) at baseline and incident cognitive impairment after 3 and 5 years. All models were adjusted for sex, *APOE* e4 genotype (rs429358 and rs7412), hypertension, diabetes type 1 and 2, history of cardiovascular diseases, depression, alcohol intake, smoking status, physical activity, years of full time education, BMI, homocysteine in the folate and vitamin B12 models and renal impairment in the homocysteine models.

In sensitivity analyses, all models were re-run after excluding participants diagnosed with Alzheimer's disease/dementia or living in institutions at baseline, use of folic acid/ vitamin B12 containing supplements, with RBC folate concentrations > 4000 nmol/L, plasma vitamin B12 concentrations >1000 pmol/L or tHcy concentrations >40 µmol/L.

6.4. Results

Population characteristics by 1-C metabolism biomarkers

1-C metabolism biomarkers and SMMSE data were available for 752-765 participants at baseline, 414-450 at 3 years and 311-318 at 5 years. Attention-specific scores (CDR) were available for 705-718 participants at baseline, 528-538 at 1.5 years and 391-400 at 3 years depending on the 1-C metabolism assay used. The median concentration for tHcy, RBC folate and plasma vitamin B12 was 16.7 µmol/L (IQR:13.5-21.4), 863 (IQR:451-1287) nmol/L and 232 pmol/L (IQR:170-324), respectively in the Newcastle 85+ Study [(169)]. A concentration >15 µmol/L of tHcy is commonly used to define hyperhomocysteinemia and, concentrations <340 nmol/L and <148 pmol/L to define inadequacy of RBC folate and plasma vitamin B12, respectively. Sixty-three percent of the cohort (n=482) had tHcy concentrations >15 µmol/L, 4% (n=26) had RBC folate concentrations <340 nmol/L and 17% (n=125) had plasma vitamin B12 concentrations < 148 pmol/L [(169)]. **Table 6.1-Table 6.3** show the population characteristics at baseline and cognitive scores at follow-up by quartile of RBC folate (Q1: <612, Q2: 612-870, Q3: 870-1280, Q4: >1280 nmol/L), plasma vitamin B12 (Q1: >170, Q2: 170-232, Q3: 232-325, Q4: >325 pmol/L) and tHcy (Q1: >13.5, Q2: 13.5-16.7, Q3: 16.7-21.4, Q4: >21.4 µmol/L), respectively. There were fewer *APOE* ε4 carriers in quartile 2 (17%) than in other quartiles (28-32%) of RBC folate (p=0.002), and fewer individuals in quartile 1 with history of cardiovascular diseases (45%) than in other quartiles (57%-63%) (p<0.001). As RBC folate increased, so did plasma vitamin B12 and vice-versa. There was an inverse association between tHcy and the other 1-C metabolism biomarkers. There were more men in tHcy

quartile 3 (50%) than in other quartiles (p<0.001). Furthermore, participants in quartile 4 of tHcy concentrations (>21.3 µmol/L) were less physically active and more likely to be renally impaired compared with those in other quartiles (**Table 6.3**).

Longitudinal associations with cognitive performance

Participants lost to follow-up (died or unable to complete any cognitive test) after 5 years (n=501) had slightly higher median tHcy (p=0.01) and plasma vitamin B12 concentrations (p=0.05), were more likely to be *APOE* ε4 carriers (p=0.01), less likely to drink alcohol (p<0.001), less physically active (p<0.001), more likely to have a history of CVD (p=0.003), diabetes type 1 and 2 (p=0.01) and Alzheimer's disease/dementia (p<0.001), and more likely to live in institutions (p<0.001) at baseline compared with those who had SMMSE data 5 years later. Furthermore, more participants lost to follow-up during every phase were cognitively impaired than those who continued on the study.

Table 6.4 shows the associations between RBC folate, plasma vitamin B12 and tHcy quartiles and attention specific and global cognitive decline over 3 and 5 years, respectively using linear mixed models.

Participants in the highest quartile of RBC folate concentrations had 1 more point on the SMMSE at baseline than those in quartile 1 (β=+1.02, SE=0.43, p=0.02) after adjustment for sex, alcohol intake, smoking status, *APOE* genotype, education, BMI, depression, diabetes type 1 and 2, hypertension, history of cardiovascular disease, physical activity and tHcy (**Table 6.4**). Plasma vitamin B12 concentration measured at baseline was not predictive of global cognition (SMMSE) in the non-adjusted or fully adjusted models. Conversely, participants in the highest quartile of tHcy had 1 point less on the SMMSE score than those in the lowest quartile at baseline (β=-1.05, SE=0.46, p=0.02). Folate, vitamin B12 and tHcy were not predictive of any attention-specific measures (PoA, CoA and RTV) over 3 years (**Table 6.4**).

Prevalent and incident cognitive impairment

Binary logistic regression models, adjusted for the same covariates, showed that participants in quartile 4 of tHcy had an increased risk (OR: 2.15, CI: 1.12-4.11, p=0.02) of prevalent cognitively impairment (defined as SMMSE≤25 points in SMMSE) than those in quartile 4, but not for incident impairment after 3 or 5 years (**Supplemental Table 6.1**).

Rate of cognitive decline by 1-C metabolism biomarkers

All domains of cognitive performance declined significantly (lower scores of SMMSE and CoA and higher scores in PoA and RTV) with time. SMMSE and CoA decreased on average by 1.68 (SE: 0.18, p<0.001) and by 1.27 ms (SE: 0.3, p<0.001) respectively, PoA and RTV (ln) increased by 105 ms (SE:15, p<0.001) and by 0.021 (SE:0.008), p=0.01) respectively, for every phase. There were no significant changes in the rate of global cognitive decline (SMMSE) by quartiles of RBC folate, plasma vitamin B12 and tHcy (**Table 6.4**). There were also no significant differences in the rate of decline in attention-specific scores (CDR) except for a slower decline in focused attention (PoA) (e.g. Q4 vs. Q1: β=-100 SE=44, p=0.02) and a trend for slower decline of sustained attention (CoA) (e.g. Q4 vs Q1: β=+1.58 SE=0.87, p=0.07) in the higher quartiles of tHcy concentration (**Table 6.4** and **Supplemental Figure 6.1**-**Supplemental Figure 6.3**).

Sensitivity analysis

Sensitivity analyses excluding those with diagnosed Alzheimer's disease/dementia (n=52-56), living in institutions at baseline (n=61-62), use of folic acid or vitamin B12 containing supplements (n=35-37), with RBC folate concentrations >4000 nmol/L (n=6), plasma vitamin B12 concentrations >1000 pmol/L (n=16) or tHcy concentrations >40 μmol/L (n=16) did not generally change the results. However, higher homocysteine quartiles were significantly associated with higher scores in PoA (poorer performance) over 5 years when participants diagnosed with dementia or Alzheimer's disease at baseline were excluded. The interaction between quartiles of tHcy concentration and time for focused attention (PoA) was no longer present when these same participants were excluded from the analysis.

Table 6.1. Population characteristics and cognitive test scores at baseline and at follow-up in the Newcastle 85+ Study by quartile of red blood cell folate concentration.

	Q1 (<612 nmol/L**)**	**Q2** (612-870 nmol/L)	**Q3** (870-1280 nmol/L)	**Q4** (>1280 nmol/L)	p^1
Men (%, n)	41 (77)	38 (72)	40 (75)	38 (72)	0.86
BMI (kg/m^2) (mean, SD)	24.2 (4.3)	24.7 (4.5)	24.3 (4.1)	24.4 (4.3)	0.78[2]
APOE ε4 carriers (%, n)	32 (49)	17 (25)	30 (47)	28 (42)	0.02
Plasma Vitamin B12 (pmol/L)	201 (135-280)	216 (159-275)	259 (193-371)	278 (205-391)	<0.001
Total Homocysteine (µmol/L)	19.9 (16.3-24.6)	18.3 (14.9-22.9)	15.6 (13.0-19.6)	13.8 (11.1-17.4)	<0.001
Alcohol Drinkers (%, n)	70 (92)	73 (94)	80 (96)	66 (89)	0.10
Smokers (%, n)	9 (16)	4 (8)	4 (8)	5 (10)	0.35
Physical Activity (High) (%, n)	38 (71)	33 (62)	34 (63)	37 (68)	0.75
Education (≥12 y) (%, n)	11 (20)	11 (20)	14 (25)	14 (26)	0.18
History of Cardiovascular disease (%, n)	45 (84)	57 (108)	66 (123)	63 (119)	<0.001
Diabetes type 1 or 2 (%, n)	9 (16)	16 (31)	13 (25)	17 (32)	0.08
Hypertension (%, n)	54 (100)	59 (112)	54 (101)	65 (122)	0.12
Renal Impairment (%, n)	20 (38)	26 (49)	22 (41)	28 (52)	0.36
Depression (Severe) (%, n)	4 (7)	10 (19)	11 (19)	9 (16)	0.25
Global cognition (SMMSE)					
Baseline	27 (24-29)	28 (25-29)	28 (25-29)	28 (25-29)	0.17
Impaired (≤25) (%, n)	31 (58)	25 (48)	26 (49)	25 (48)	0.52
3 years	27 (23-29)	28 (24-29)	26 (23-29)	27 (25-29)	0.19
Impaired (≤25) (%, n)	37 (41)	35 (35)	40 (48)	25 (27)	0.10
5 years	28 (23-29)	27 (23-29)	27 (23-28)	28 (25-29)	0.17
Impaired (≤25) (%, n)	32 (26)	37 (27)	43 (33)	26 (21)	0.16
Focused attention (PoA, ms)					
Baseline	1499 (1341-1691)	1494 (1371-1752)	1484 (1360-1719)	1473 (1341-1667)	0.54
1.5 years	1546 (1398-1745)	1538 (1377-1740)	1533 (1399-1731)	1515 (1366-1718)	0.84
3 years	1594 (1409-1777)	1567 (1378-1775)	1550 (1420-1782)	1490 (1367-1688)	0.16

	Q1 (<612 nmol/L**)**	**Q2** (612-870 nmol/L)	**Q3** (870-1280 nmol/L)	**Q4** (>1280 nmol/L)	p^1
Sustained attention (CoA, ms)					
Baseline	87.8 (80.7-91.4)	87.5 (80.8-91.0)	85.7 (77.0-91.0)	87.7 (82.3-91.3)	0.20
1.5 years	86.6 (79.4-90.4)	88.0 (81.9-91.6)	87.0 (78.4-90.5)	86.5 (80.8-90.9)	0.24
3 years	86.7 (81.1-91.0)	86.8 (79.4-90.7)	86.4 (78.6-90.7)	87.0 (82.2-91.5)	0.73
Reaction time variability (RTV)					
Baseline	59.1 (50.6-71.4)	61.2 (54.6-71.7)	61.5 (53.2-73.1)	57.6 (50.3-68.6)	0.06
1.5 years	60.0 (53.8-71.0)	60.0 (52.1-68.2)	60.2 (52.2-72.0)	57.6 (52.1-67.0)	0.68
3 years	59.9 (50.8-70.0)	59.8 (49.4-69.6)	61.4 (51.8-72.3)	59.3 (50.3-66.7)	0.60

Lower scores in PoA and RTV and, higher scores in CoA reflect better performance. Continuous variables are presented as medians and IQR unless otherwise stated. History of cardiovascular disease includes cardiac, cerebrovascular and peripheral vascular diseases. [1] No quartile difference by Chi-squared test ($\chi 2$) for categorical or by Kruskal-Wallis test for non-parametric continuous variables. [2] No BMI difference by one-way ANOVA. CoA, continuity of attention; IQR, interquartile range; ms, milliseconds; PoA, power of attention; Q, quartile; RTV, reaction time variability; SD, standard deviation; SMMSE, Standardized Mini-mental State Examination.

Table 6.2. Population characteristics and cognitive test scores at baseline and at follow-up in the Newcastle 85+ Study by quartile of plasma vitamin B12 concentration.

	Q1 (<170 pmol/L)	**Q2** (170-232 pmol/L)	**Q3** (232-325 pmol/L)	**Q4** (>325 pmol/L)	p^1
Men (%, n)	42 (79)	41 (77)	39 (74)	35 (66)	0.56
BMI (kg/m^2) (mean, SD)	25.1 (4.5)	23.8 (4.1)	25.0 (4.3)	23.7 (4.3)	0.001[2]
APOE ε4 carriers (%, n)	26 (40)	24 (38)	23 (35)	32 (50)	0.30
Red blood cell folate (nmol/L)	683 (479-992)	838 (605-1159)	913 (690-1393)	1058 (745-1608)	<0.001
Total Homocysteine (µmol/L)	19.7 (15.9-25.1)	17.3 (14.5-21.8)	15.9 (13.3-19.8)	13.9 (11.1-18.2)	<0.001
Alcohol Drinkers (%, n)	73 (103)	72 (91)	80 (91)	65 (86)	0.07
Smokers (%, n)	6 (11)	5 (10)	6 (12)	5 (9)	0.77
Physical Activity (High) (%, n)	38 (72)	37 (68)	34 (63)	33 (62)	0.55
Education (≥12 y) (%, n)	10 (19)	13 (23)	14 (27)	12 (22)	0.15
History of Cardiovascular disease (%, n)	51 (96)	60 (113)	62 (117)	57 (108)	0.10
Diabetes type 1 or 2 (%, n)	12 (23)	18 (33)	12 (22)	13 (25)	0.32
Hypertension (%, n)	58 (111)	54 (100)	61 (114)	59 (110)	0.55
Renal Impairment (%, n)	23 (44)	27 (51)	23 (44)	22 (41)	0.65
Depression (Severe) (%, n)	8 (14)	8 (14)	10 (18)	9 (15)	0.90
Dementia/ Alzheimer (%, n)	6 (12)	8 (14)	8 (15)	6 (11)	0.83
Global cognition (SMMSE)					
Baseline	28 (25-29)	27 (25-29)	28 (25-29)	28 (25-29)	0.73
Impaired (≤25) (%, n)	28 (53)	26 (49)	27 (50)	27 (51)	0.99
3 years	27 (23-29)	27 (25-29)	27 (24-29)	27 (24-29)	0.94
Impaired (≤25) (%, n)	40 (48)	32 (35)	31 (33)	35 (35)	0.47
5 years	27 (22-29)	27 (24-29)	27 (23-28)	28 (24-29)	0.95
Impaired (≤25) (%, n)	38 (33)	35 (28)	32 (24)	33 (23)	0.83
Focused attention (PoA, ms)					
Baseline	1502 (1351-1723)	1497 (1370-1710)	1482 (1356-1676)	1467 (1342-1705)	0.75
1.5 years	1537 (1370-1848)	1519 (1390-1799)	1548 (1402-1694)	1506 (1373-1725)	0.95
3 years	1503 (1361-1744)	1560 (1389-1778)	1555 (1425-1732)	1547 (1405-1757)	0.69

	Q1 (<170 pmol/L)	**Q2** (170-232 pmol/L)	**Q3** (232-325 pmol/L)	**Q4** (>325 pmol/L)	p^1
Sustained attention (CoA, ms)					
Baseline	87.8 (80.7-91.6)	86.8 (81.5-90.9)	87.5 (18.7-91.7)	86.6 (78.5-90.8)	0.54
1.5 years	86.8 (81.2-90.9)	87.3 (82.0-90.8)	87.9 (79.6-91.0)	85.8 (78.9-90.7)	0.63
3 years	86.9 (81.7-91.3)	87.5 (81.0-90.4)	88.3 (81.1-91.8)	85.3 (79.6-90.0)	0.09
Reaction time variability (RTV)					
Baseline	60.9 (52.0-70.4)	60.2 (51.0-70.3)	59.6 (52.2-69.6)	58.9 (51.2-72.2)	1.00
1.5 years	60.0 (51.2-67.7)	60.1 (53.7-70.8)	60.4 (52.1-68.3)	57.3 (51.1-68.8)	0.45
3 years	59.9 (49.5-67.4)	59.8 (51.5-71.6)	59.2 (51.3-67.8)	60.9 (50.6-71.5)	0.74

Lower scores in PoA and RTV and, higher scores in CoA reflect better performance. Continuous variables are presented as medians and IQR unless otherwise stated. History of cardiovascular disease includes cardiac, cerebrovascular and peripheral vascular diseases. [1] No quartile difference by Chi-squared test (χ2) for categorical or by Kruskal-Wallis test for non-parametric continuous variables. [2] No BMI difference by one-way ANOVA. CoA, continuity of attention; IQR, interquartile range; ms, milliseconds; PoA, power of attention; Q, quartile; RTV, reaction time variability; SD, standard deviation; SMMSE, Standardized Mini-mental State Examination.

Table 6.3. Population characteristics and cognitive test scores at baseline and at follow-up in the Newcastle 85+ Study by quartile of total homocysteine concentration.

	Q1 (<13.5 µmol/L)	**Q2** (13.5-16.7 µmol/l)	**Q3** (16.7-21.4 µmol/l)	**Q4** (>21.4 µmol/L)	p^*
Men (%, n)	32 (61)	32 (62)	50 (96)	43 (83)	<0.001
BMI (kg/m^2) (mean, SD)	24.3 (4.4)	24.3 (4.3)	24.9 (4.5)	24.5 (4.3)	0.44†
APOE ε4 carriers (%, n)	23 (35)	28 (46)	29 (44)	26 (40)	0.70
Red blood cell folate (nmol/L)	1272 (896-1748)	940 (675-1279)	779 (573-1084)	680 (477-898)	<0.001
Plasma vitamin B12 (pmol/L)	297 (225-430)	230 (185-303)	225 (161-293)	186 (134-262)	<0.001
Alcohol Drinkers (%, n)	69 (86)	73 (101)	80 (102)	68 (90)	0.14
Smokers (%, n)	5 (9)	3 (5)	7 (14)	8 (15)	0.01
Physical Activity (High) (%, n)	38 (72)	34 (65)	38 (72)	31 (58)	0.02
Education (≥12 y) (%, n)	15 (28)	13 (25)	12 (22)	9 (17)	0.57
History of Cardiovascular disease (%, n)	56 (106)	58 (112)	59 (113)	59 (113)	0.88
Diabetes type 1 or 2 (%, n)	14 (27)	13 (25)	13 (25)	16 (31)	0.80
Hypertension (%, n)	56 (107)	52 (99)	59 (113)	63 (121)	0.14
Renal Impairment (%, n)	8 (15)	15 (29)	22 (41)	51 (98)	<0.001
Depression (Severe) (%, n)	9 (16)	11 (19)	5 (9)	10 (18)	0.52
Dementia/ Alzheimer (%, n)	9 (17)	8 (15)	6 (12)	7 (13)	0.77
Global cognition (SMMSE)					
Baseline	28 (26-29)	28 (26-29)	28 (25-29)	27 (24-29)	0.01
Impaired (≤25) (%, n)	24 (46)	23 (45)	29 (55)	32 (61)	0.19
3 years	27 (24-29)	27 (24-29)	27 (25-29)	27 (22-29)	0.46
Impaired (≤25) (%, n)	31 (35)	30 (34)	35.0 (43)	43 (42)	0.17
5 years	28 (25-28)	27 (23-29)	28 (25-29)	27 (23-29)	0.80
Impaired (≤25) (%, n)	32 (28)	38 (31)	28 (24)	41 (26)	0.32
Focused attention (PoA, ms)					
Baseline	1450 (1326-1662)	1486 (1354-1723)	1476 (1351-1659)	1517 (1409-1753)	0.03
1.5 years	1532 (1371-1731)	1519 (1371-1711)	1513 (1379-1724)	1601 (1444-1782)	0.13
3 years	1492 (1369-1689)	1546 (1383-1749)	1564 (1442-1786)	1559 (1406-1800)	0.25

	Q1 (<13.5 µmol/L)	**Q2** (13.5-16.7 µmol/l)	**Q3** (16.7-21.4 µmol/l)	**Q4** (>21.4 µmol/L)	*p**
Sustained attention (CoA, ms)					
Baseline	87.8 (80.7-91.7)	87.6 (81.4-91.3)	87.3 (79.2-90.8)	86.3 (78.3-91.3)	0.44
1.5 years	86.0 (81.7-90.8)	88.2 (82.7-91.5)	88.0 (81.5-90.8)	85.1 (76.8-90.2)	0.02
3 years	86.8 (80.2-91.8)	87.5 (81.0-91.7)	87.0 (81.3-90.7)	86.3 (76.5-90.0)	0.35
Reaction time variability (RTV)					
Baseline	57.1 (50.8-67.3)	61.8 (52.0-72.1)	60.3 (51.5-72.8)	61.2 (53.0-71.8)	0.08
1.5 years	60.5 (53.1-71.1)	57.0 (50.8-67.0)	59.4 (52.9-67.8)	62.9 (54.0-71.4)	0.04
3 years	55.8 (49.6-67.8)	60.8 (52.0-68.6)	60.7 (51.2-72.5)	61.8 (51.6-69.7)	0.34

Lower scores in PoA and RTV and, higher scores in CoA reflect better performance. Continuous variables are presented as medians and IQR unless otherwise stated. History of cardiovascular disease includes cardiac, cerebrovascular and peripheral vascular diseases. * No quartile difference by Chi-squared test (χ2) for categorical or by Kruskal-Wallis test for non-parametric continuous variables. † No BMI difference by one-way ANOVA. CoA, continuity of attention; IQR, interquartile range; ms, milliseconds; PoA, power of attention; Q, quartile; RTV, reaction time variability; SD, standard deviation; SMMSE, Standardized Mini-mental State Examination.

Table 6.4. Association between folate, vitamin B12 and homocysteine and attention specific and global cognitive decline in the Newcastle 85+ Study.

1-C Biomarker	Change over Time, β (SE); *p* value	Intercept, β (SE); *p* value	Biomarker x Time, β (SE); *p* value
Global cognitive function (SMMSE)			
Red blood cell folate (nmol/L)			
Q2 (612-870)		+0.57 (0.42); 0.17	-0.38 (0.51); 0.46
Q3 (870-1280)	-1.69 (0.18); <0.001	+0.61 (0.42); 0.15	-0.88 (0.49); 0.08
Q4 (>1280)		+1.02 (0.43); 0.02	+0.13 (0.51); 0.80
Plasma vitamin B12 (pmol/L)			
Q2 (170-232)		+0.62 (0.42); 0.14	+0.50 (0.49); 0.30
Q3 (232-325)	-1.68 (0.18); <0.001	-0.15 (0.43); 0.73	-0.55 (0.50); 0.27
Q4 (>325)		+0.54 (0.43); 0.21	+0.15 (0.51); 0.77
Total homocysteine (µmol/L)			
Q2 (13.5-16.7)		-0.53 (0.41); 0.20	+0.61 (0.52); 0.24
Q3 (16.7-21.4)	-1.68 (0.18); <0.001	-0.74 (0.43); 0.08	-0.09 (0.51); 0.86
Q4 (>21.4)		-1.05 (0.46); 0.02	-0.35 (0.53); 0.28
Focused attention (PoA, ms)			
Red blood cell folate (nmol/L)			
Q2 (612-870)		+19 (62); 0.76	-10 (43); 0.82
Q3 (870-1280)	+104 (15); <0.001	+20 (63); 0.75	+98 (42); 0.02
Q4 (>1280)		-45 (63); 0.48	+27 (43); 0.52
Plasma vitamin B12 (pmol/L)			
Q2 (170-232)		-95 (62); 0.13	+40 (42); 0.34
Q3 (232-325)	+105 (15); <0.001	-41 (62); 0.52	+26 (43); 0.55
Q4 (>325)		-114 (64); 0.07	+35 (43); 0.42
Total homocysteine (µmol/L)			
Q2 (13.5-16.7)		+108 (61); 0.08	-110 (42); 0.01
Q3 (16.7-21.4)	+105 (15); <0.001	+53 (63); 0.40	-93 (42); 0.03
Q4 (>21.4)		+81 (68); 0.23	-100 (44); 0.02
Sustained Attention (CoA, ms)			
Red blood cell folate (nmol/L)			
Q2 (612-870)		0.91 (1.20); 0.45	-0.34 (0.84); 0.69
Q3 (870-1280)	-1.26 (0.30); <0.001	-1.27 (1.22); 0.30	-0.45 (0.83); 0.58
Q4 (>1280)		+1.00 (1.23); 0.41	-1.07 (0.84); 0.21
Plasma vitamin B12 (pmol/L)			
Q2 (170-232)		+0.76 (1.21); 0.53	-0.45 (0.81); 0.58
Q3 (232-325)	-1.27 (0.30); <0.001	-1.00 (1.22); 0.41	-0.62 (0.83); 0.45
Q4 (>325)		-0.32 (1.24); 0.80	-0.75 (0.84); 0.37

1-C Biomarker	Change over Time, β (SE); *p* value	Intercept, β (SE); *p* value	Biomarker x Time, β (SE); *p* value
Total homocysteine (µmol/L)			
Q2 (13.5-16.7)		+0.38 (1.18); 0.75	+1.65 (0.82); 0.04
Q3 (16.7-21.4)	-1.27 (0.30); <0.001	+1.58 (1.22); 0.20	+2.19 (0.83); 0.01
Q4 (>21.4)		+0.04 (1.32); 0.98	+1.58 (0.87); 0.07
Reaction time variability (RTV)			
Red blood cell folate (nmol/L)			
Q2 (612-870)		+0.017 (0.026); 0.52	-0.037 (0.022); 0.09
Q3 (870-1280)	+0.021 (0.008); 0.01	+0.043 (0.027); 0.10	+0.001 (0.021); 0.97
Q4 (>1280)		-0.019 (0.027); 0.49	+0.028 (0.022); 0.20
Plasma vitamin B12 (pmol/L)			
Q2 (170-232)		+0.015 (0.026); 0.58	+0.001 (0.021); 0.95
Q3 (232-325)	+0.021 (0.008); 0.01	+0.034 (0.027); 0.20	-0.017 (0.021); 0.44
Q4 (>325)		+0.010 (0.027); 0.72	-0.015 (0.022); 0.49
Total homocysteine (µmol/L)			
Q2 (13.5-16.7)		+0.016 (0.026); 0.53	+0.035 (0.028); 0.22
Q3 (16.7-21.4)	+0.021 (0.008); 0.01	+0.018 (0.027); 0.49	+0.030 (0.030); 0.31
Q4 (>21.4)		+0.034 (0.029); 0.24	+0.042 (0.031); 0.18

For all models, quartile 1 (<612 nmol/L red blood cell folate, <170 pmol/L plasma vitamin B12 and <13.5 total homocysteine, respectively) was used as the reference (0.00). Models are adjusted for alcohol intake, smoking status, *APOE* genotype (rs429358 and rs7412), sex, education, BMI, depression, hypertension, diabetes type 1 and 2, history of cardiovascular diseases and physical activity. Red blood cell folate and plasma vitamin B12 models were additionally adjusted for total homocysteine and the homocysteine model for renal impairment. Higher scores in the SMMSE and CoA and, lower scores in PoA and RTV tests represent better performance. CoA, continuity of attention; ms, milliseconds; PoA, power of attention; Q, quartile; RTV, reaction time variability; SE, standard error; SMMSE, Standardized Mini-mental State Examination.

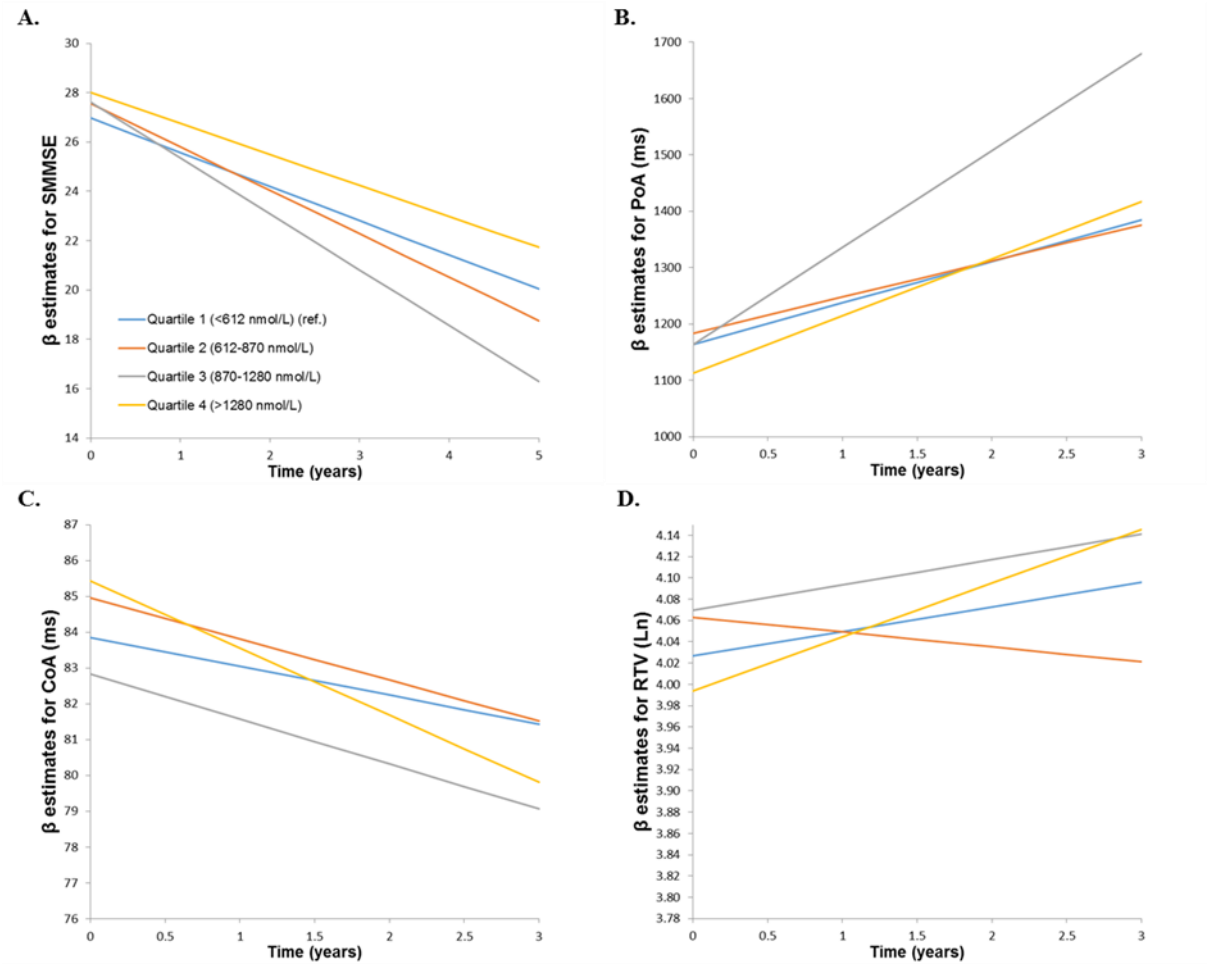
6.5. Supplemental material

Supplemental Table 6.1. Association between folate, vitamin B12 and homocysteine and prevalent cognitive impairment at baseline and incident cognitive impairment after 5 years (SMMSE<26).

	Prevalent Cognitive Impairment				Incident Cognitive Impairment			
	Model 1		Model 2		Model 1		Model 2	
	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
Red blood cell folate (nmol/l)								
Q2 (612-870)	0.77 (0.44-1.33)	0.35	0.73 (0.40-1.31)	0.29	1.68 (0.68-4.14)	0.26	1.68 (0.61-4.65)	0.31
Q3 (870-1280)	0.74 (0.43-1.28)	0.29	0.71 (0.38-1.30)	0.26	2.40 (1.02-5.65)	0.05	2.59 (0.96-7.02)	0.06
Q4 (>1280)	0.84 (0.49-1.45)	0.53	0.73 (0.40-1.34)	0.31	1.10 (0.42-2.87)	0.84	0.84 (0.27-2.67)	0.88
Plasma vitamin B12 (pmol/l)								
Q2 (170-232)	0.80 (0.46-1.40)	0.44	0.64 (0.35-1.18)	0.16	1.15 (0.52-2.53)	0.73	1.41 (0.56-3.53)	0.46
Q3 (232-325)	1.06 (0.62-1.81)	0.84	1.03 (0.57-1.87)	0.93	0.64 (0.26-1.54)	0.32	0.61 (0.23-1.66)	0.34
Q4 (>325)	0.89 (0.51-1.54)	0.66	0.67 (0.36-1.23)	0.19	0.85 (0.36-2.03)	0.72	1.12 (0.40-4.11)	0.84
Total homocysteine (µmol/l)								
Q2 (13.5-16.7)	0.99 (0.56-1.75)	0.96	1.05 (0.57-1.96)	0.87	1.12 (0.50-2.54)	0.79	1.71 (0.68-4.30)	0.26
Q3 (16.7-21.4)	1.18 (0.67-2.09)	0.57	1.40 (0.75-2.60)	0.29	0.79 (0.34-1.88)	0.60	0.97 (0.37-2.56)	0.96
Q4 (>21.4)	1.55 (0.89-2.69)	0.13	2.15 (1.12-4.11)	0.02	1.10 (0.46-2.64)	0.83	1.81 (0.63-5.25)	0.27

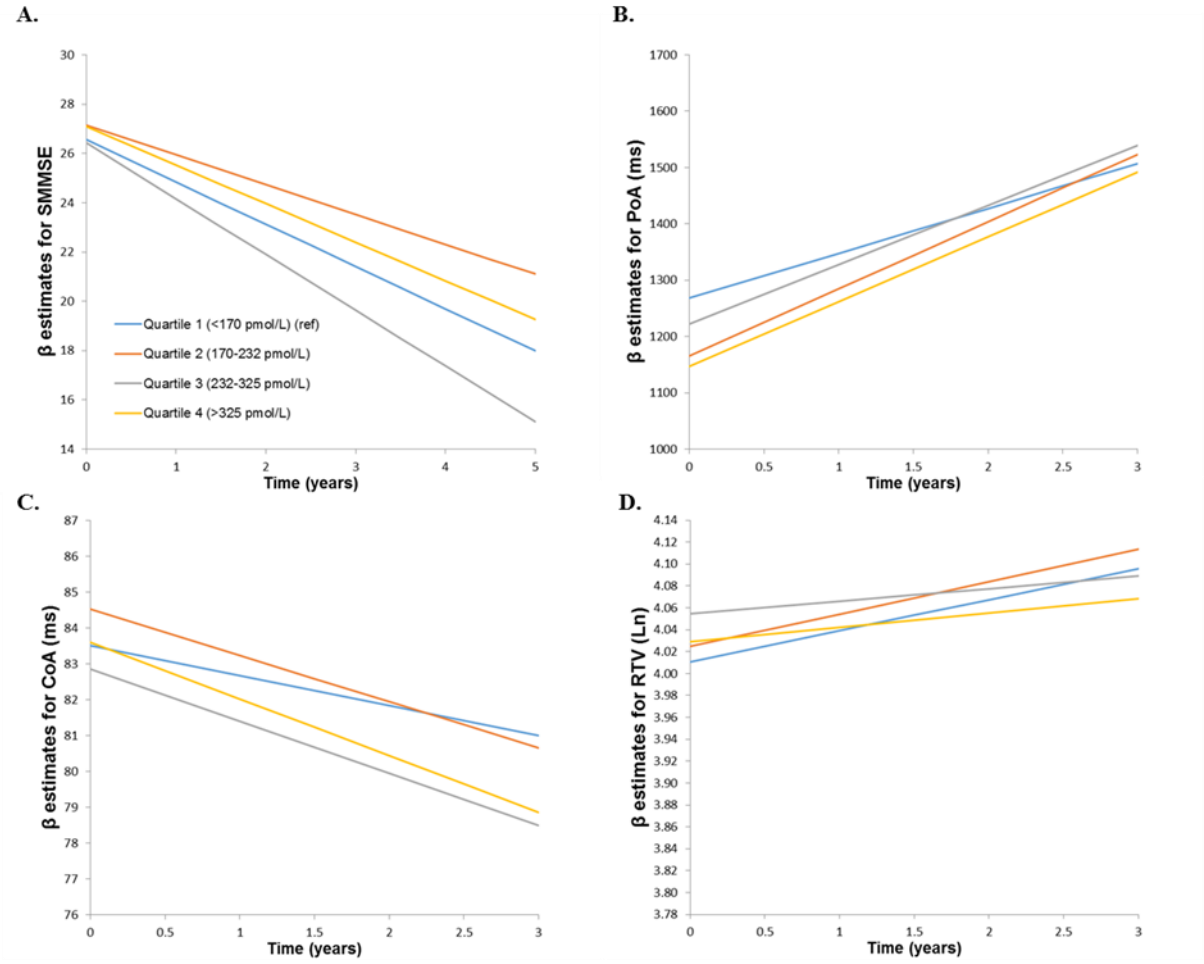
Quartile 1 (<612 nmol/L red blood cell folate, <170 pmol/L plasma vitamin B12 and <13.5 total homocysteine, respectively) was used as the reference category (1.00) for all models. Model 1 is unadjusted, Model 2 is adjusted for alcohol intake, smoking status, *APOE* genotype (rs429358 and rs7412), sex, education, BMI, depression, hypertension, diabetes type 1 and 2, history of cardiovascular diseases and physical activity. Red blood cell folate and plasma vitamin B12 models were additionally adjusted for total homocysteine and the homocysteine model for renal impairment. CI; confidence interval; OR, odds ratio; SMMSE, Standardized Mini-mental State Examination.

Supplemental Figure 6.1. Linear decline in cognition by quartiles of red blood cell folate concentration.



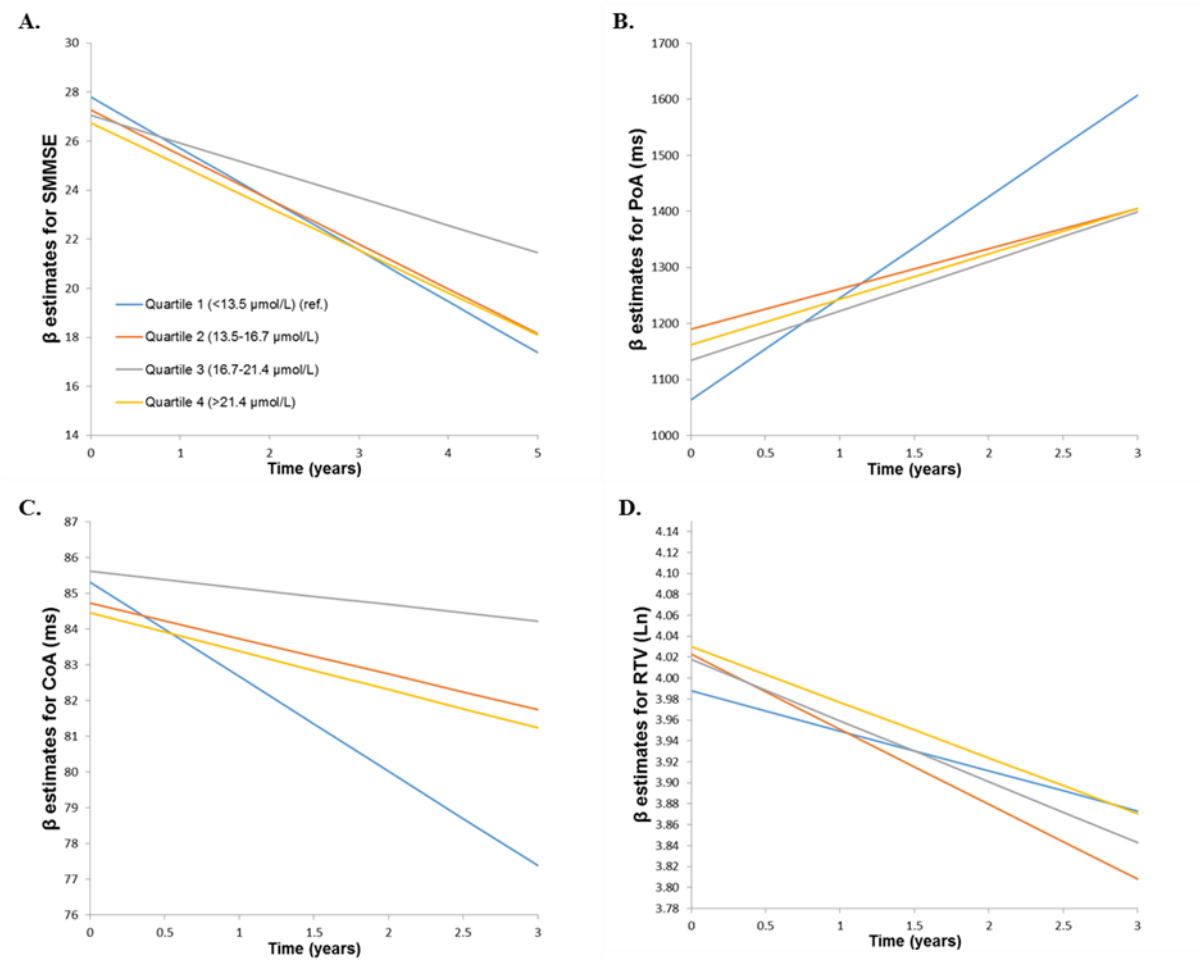
A. global cognition (SMMSE), **B.** focused attention (PoA), **C.** sustained attention (CoA) and **D.** reaction time variability. β estimates were derived from linear mixed models adjusted for alcohol intake, smoking status, *APOE* genotype (rs429358 and rs7412), sex, education, BMI, depression, hypertension, diabetes type 1 and 2, history of cardiovascular diseases, physical activity and total homocysteine. Quartile 1 was used as the reference (0.00). Higher scores in the SMMSE and CoA and, lower scores in PoA and RTV tests represent better performance. CoA, continuity of attention; Ln, natural logarithm; ms, milliseconds; PoA, power of attention; RTV, reaction time variability; SMMSE, Standardized Mini-mental State Examination.

Supplemental Figure 6.2. Linear decline in cognition by quartiles of plasma vitamin B12 concentration.



A. global cognition (SMMSE), **B**. focused attention (PoA), **C.** sustained attention (CoA) and **D.** reaction time variability. β estimates were derived from linear mixed models adjusted for alcohol intake, smoking status, *APOE* genotype (rs429358 and rs7412), sex, education, BMI, depression, hypertension, diabetes type 1 and 2, history of cardiovascular diseases, physical activity and total homocysteine. Quartile 1 was used as the reference (0.00). Higher scores in the SMMSE and CoA and, lower scores in PoA and RTV tests represent better performance. CoA, continuity of attention; Ln, natural logarithm; ms, milliseconds; PoA, power of attention; RTV, reaction time variability; SMMSE, Standardized Mini-mental State Examination.

Supplemental Figure 6.3. Linear decline in cognition by quartiles of total homocysteine concentration.



A. global cognition (SMMSE), **B.** focused attention (PoA), **C.** sustained attention (CoA) and **D.** reaction time variability. β estimates were derived from linear mixed models adjusted for alcohol intake, smoking status, *APOE* genotype (rs429358 and rs7412), sex, education, BMI, depression, hypertension, diabetes type 1 and 2, history of cardiovascular diseases, physical activity and renal impairment. Quartile 1 was used as the reference (0.00). Higher scores in the SMMSE and CoA and, lower scores in PoA and RTV tests represent better performance. CoA, continuity of attention; Ln, natural logarithm; ms, milliseconds; PoA, power of attention; RTV, reaction time variability; SMMSE, Standardized Mini-mental State Examination.

6.6. Summary

Although the biological rationale for the association between folate, vitamin B12 and homocysteine with cognitive function seems plausible, conflicting results have been reported. This chapter aimed to determine the associations between 1-C metabolism biomarkers (folate, vitamin B12 and homocysteine), and cognitive impairment at baseline and the rate of cognitive decline over 5 years in 765 very old adults. Global cognition was measured by the SMMSE at baseline, and at 3 and 5 years of follow-up and, attention-specific cognition with the CDR system at baseline, and at 1.5 and 3 years of follow-up. Baseline RBC folate, plasma vitamin B12 and tHcy concentrations were determined by immunoassay. Linear mixed models were used to estimate the associations between quartiles of 1-C metabolism biomarkers and cognition over 3 (CDR) and 5 years (SMMSE). Compared to participants in the lowest quartile of RBC folate concentrations (<612 nmol/L), those in the highest quartile of RBC folate concentrations (>1280 nmol/L) had 1 more point on the SMMSE over 5 years (β=+1.02, SE=0.43, p=0.02). Those in quartile 4 of tHcy (>21.4 µmol/L) had 1 point less in the SMMSE over 5 years than those in the lowest quartile (<13.5 µmol/L) (β=-1.05, SE=0.46, p=0.02). Plasma vitamin B12 was not predictive of global or attention-specific cognition at baseline and at follow-up. None of the 1-C metabolism biomarkers except tHcy was associated with the rate of decline in attention scores over 3 years. RBC folate and tHcy but not plasma vitamin B12 were associated with better global cognition in the very old at baseline and over 5 years.

Chapter 7 is the last experimental chapter in this thesis and will examine associations between RBC folate, plasma vitamin B12 and tHcy concentrations at baseline, and all-cause and cardiovascular mortality over 9 years mortality follow-up in very old adults.

CHAPTER SEVEN

7. One-carbon metabolism biomarkers and all-cause and cardiovascular mortality in the very old

[Mendonça N et al. (2017) Elevated total homocysteine and plasma vitamin B12 concentrations are associated with all-cause and cardiovascular mortality in the very old: The Newcastle 85+ Study. *J Gerontol A Biol Sci Med Sci. In review*]

Key words: 'aged, 80 and over', Newcastle 85+, homocysteine, folate, vitamin B12, mortality.

7.1. Abstract

Folate and vitamin B12 are key to the correct functioning of one-carbon (1-C) metabolism. The current evidence on associations between 1-C metabolism biomarkers and mortality is inconclusive and generally based on younger populations. This study aimed to determine the associations between biomarkers of 1-C metabolism and all-cause and cardiovascular (CVD) mortality in the very old. The Newcastle 85+ Study is a prospective longitudinal study of participants aged 85 years at recruitment living in Northeast England. Baseline red blood cell folate (RBC folate), plasma vitamin B12 and total homocysteine (tHcy) concentrations were available for 752-766 participants. Associations between biomarkers of 1-C metabolism and all-cause and CVD mortality for up to 9 years were assessed by Cox proportional hazard models and confirmed by restricted cubic splines. Participants with higher tHcy concentrations had twice the risk of death from any cause than those with lower concentrations (e.g. Q4 vs. Q1, HR: 2.05, 95% CI: 1.51-2.77, p<0.001) after adjustment for sociodemographic, lifestyle and health variables. Women with elevated plasma vitamin B12 concentrations (>500 pmol/L) had increased risk of all-cause mortality (HR: 1.70, 95% CI: 1.13-2.56, p=0.011) compared with those with concentrations 148-500 pmol/L. Higher concentrations of tHcy and plasma vitamin B12 were associated with increased risk of all-cause and CVD mortality in the very old. This confirms findings for tHcy in younger populations but the adverse relationships between elevated plasma vitamin B12 concentrations and mortality in this population are novel and require further investigation.

7.2. Introduction

Given the close involvement in 1-C metabolism, it is no surprise that folate and vitamin B12 have been associated with cardiovascular disease (CVD), cancer and all-cause mortality. However, the current evidence for links between 1-C metabolism biomarkers and mortality is inconclusive and most is based on studies in younger populations. A recent meta-analysis pooling data from 30 randomized controlled trials (RCTs) reported that folic acid supplementation reduced CVD risk by 4% and the risk of stroke by 10% [(149)]. Part of this effect might be due to reduction of homocysteine concentrations as supplementation with folic acid typically lowers homocysteine concentrations by 13-25% with a further reduction of 7% with the addition of vitamin B12 [(124)]. However several studies did not find a

significant association between folate status and all-cause and cardiovascular-specific mortality [151,197-201]. With regards to vitamin B12, it is hypothesized that a very high vitamin B12 concentration may be primarily due to disruption and release into the circulation of vitamin B12 stores, especially from the liver, but no clear mechanism has been determined [114,160]. Some have found that high concentrations of vitamin B12 are associated with all-cause mortality [152,153] while others did not [156-159].

This chapter aimed to examine associations between red blood cell folate (RBC folate), plasma vitamin B12 and total homocysteine (tHcy) concentrations at baseline, and all-cause and cardiovascular mortality over 9 years in a large sample of very old adults.

7.3. Methods

Participants

This chapter uses data from the Newcastle 85+ Study and details were reported in *Chapter 2, General Methods - 2.1 The Newcastle 85+ Study*.

Biomarkers of 1-C metabolism

Blood samples collection, tHcy, RBC folate and plasma vitamin B12 analyses, and genotyping were described in *Chapter 2, General Methods - 2.3 Blood collection, one-carbon metabolism biomarkers and genotyping.* tHcy, RBC folate and plasma vitamin B12 concentrations were categorised into the following quartiles: RBC folate - Quartile 1 (Q1) <612, Q2:612-870, Q3:870-1280, Q4>1280 nmol/L); tHcy - Q1 <13.5, Q2:13.5-16.7, Q3:16.7-21.4, Q4>21.4 µmol/L; plasma vitamin B12 - Q1 <170, Q2:170-232, Q3: 232-325, Q4>325 pmol/L. Sex-specific quartiles were used for sex-specific models. For plasma vitamin B12 concentrations we also used pre-defined thresholds of <148, 148-500 and >500 pmol/L. Plasma vitamin B12 concentration <148 pmol/L is commonly used to diagnose deficiency [112] while a concentration >600 pmol/L is used to identify high plasma vitamin B12 concentrations [154,155]. Further, an upper limit of 569 pmol/L is used by the department of clinical biochemistry at Newcastle Royal Victoria Infirmary where samples were analysed [202]. However, because there were not enough participants with plasma vitamin B12 concentrations >600 pmol/L, >500 pmol/L was used.

All-cause and cardiovascular mortality

Information on dates and cause of death (International classification of diseases and related disorders, 10th revision [203]) were obtained from the Health and Social Care Information Service (now NHS Digital), UK. Deaths coded as I00-I99 (Chapter IX) were used to determine CVD mortality [203]. The time to event of interest (all-cause mortality or cardiovascular-specific mortality) was calculated as the time between blood draw (2006-2007) and time of death (or censored at 28 April 2015). Mortality follow-up was conducted over 9 years.

Health assessment and disease count

Details on the variables recorded by the multidimensional health questionnaires, the GP medical records review and the disease count were also described in *Chapter 2, General Methods - 2.4 Other socioeconomic, health and lifestyle variables*. The Standardized Mini-Mental State Examination (SMMSE) 30-point scale was used to assess global cognitive function.

Statistical analysis

Longitudinal associations between quartiles of 1-C metabolism biomarkers and mortality (all-cause and CVD) were assessed with Kaplan-Meier curves (unadjusted) and Cox proportional hazards models. Model 1 was adjusted for sex, years of full time education and housing (community-dwelling/ institutionalized), and model 2 was further adjusted for BMI, physical activity (low/ medium/ high), smoking status (never/ former/ current), current alcohol drinkers (yes/no), disease count, SMMSE and the other 1-C metabolism biomarkers. tHcy models were also adjusted for renal impairment (yes/no). The fully adjusted CVD mortality model was also adjusted for history of CVD, hypertension and diagnosis of diabetes type 1 and 2 instead of disease count.

A fully-adjusted Cox proportional hazards model using pre-defined thresholds of plasma vitamin B12 concentrations was also fitted in sensitivity analysis.

To confirm findings and to assess the linearity of relationships, restricted cubic splines were fitted to the fully adjusted Cox proportional hazards models with 5 knots at default locations based on quantiles. For example, the quantiles for the tHcy model for all

participants were 10, 14, 17, 21 and 32 µmol/L; for the RBC folate model they were 370, 639, 868, 1205 and 2244 nmol/L; and 102, 178, 232, 307 and 584 pmol/L for plasma vitamin B12. Multicollinearity was checked with tolerance, variation inflation factor and eigenvalues. The proportional hazards assumption for 1-C metabolism biomarkers quartiles was tested by visual inspection of log(-log(survival) vs log(time) plots and Schoenfeld residuals. Analyses were repeated after further adjustment for alanine aminotransferase (ALT), alkaline phosphatase and bilirubin concentrations, or further adjustment for high sensitivity C-reactive protein (hs-CRP), or excluding participants who died during the first year of follow-up, or who were taking folic acid or vitamin B12 supplements at baseline.

All analyses were carried out in IBM SPSS statistics v22.0 except for checking the Schoenfeld residuals and plotting the restricted cubic splines which used R v3.2.2 (packages "survival" and "rms"). General statistical methods are presented in *Chapter 2, General Methods - 2.5 General statistical analysis.*

7.4. Results

Over the 4082 person-years of follow-up, 73% (n=564) (13.8 deaths per 100 person-years) of those that had 1-C metabolism biomarkers measured at baseline died. Of these, 53% (n=299) (7.3 deaths per 100 person-years) died from CVD. The median survival time was 5.5 (IQR: 2.7-8.0) years.

Data on biomarkers of 1-C metabolism were more likely to be missing from women (p=0.004), those who lived in institutions (p<0.001), who were less physically active (p<0.001) and who had a lower score in the SMMSE (p=0.030).

Total homocysteine

There were more men, more active smokers (p=0.01), more renally impaired participants (p<0.001) and with lower concentrations of ALT (p<0.001) in higher quartiles of tHcy (p<0.001). tHcy quartiles were inversely associated with RBC folate and plasma vitamin B12 concentrations in a dose-dependent manner (p<0.001) (**Figure 7.1**).

Survival was longest in Q1 of tHcy concentrations (median: 7.1, 95% CI: 5.9-8.2 years) and lowest in Q4 (median: 4.0, 95% CI: 3.3-4.7) (p<0.001) in unadjusted models (**Figure 7.1** and **Supplemental Table 7.1**). These differences emerged after the first year of follow-up

and were present in women (p=0.001) but not in men (**Figure 7.1**). Participants in Q4 of tHcy concentration had twice the risk of death from any cause than those in the lowest quartile of tHcy (Q1) in models with all participants (e.g. HR: 2.05, 95% CI: 1.51-2.77, p<0.001) (**Table 7.4**), and in both men (**Table 7.5**) and women (**Table 7.6**) after adjustment for sex, education, housing, BMI, physical activity, smoking, alcohol intake, disease count, SMMSE, renal impairment, RBC folate and plasma vitamin B12. Visual inspection of restricted cubic splines for tHcy in the fully adjusted Cox proportional hazards model confirmed these findings (**Figure 7.4.**-**Figure 7.6**) Results were similar for cardiovascular mortality (**Figure 7.1** and **Supplemental Figure 7.1**-**Supplemental Figure 7.3** and **Table 7.4**-**Table 7.6**). All relationships between tHcy and mortality were linear except for all-cause mortality in men (p=0.038)

Red blood cell folate

Those in higher quartiles of RBC folate had a slightly higher chronic disease count (p=0.01), more history of CVD (<0.001) and higher ALT concentration (p<0.001) than those in lower quartiles (**Table 7.2**).

Hazard ratios for all-cause and cardiovascular mortality with RBC folate concentration quartiles were not significantly different in unadjusted (**Figure 7.2**) or fully-adjusted Cox proportional hazards models (**Table 7.4**-**Table 7.6**). All relationships between RBC folate and mortality were linear (**Figure 7.4.**-**Figure 7.6** and **Supplemental Figure 7.1**-**Supplemental Figure 7.3**).

Plasma vitamin B12

There were no significant differences in the prevalence of chronic diseases or risk factors between quartiles of plasma vitamin B12 except for BMI (p=0.001) and ALT concentration (p=0.034) (**Table 7.3**).

Like RBC folate, individuals in different quartiles of plasma vitamin B12 concentration had similar survival rates in unadjusted Kaplan-Meier estimates (**Figure 7.3**) and fully-adjusted Cox models (**Table 7.4**-**Table 7.6**). However, women in Q4 (>336 pmol/L) had a higher risk of cardiovascular death compared with those in Q1 (<174 pmol/L) (HR: 1.69, 95% CI: 1.01-2.85, p=0.046) (**Table 7.6**) although this association was no longer significant

(p=0.078) when those who died in the first year of follow-up were excluded. Visual inspection of restricted cubic spline curves showed a linear relationship with mortality.

Seventeen percent (n=128) of participants had plasma vitamin B12 concentrations <148 pmol/L, 76% (n=569) with concentrations 148-500 pmol/L and 7.4% (n=56) with concentrations >500 pmol/L. Cox proportional hazards models using these cut-offs showed that women with >500 pmol/L of plasma vitamin B12 had increased risk of all-cause mortality than those with <148 pmol/L (HR:2.06, 95% CI:1.23-3.47, p=0.006) and 148-500 pmol/L (HR:1.70, 95% CI:1.13-2.56, p=0.011) and increased risk of CVD mortality (**Supplemental Table 7.2**). **Figure 7.4.**-**Figure 7.6** confirmed these findings. The relationship was weaker when those who died during the first year of follow-up were excluded. All relationships between plasma vitamin B12 and mortality were linear except for cardiovascular mortality in women (p=0.016) (**Figure 7.4.**-**Figure 7.6** and **Supplemental Figure 7.1**-**Supplemental Figure 7.3**).

Table 7.1. Population characteristics in the Newcastle 85+ Study by quartiles of total homocysteine.

	Q1 (<13.5 µmol/L)	**Q2** (13.5-16.7 µmol/l)	**Q3** (16.7-21.4 µmol/l)	**Q4** (>21.4 µmol/L)	p^1
Men (%, n)	32 (61)	32 (62)	50 (96)	43 (83)	<0.001
Education (≥12 y) (%, n)	15 (28)	13 (25)	12 (22)	9 (17)	0.57
Housing (Institutional) (%, n)	8 (15)	9 (17)	7 (13)	9 (17)	0.86
BMI (mean, SD)	24.3 (4.4)	24.3 (4.3)	24.9 (4.5)	24.5 (4.3)	0.44[2]
Red blood cell folate (nmol/L)	1272 (896-1748)	940 (675-1279)	779 (573-1084)	680 (477-898)	<0.001
Plasma vitamin B12 (pmol/L)	297 (225-430)	230 (185-303)	225 (161-293)	186 (134-262)	<0.001
Alcohol drinkers (%, n)	69 (86)	73 (101)	80 (102)	68 (90)	0.14
Smokers (%, n)	5 (9)	3 (5)	7 (14)	8 (15)	0.01
Physical activity (High) (%, n)	38 (72)	34 (65)	38 (72)	31 (58)	0.02
Self-rated health (fair or poor) (%, n)	23 (43)	20 (37)	19 (36)	26 (48)	0.45
Disease count (mean, SD)	2.1 (1.2)	2.2 (1.2)	2.3 (1.2)	2.5 (1.3)	0.08[2]
History of Cardiovascular disease (%, n)	56 (106)	58 (112)	59 (113)	59 (113)	0.88
Diabetes type 1 or 2 (%, n)	14 (27)	13 (25)	13 (25)	16 (31)	0.80
Hypertension (%, n)	56 (107)	52 (99)	59 (113)	63 (121)	0.14
SMMSE (0-30)	28 (26-29)	28 (26-29)	28 (25-29)	27 (24-29)	0.01
Renal impairment (%, n)	8 (15)	15 (29)	22 (41)	51 (98)	<0.001
ALT (U/L)	18 (15-23)	17 (14-21)	16 (13-20)	15 (12-19)	<0.001
hs-CRP (mg/L)	2.2 (1.0-5.3)	2.4 (1.3-5.8)	2.6 (1.3-6.1)	3.3 (1.4-6.2)	0.075

Continuous variables are presented as medians and interquartile range unless otherwise stated. History of cardiovascular disease includes cardiac, cerebrovascular and peripheral vascular diseases. [1] No quartile difference by Chi-squared test ($\chi 2$) for categorical or by Kruskal-Wallis test for non-parametric continuous variables. [2] No BMI or disease count difference by one-way ANOVA. ALT, alanine aminotransferase; BMI, body mass index; hs-CRP, high sensitivity C-reactive protein; ms, milliseconds; Q, quartile; SD, standard deviation; SMMSE, standardized mini-mental state examination.

Table 7.2. Population characteristics in the Newcastle 85+ Study by quartiles of red blood cell folate.

	Q1 (<612 nmol/L**)**	**Q2** (612-870 nmol/L)	**Q3** (870-1280 nmol/L)	**Q4** (>1280 nmol/L)	*p*[1]
Men (%, n)	41 (77)	38 (72)	40 (75)	38 (72)	0.86
Education (≥12 y) (%, n)	11 (20)	11 (20)	14 (25)	14 (26)	0.18
Housing (Institutional) (%, n)	12 (22)	7 (14)	7 (12)	7 (14)	0.23
BMI (mean, SD)	24.2 (4.3)	24.7 (4.5)	24.3 (4.1)	24.4 (4.3)	0.78[2]
Plasma Vitamin B12 (pmol/L)	201 (135-280)	216 (159-275)	259 (193-371)	278 (205-391)	<0.001
Total Homocysteine (µmol/L)	19.9 (16.3-24.6)	18.3 (14.9-22.9)	15.6 (13.0-19.6)	13.8 (11.1-17.4)	<0.001
Alcohol drinkers (%, n)	70 (92)	73 (94)	80 (96)	66 (89)	0.10
Smokers (%, n)	9 (16)	4 (8)	4 (8)	5 (10)	0.35
Physical activity (High) (%, n)	38 (71)	33 (62)	34 (63)	37 (68)	0.75
Self-rated health (fair or poor) (%, n)	20 (37)	22 (42)	20 (37)	23 (43)	0.88
Disease count (mean, SD)	2.0 (1.2)	2.3 (1.3)	2.4 (1.2)	2.4 (1.2)	0.01
History of Cardiovascular disease (%, n)	45 (84)	57 (108)	66 (123)	63 (119)	<0.001
Diabetes type 1 or 2 (%, n)	9 (16)	16 (31)	13 (25)	17 (32)	0.08
Hypertension (%, n)	54 (100)	59 (112)	54 (101)	65 (122)	0.12
SMMSE (0-30)	27 (24-29)	28 (25-29)	28 (25-29)	28 (25-29)	0.17
Renal impairment (%, n)	20 (38)	26 (49)	22 (41)	28 (52)	0.36
ALT (U/L)	15 (12-20)	16 (13-21)	17 (14-21)	18 (15-22)	<0.001
hs-CRP (mg/L)	2.9 (1.3-6.9)	2.8 (1.2-6.2)	2.5 (1.3-5.8)	2.5 (1.0-5.5)	0.441

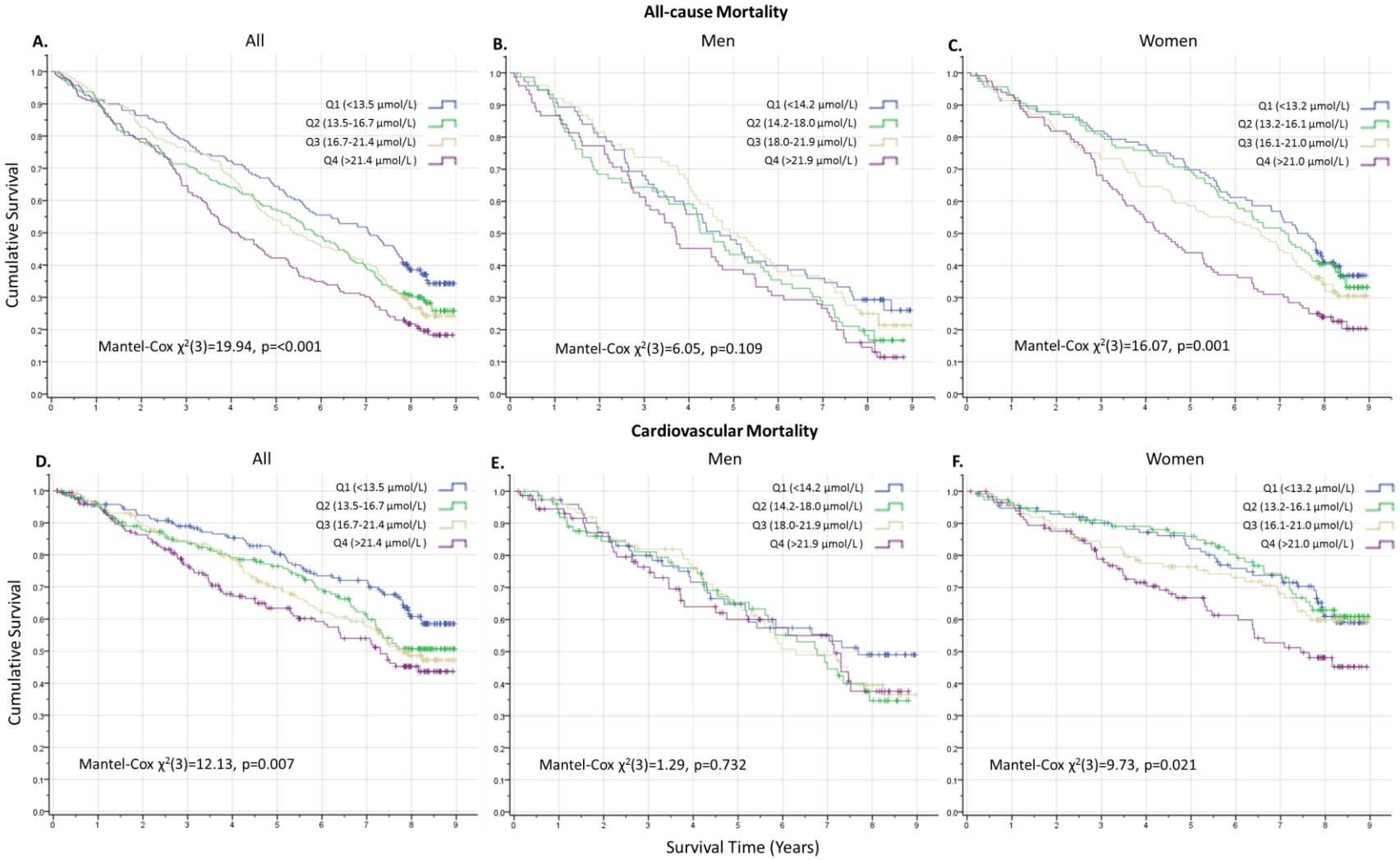
Continuous variables are presented as medians and interquartile range unless otherwise stated. History of cardiovascular disease includes cardiac, cerebrovascular and peripheral vascular diseases. [1] No quartile difference by Chi-squared test ($\chi 2$) for categorical or by Kruskal-Wallis test for non-parametric continuous variables. [2] No BMI or disease count difference by one-way ANOVA. ALT, alanine aminotransferase; BMI, body mass index; hs-CRP, high sensitivity C-reactive protein; ms, milliseconds; Q, quartile; SD, standard deviation; SMMSE, standardized mini-mental state examination.

Table 7.3. Population characteristics in the Newcastle 85+ Study by quartiles of plasma vitamin B12.

	Q1 (<170 pmol/L)	**Q2** (170-232 pmol/L)	**Q3** (232-325 pmol/L)	**Q4** (>325 pmol/L)	*p*[1]
Men (%, n)	42 (79)	41 (77)	39 (74)	35 (66)	0.56
Education (≥12 y) (%, n)	10 (19)	13 (23)	14 (27)	12 (22)	0.15
Housing (Institutional) (%, n)	6 (12)	7 (13)	8 (15)	12 (22)	0.23
BMI (mean, SD)	25.1 (4.5)	23.8 (4.1)	25.0 (4.3)	23.7 (4.3)	0.001[2]
Red blood cell folate (nmol/L)	683 (479-992)	838 (605-1159)	913 (690-1393)	1058 (745-1608)	<0.001
Total Homocysteine (µmol/L)	19.7 (15.9-25.1)	17.3 (14.5-21.8)	15.9 (13.3-19.8)	13.9 (11.1-18.2)	<0.001
Alcohol Drinkers (%, n)	73 (103)	72 (91)	80 (91)	65 (86)	0.07
Smokers (%, n)	6 (11)	5 (10)	6 (12)	5 (9)	0.77
Physical Activity (High) (%, n)	38 (72)	37 (68)	34 (63)	33 (62)	0.55
Self-rated health (fair or poor) (%, n)	21 (40)	22 (40)	18 (34)	25 (45)	0.52
Disease count (mean, SD)	2.2 (1.3)	2.4 (1.2)	2.2 (1.2)	2.3 (1.2)	0.51
History of Cardiovascular disease (%, n)	51 (96)	60 (113)	62 (117)	57 (108)	0.10
Diabetes type 1 or 2 (%, n)	12 (23)	18 (33)	12 (22)	13 (25)	0.32
Hypertension (%, n)	58 (111)	54 (100)	61 (114)	59 (110)	0.55
SMMSE (0-30)	28 (25-29)	27 (25-29)	28 (25-29)	28 (25-29)	0.73
Renal Impairment (%, n)	23 (44)	27 (51)	23 (44)	22 (41)	0.65
ALT (U/L)	16 (13-20)	16 (13-21)	17 (14-21)	17 (14-22)	0.03
hs-CRP (mg/L)	2.7 (1.2-6.3)	3.0 (1.4-5.6)	2.5 (1.2-5.6)	2.3 (1.0-7.0)	0.668

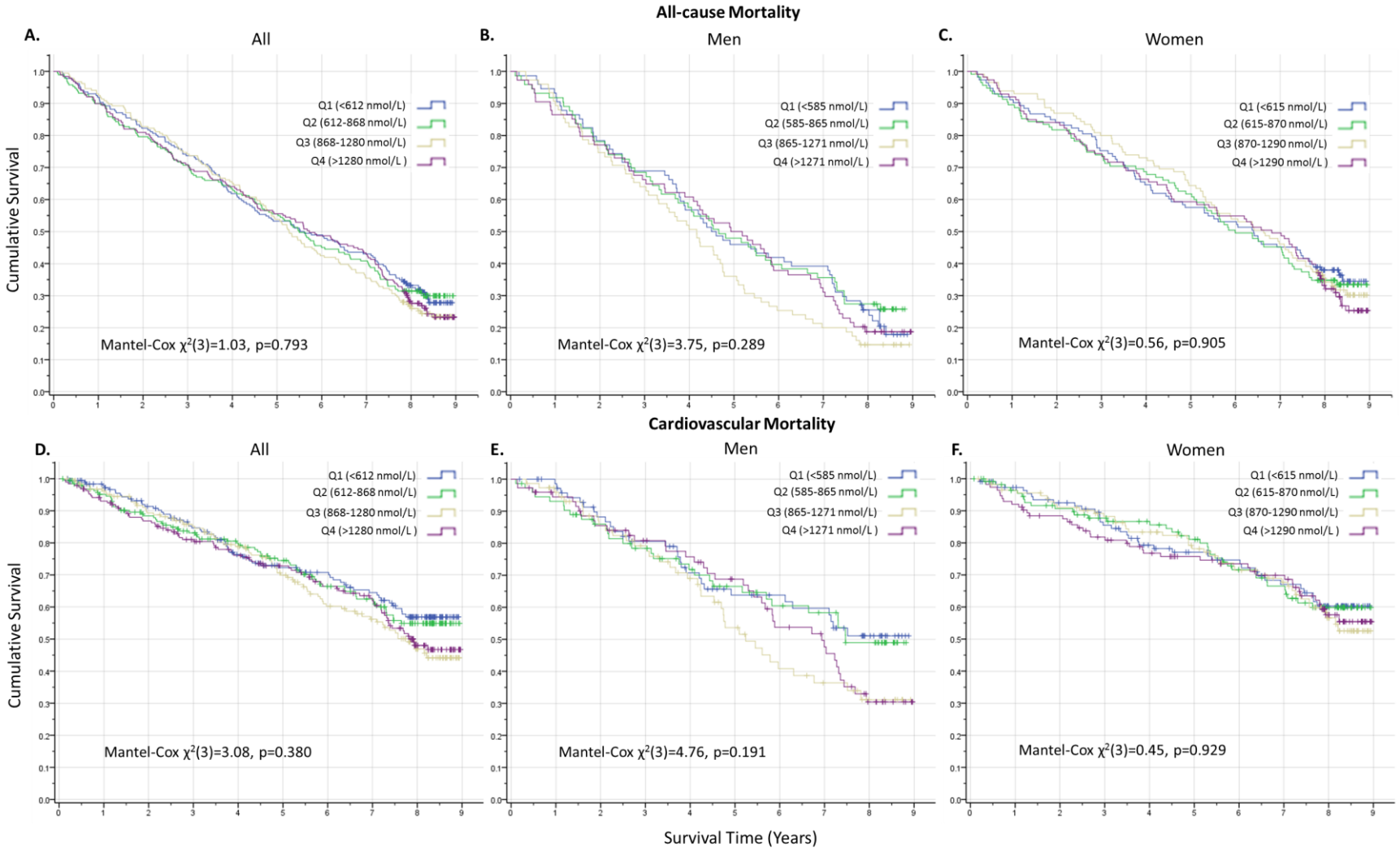
Continuous variables are presented as medians and interquartile range unless otherwise stated. History of cardiovascular disease includes cardiac, cerebrovascular and peripheral vascular diseases. [1] No quartile difference by Chi-squared test ($\chi 2$) for categorical or by Kruskal-Wallis test for non-parametric continuous variables. [2] No BMI or disease count difference by one-way ANOVA. ALT, alanine aminotransferase; BMI, body mass index; hs-CRP, high sensitivity C-reactive protein; ms, milliseconds; Q, quartile; SD, standard deviation; SMMSE, standardized mini-mental state examination.

Figure 7.1. Kaplan-Meier plot of the probability of survival for all-cause and cardiovascular mortality by quartiles of total homocysteine.



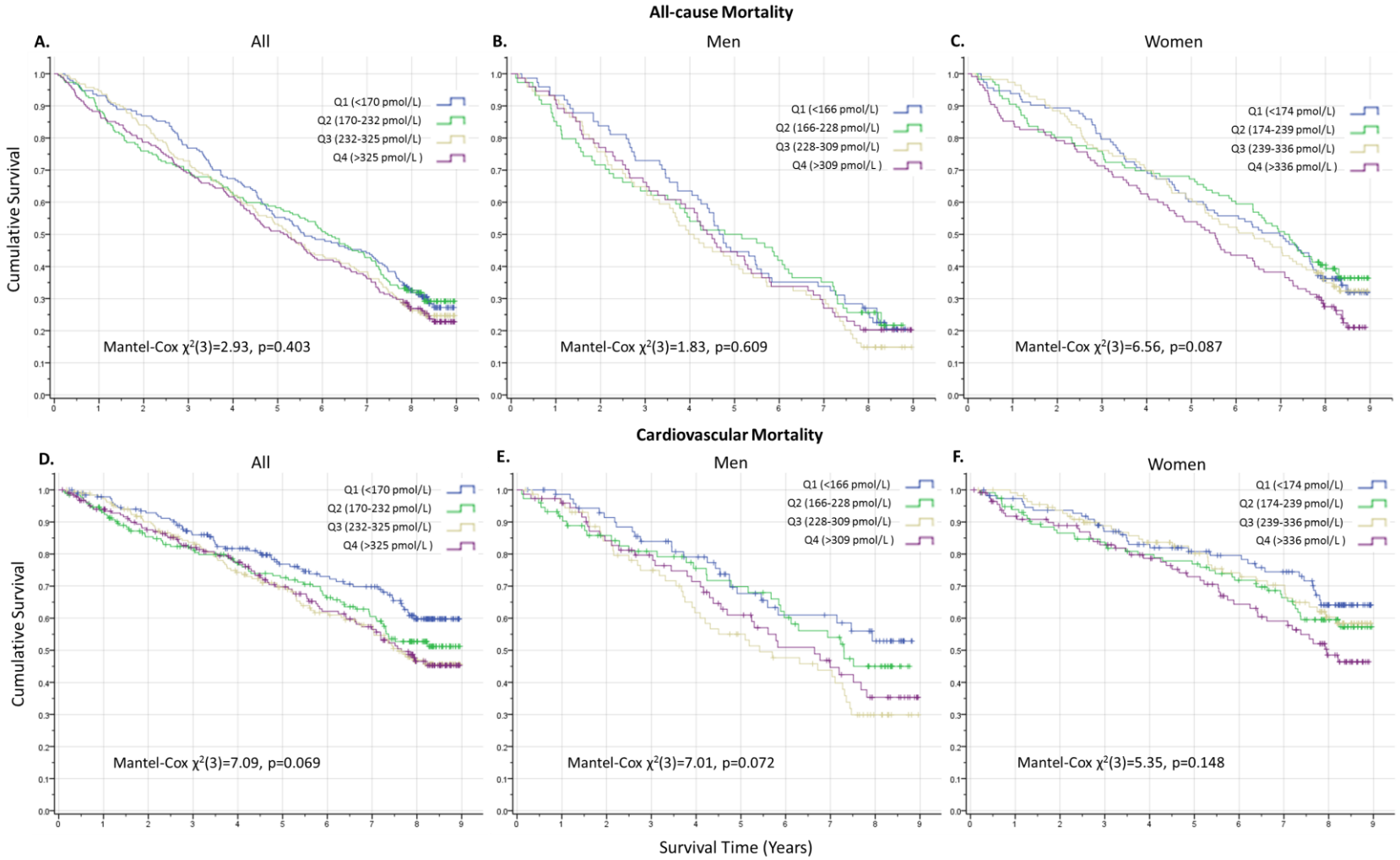
Total homocysteine quartiles for all-cause mortality in **A.** all, **B.** men and **C.** women and for cardiovascular mortality in **D.** all, **E.** men and **F.** women. Q, quartile. Crosses indicate censoring.

Figure 7.2. Kaplan-Meier plot of the probability of survival for all-cause and cardiovascular mortality by quartiles of red blood cell folate.



Red blood cell folate quartiles for all-cause mortality in **A.** all, **B.** men and **C.** women and for cardiovascular mortality in **D.** all, **E.** men and **F.** women. Q, quartile. Crosses indicate censoring.

Figure 7.3. Kaplan-Meier plot of the probability of survival for all-cause and cardiovascular mortality by quartiles of plasma vitamin B12.



Plasma vitamin B12 quartiles for all-cause mortality in **A.** all, **B.** men and **C.** women and for cardiovascular mortality in **D.** all, **E.** men and **F.** women. Q, quartile. Crosses indicate censoring.

Table 7.4. Hazard ratios for all-cause and cardiovascular mortality by total homocysteine, red blood cell folate and plasma vitamin B12 quartiles in all participants.

	All-cause mortality						Cardiovascular mortality					
	Model 1			Model 2			Model 1			Model 2		
Quartiles	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*
Total Homocysteine (μmol/L)												
<13.5		1.00 (reference)			1.00 (reference)			1.00 (reference)			1.00 (reference)	
13.5-16.7	1.36	1.05-1.76	0.018	1.78	1.36-2.33	<0.001	1.39	0.98-1.96	0.067	1.79	1.24-2.58	0.002
16.7-21.4	1.23	0.94-1.61	0.106	1.43	1.08-1.88	0.011	1.33	0.95-1.88	0.101	1.65	1.14-2.40	0.008
>21.4	1.67	1.30-2.15	<0.001	2.05	1.51-2.77	<0.001	1.68	1.20-2.37	0.003	2.29	1.51-3.48	<0.001
Red Blood Cell Folate (nmol/L)												
<612		1.00 (reference)			1.00 (reference)			1.00 (reference)			1.00 (reference)	
612-868	1.12	0.87-1.44	0.375	1.03	0.80-1.34	0.796	1.11	0.79-1.57	0.553	0.89	0.62-1.27	0.515
868-1280	1.24	0.97-1.58	0.093	1.18	0.92-1.53	0.199	1.35	0.96-1.88	0.084	1.08	0.76-1.54	0.661
>1280	1.24	0.97-1.59	0.086	1.16	0.89-1.50	0.277	1.37	0.98-1.91	0.067	1.12	0.79-1.60	0.521
Plasma Vitamin B12 (pmol/L)												
<170		1.00 (reference)			1.00 (reference)			1.00 (reference)			1.00 (reference)	
170-232	1.03	0.80-1.32	0.819	0.99	0.77-1.28	0.923	1.36	0.96-1.92	0.083	1.26	0.87-1.81	0.217
232-325	1.06	0.83-1.36	0.621	1.01	0.78-1.31	0.961	1.41	1.00-2.00	0.048	1.25	0.86-1.81	0.237
>325	1.14	0.89-1.46	0.292	1.09	0.83-1.42	0.537	1.48	1.05-2.08	0.026	1.43	0.98-2.08	0.067

Model 1 is adjusted for sex, education and housing; Model 2 is additionally adjusted for body mass index, physical activity, smoking, alcohol intake, disease count, mini-mental state examination score and the other 1-C metabolism biomarkers. The cardiovascular mortality model 2 was also adjusted for history of cardiovascular diseases, hypertension and diagnosis of diabetes type 1 and 2 instead of disease count. The total homocysteine model is additionally adjusted for renal impairment. CI, confidence interval; HR, hazard ratio.

Table 7.5. Hazard ratios for all-cause and cardiovascular mortality by total homocysteine, red blood cell folate and plasma vitamin B12 quartiles in men.

	All-cause mortality						Cardiovascular mortality					
	Model 1			Model 2			Model 1			Model 2		
Quartiles	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*
Total Homocysteine (µmol/L)												
<14.2		1.00 (reference)			1.00 (reference)			1.00 (reference)			1.00 (reference)	
14.2-18.0	1.43	0.98-2.08	0.062	1.56	1.04-2.33	0.031	1.37	0.84-2.25	0.211	1.62	0.94-2.79	0.081
18.0-21.9	1.10	0.75-1.62	0.626	1.11	0.73-1.71	0.621	1.26	0.78-2.05	0.348	1.62	0.93-2.82	0.091
>21.9	1.63	1.12-2.38	0.011	1.80	1.13-2.89	0.014	1.45	0.87-2.40	0.152	2.09	1.09-4.01	0.026
Red Blood Cell Folate (nmol/L)												
<585		1.00 (reference)			1.00 (reference)			1.00 (reference)			1.00 (reference)	
585-865	0.91	0.62-1.36	0.656	0.94	0.63-1.41	0.762	0.89	0.53-1.51	0.671	0.73	0.42-1.27	0.261
865-1271	1.33	0.91-1.94	0.137	1.34	0.90-2.00	0.149	1.39	0.84-2.30	0.201	1.09	0.63-1.87	0.763
>1271	1.14	0.78-1.67	0.497	1.16	0.77-1.74	0.479	1.36	0.83-2.23	0.227	1.24	0.73-2.10	0.427
Plasma Vitamin B12 (pmol/L)												
<166		1.00 (reference)			1.00 (reference)			1.00 (reference)			1.00 (reference)	
166-228	0.99	0.68-1.43	0.943	1.08	0.72-1.60	0.720	1.18	0.70-1.99	0.546	1.08	0.62-1.89	0.776
228-309	1.19	0.83-1.71	0.351	1.25	0.84-1.86	0.278	1.80	1.10-2.95	0.020	1.60	0.92-2.75	0.094
>309	1.04	0.72-1.51	0.831	0.99	0.64-1.53	0.976	1.46	0.88-2.41	0.146	1.17	0.65-2.10	0.609

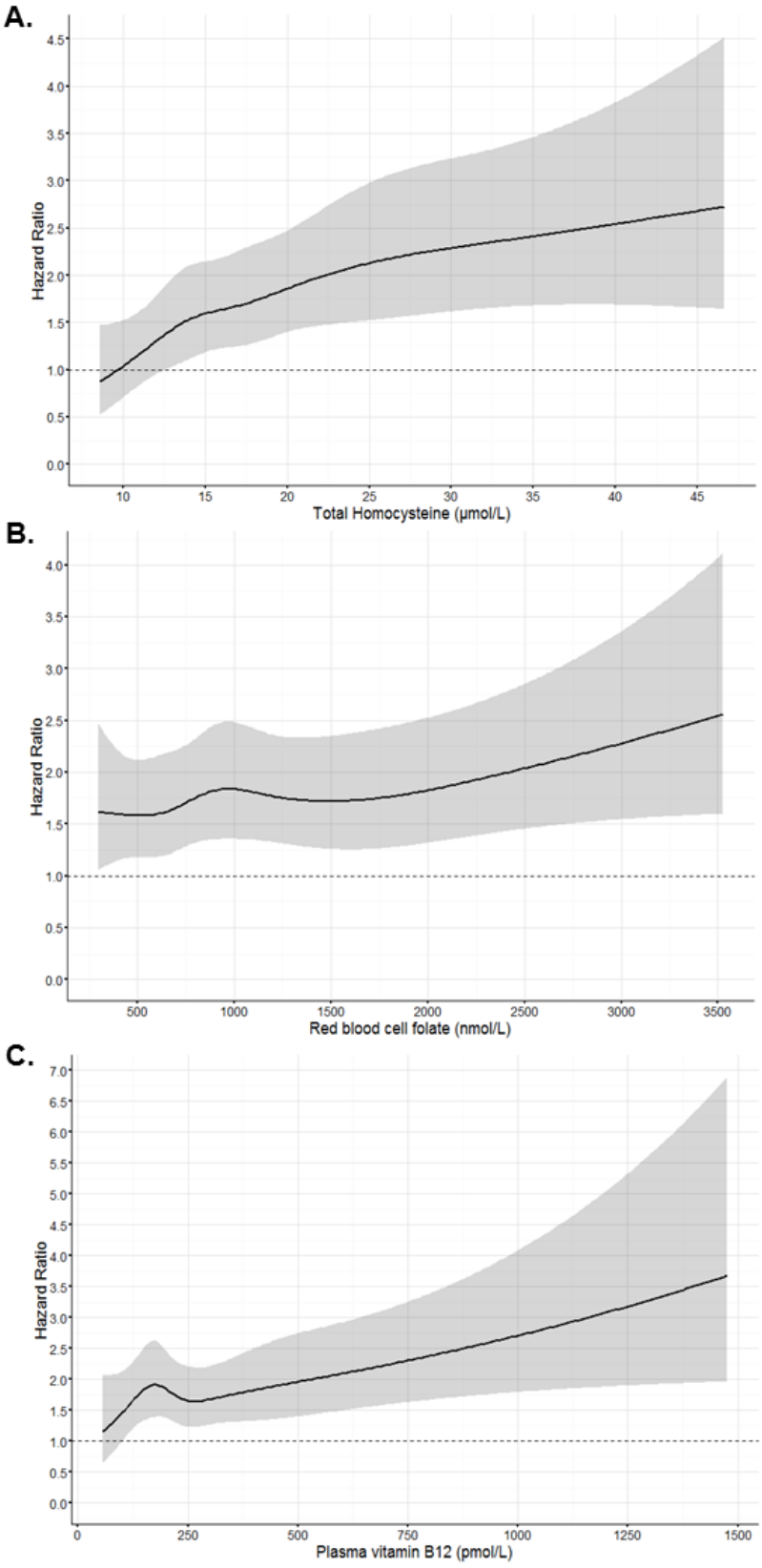
Model 1 is adjusted for education and housing; Model 2 is additionally adjusted for body mass index, physical activity, smoking, alcohol intake, disease count, mini-mental state examination score and the other 1-C metabolism biomarkers. The cardiovascular mortality model 2 was also adjusted for history of cardiovascular diseases, hypertension and diagnosis of diabetes type 1 and 2 instead of disease count. The homocysteine model is additionally adjusted for renal impairment. CI, confidence interval; HR, hazard ratio.

Table 7.6. Hazard ratios for all-cause and cardiovascular mortality by total homocysteine, red blood cell folate and plasma vitamin B12 quartiles in women.

	All-cause mortality						Cardiovascular mortality					
	Model 1			Model 2			Model 1			Model 2		
Quartiles	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*
Total Homocysteine (μmol/L)												
<13.2		1.00 (reference)			1.00 (reference)			1.00 (reference)			1.00 (reference)	
13.2-16.1	1.15	0.82-1.62	0.419	1.75	1.20-2.54	0.003	1.03	0.64-1.65	0.915	1.50	0.90-2.52	0.123
16.1-21.0	1.29	0.92-1.81	0.147	1.55	1.07-2.24	0.020	1.20	0.75-1.91	0.443	1.35	0.82-2.24	0.239
>21.0	1.81	1.30-2.50	<0.001	2.60	1.69-3.98	<0.001	1.72	1.11-2.67	0.016	2.38	1.33-4.24	0.003
Red Blood Cell Folate (nmol/L)												
<615		1.00 (reference)			1.00 (reference)			1.00 (reference)			1.00 (reference)	
615-870	1.12	0.80-1.57	0.497	0.92	0.65-1.29	0.620	1.03	0.65-1.65	0.889	0.78	0.48-1.26	0.306
870-1290	1.07	0.77-1.49	0.675	0.93	0.66-1.32	0.694	1.13	0.72-1.76	0.604	0.82	0.51-1.31	0.406
>1290	1.23	0.89-1.71	0.213	1.01	0.71-1.43	0.980	1.18	0.75-1.85	0.483	0.83	0.51-1.36	0.457
Plasma Vitamin B12 (pmol/L)												
<174		1.00 (reference)			1.00 (reference)			1.00 (reference)			1.00 (reference)	
174-239	0.93	0.67-1.31	0.689	0.88	0.62-1.24	0.454	1.31	0.82-2.09	0.256	1.28	0.78-2.11	0.324
239-336	0.95	0.68-1.33	0.774	0.91	0.63-1.30	0.603	1.06	0.65-1.74	0.807	0.92	0.54-1.57	0.772
>336	1.20	0.87-1.66	0.261	1.19	0.83-1.71	0.336	1.49	0.94-2.37	0.092	1.69	1.01-2.85	0.046

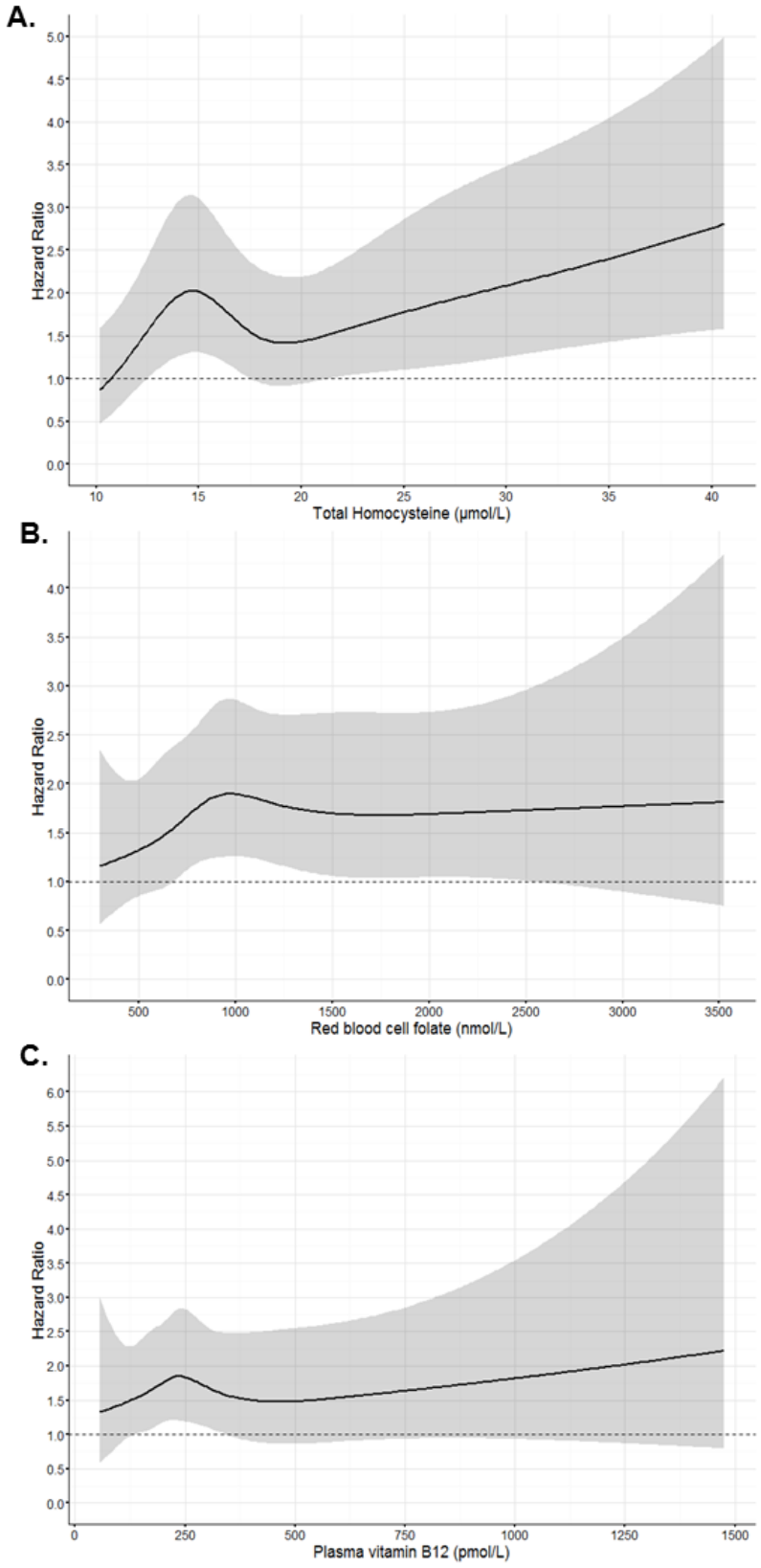
Model 1 is adjusted for education and housing; Model 2 is additionally adjusted for body mass index, physical activity, smoking, alcohol intake, disease count, mini-mental state examination score and the other 1-C metabolism biomarkers. The cardiovascular mortality model 2 was also adjusted for history of cardiovascular diseases, hypertension and diagnosis of diabetes type 1 and 2 instead of disease count. The homocysteine model is additionally adjusted for renal impairment. CI, confidence interval; HR, hazard ratio

Figure 7.4. Restricted cubic spline curves of dose-response relationship between one-carbon metabolism biomarkers and all-cause mortality in all participants.



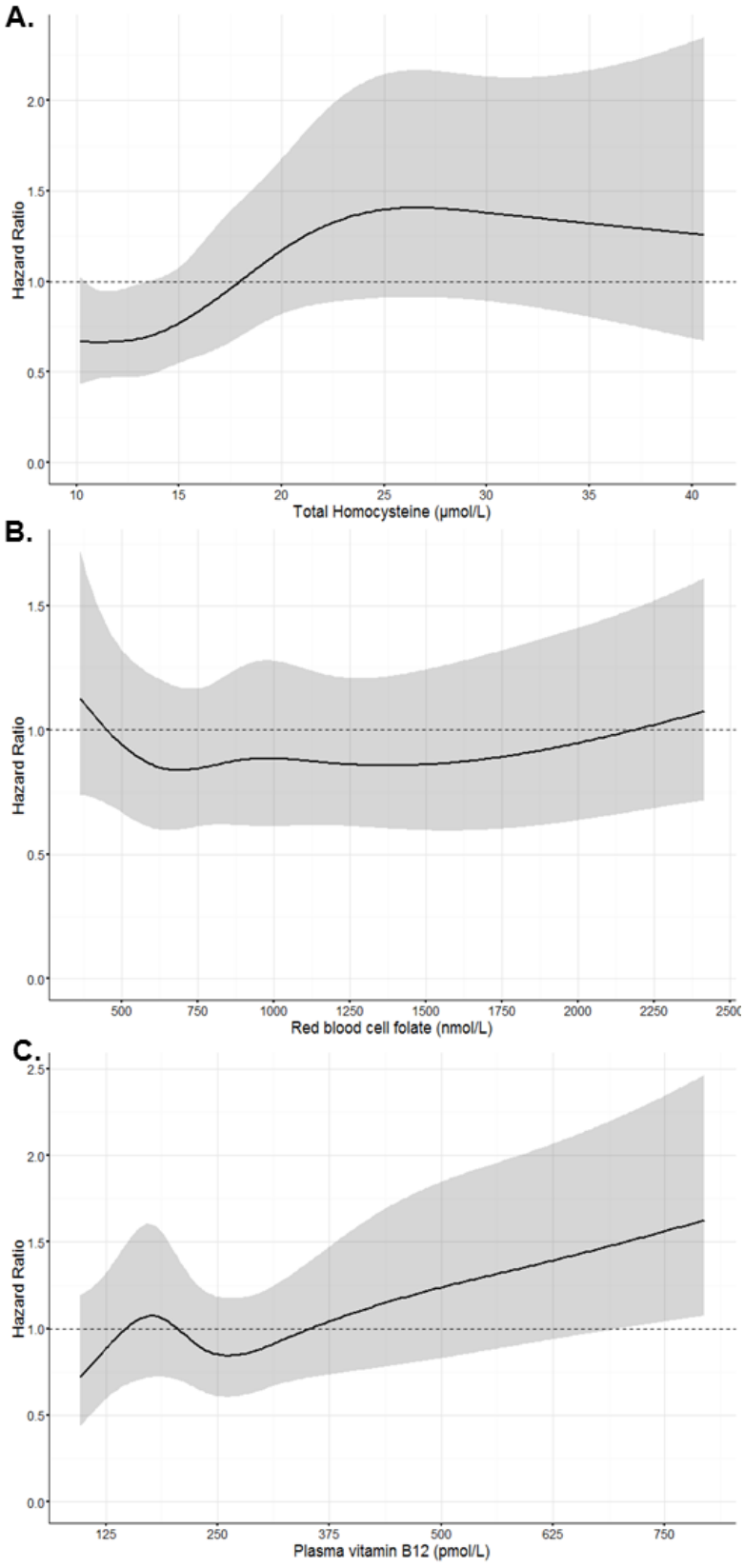
(A.) total homocysteine, **(B.)** red blood cell folate and **(C.)** plasma vitamin B12. Hazard ratios are from the fully-adjusted cox regression models.

Figure 7.5. Restricted cubic spline curves of dose-response relationship between one-carbon metabolism biomarkers and all-cause mortality in men.



(A.) total homocysteine, **(B.)** red blood cell folate and **(C.)** plasma vitamin B12. Hazard ratios are from the fully-adjusted cox regression models.

Figure 7.6. Restricted cubic spline curves of dose-response relationship between one-carbon metabolism biomarkers and all-cause mortality in women.



(A.) total homocysteine, **(B.)** red blood cell folate and **(C.)** plasma vitamin B12. Hazard ratios are from the fully-adjusted cox regression models.

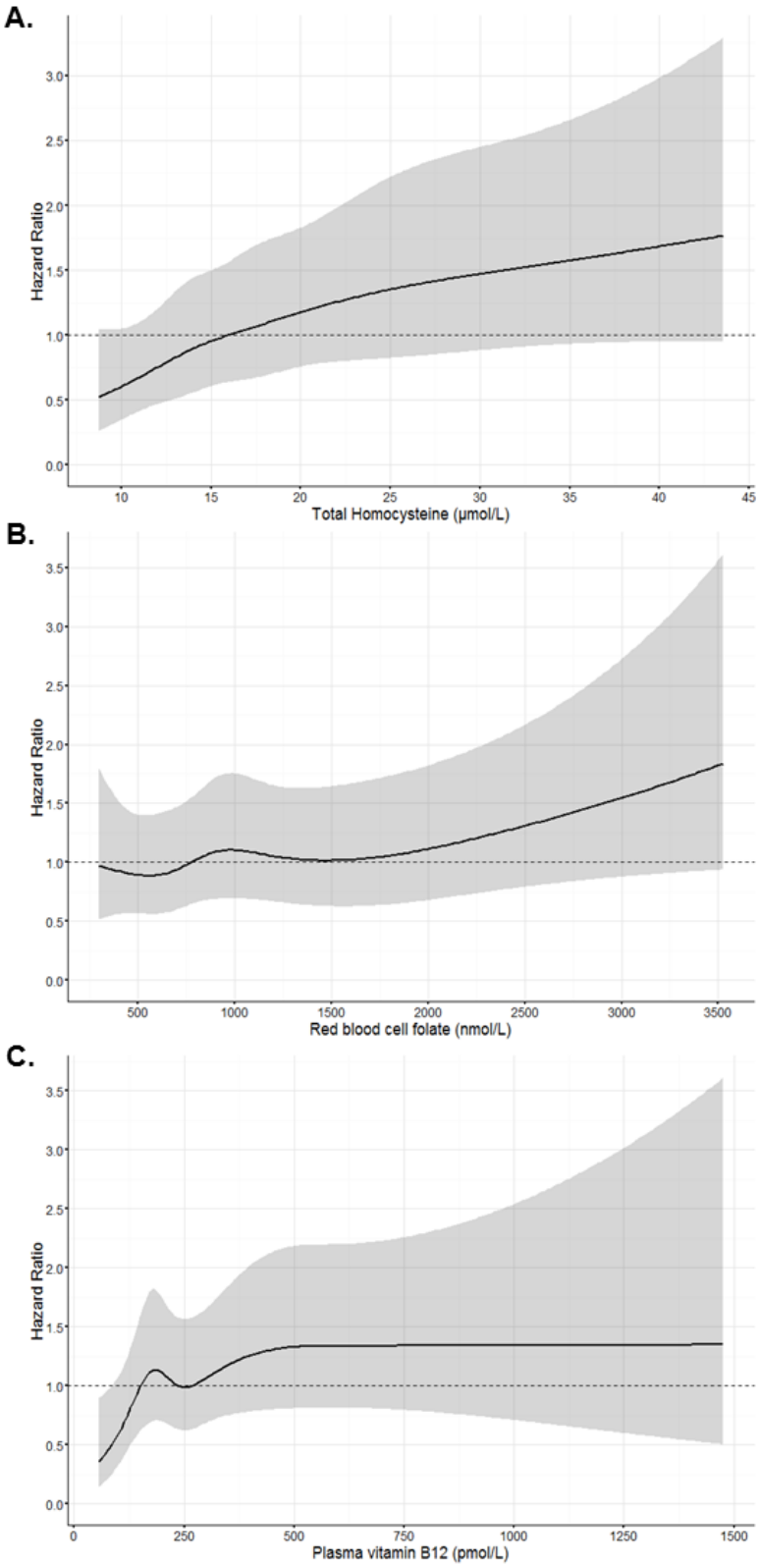
7.5. Supplemental material

Supplemental Table 7.1. Survival time and deaths by quartiles of biomarkers of one-carbon metabolism in the Newcastle 85+ Study.

Total homocysteine (µmol/l)	**Q1** (<13.5)	**Q2** (13.5-16.7)	**Q3** (16.7-21.4)	**Q4** (>21.4)
Survival time (years)	7.1 (3.3-8.3)	5.8 (2.5-8.0)	5.4 (3.2-7.9)	4.1 (2.4-7.5)
Deaths (%, n)	64 (122)	72 (138)	74 (142)	80 (154)
Cardiovascular deaths (%, n)	33 (62)	38 (73)	42 (81)	43 (82)
Red blood cell folate (nmol/L)	**Q1** (<612**)**	**Q2** (612-870)	**Q3** (870-1280)	**Q4** (>1280)
Survival time (years)	5.6 (2.9-8.1)	5.5 (2.5-8.1)	5.3 (2.9-7.9)	5.7 (2.6-7.9)
Deaths (%, n)	70 (130)	70 (133)	75 (140)	75 (141)
Cardiovascular deaths (%, n)	34 (64)	36 (68)	43 (79)	42 (80)
Plasma vitamin B12 (pmol/L)	**Q1 (**<170)	**Q2** (170-232)	**Q3** (232-325)	**Q4** (>325)
Survival time (years)	5.6 (3.3-8.2)	6.1 (2.2-8.0)	5.3 (2.6-7.9)	5.2 (2.5-7.9)
Deaths (%, n)	71 (134)	70 (130)	75 (140)	76 (142)
Cardiovascular deaths (%, n)	31 (59)	40 (74)	43 (81)	42 (78)

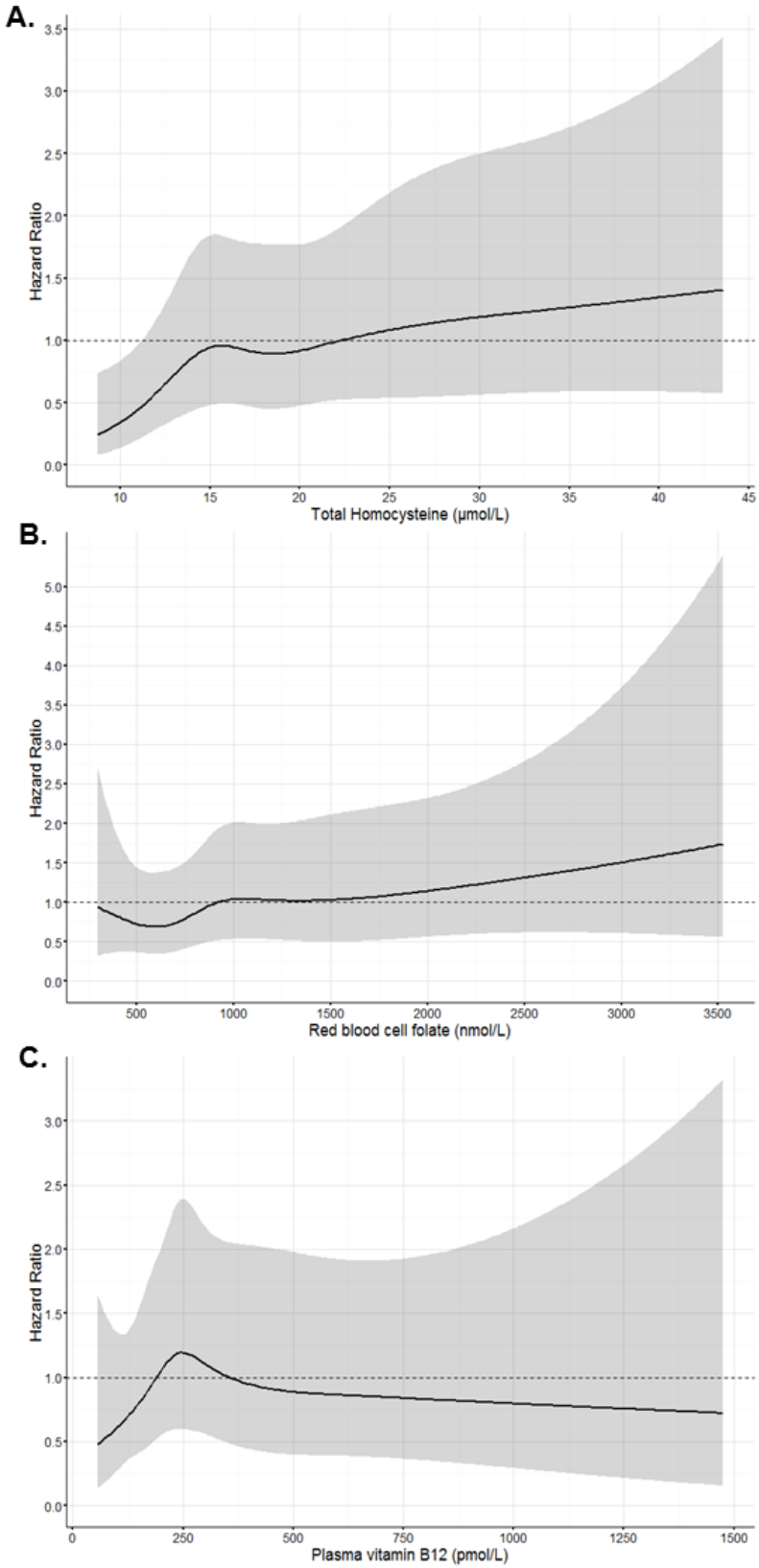
Values are medians and interquartile range unless otherwise mentioned. Q, quartile.

Supplemental Figure 7.1. Restricted cubic spline curves of dose-response relationship between one-carbon metabolism biomarkers and cardiovascular mortality in all participants.



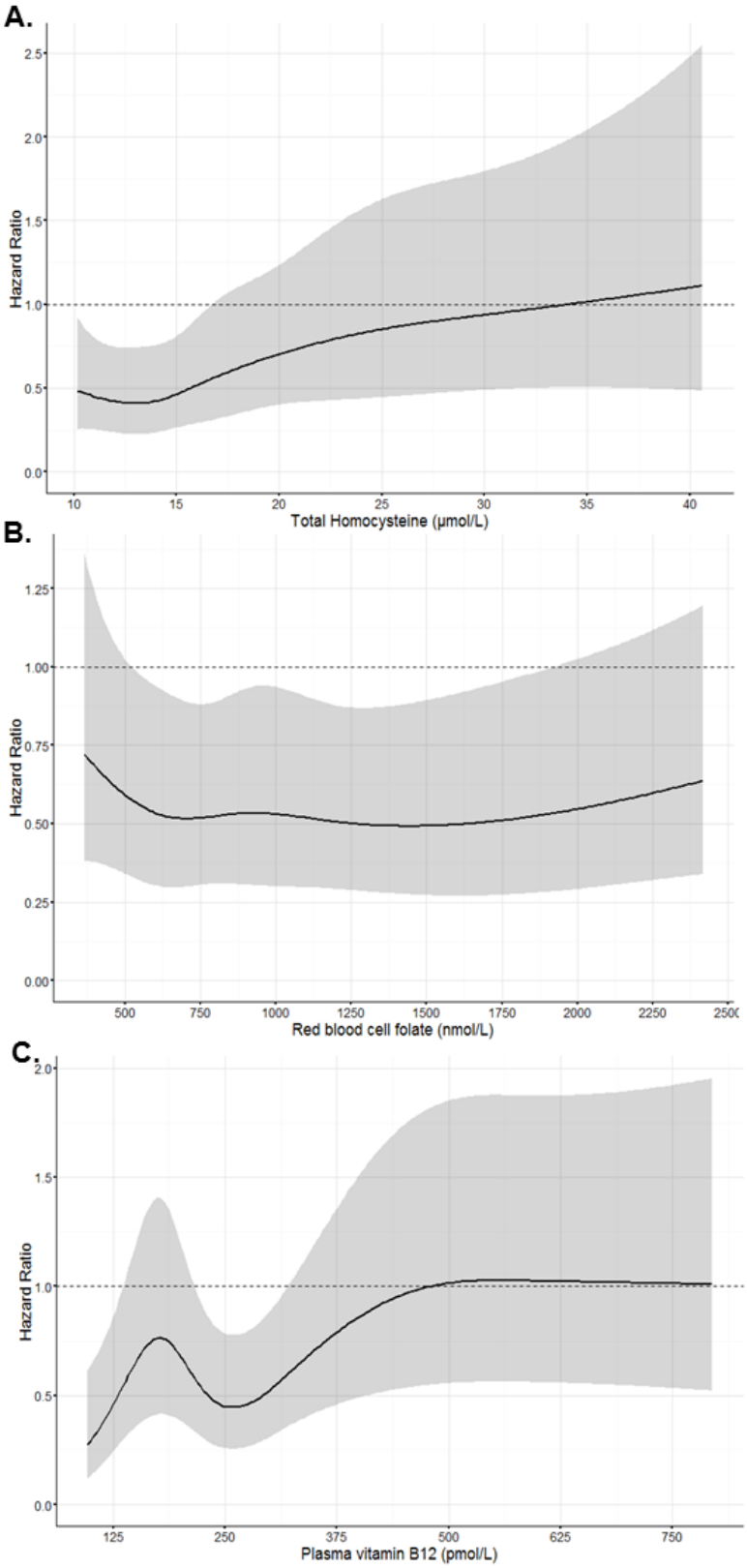
(A.) total homocysteine, **(B.)** red blood cell folate and **(C.)** plasma vitamin B12. Hazard ratios are from the fully-adjusted cox regression models.

Supplemental Figure 7.2. Restricted cubic spline curves of dose-response relationship between one-carbon metabolism biomarkers and cardiovascular mortality in men.



(A.) total homocysteine, **(B.)** red blood cell folate and **(C.)** plasma vitamin B12. Hazard ratios are from the fully-adjusted cox regression models.

Supplemental Figure 7.3. Restricted cubic spline curves of dose-response relationship between one-carbon metabolism biomarkers and cardiovascular mortality in women.



(A.) total homocysteine, **(B.)** red blood cell folate and **(C.)** plasma vitamin B12. Hazard ratios are from the fully-adjusted cox regression models.

Supplemental Table 7.2. Hazard ratios for all-cause and cardiovascular mortality by plasma vitamin B12 categories and sex.

Vit. B12 (pmol/L)		All-cause mortality						Cardiovascular mortality					
		Model 1			Model 2			Model 1			Model 2		
	n	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*
All Participants													
<148	124	0.90	0.71-1.14	0.369	0.83	0.64-1.08	0.166	0.62	0.43-0.89	0.009	0.59	0.40-0.88	0.010
148-500	549	1.00 (reference)			1.00 (reference)			1.00 (reference)			1.00 (reference)		
>500	53	1.23	0.89-1.69	0.209	1.42	1.03-1.96	0.034	1.06	0.68-1.66	0.791	1.34	0.84-2.11	0.218
Men													
<148	50	0.89	0.63-1.27	0.526	0.76	0.51-1.14	0.186	0.62	0.37-1.04	0.067	0.62	0.35-1.12	0.112
148-500	219	1.00 (reference)			1.00 (reference)			1.00 (reference)			1.00 (reference)		
>500	20	0.90	0.53-1.54	0.695	1.27	0.72-2.26	0.411	0.63	0.27-1.43	0.268	0.70	0.29-1.70	0.431
Women													
<148	74	0.90	0.65-1.24	0.512	0.82	0.58-1.17	0.279	0.61	0.37-1.02	0.058	0.51	0.29-0.90	0.021
148-500	330	1.00 (reference)			1.00 (reference)			1.00 (reference)			1.00 (reference)		
>500	33	1.67	1.12-2.49	0.012	1.70	1.13-2.56	0.011	1.66	0.97-2.84	0.065	2.04	1.17-3.55	0.013

Model 1 is adjusted for education and housing; Model 2 is additionally adjusted for body mass index, physical activity, smoking, alcohol intake, disease count, mini-mental state examination score and the other 1-C metabolism biomarkers. The cardiovascular mortality model 2 was also adjusted for history of cardiovascular diseases, hypertension, and diagnosis of diabetes type 1 and 2 instead of disease count. The homocysteine model is additionally adjusted for renal impairment. CI, confidence interval; HR, hazard ratio.

7.6. Summary

Folate and vitamin B12 are key to the correct functioning of one-carbon (1-C) metabolism. The current evidence on associations between 1-C metabolism biomarkers and mortality is inconclusive and generally based on younger populations. This chapter aimed to determine the associations between biomarkers of 1-C metabolism (tHcy, RBC folate and plasma vitamin B12) measured at baseline and all-cause and cardiovascular (CVD) mortality in 752-766 very old adults. Associations between biomarkers of 1-C metabolism and all-cause and CVD mortality for up to 9 years were assessed by Cox proportional hazard models and confirmed by restricted cubic splines. Participants with higher tHcy concentrations had twice the risk of death from any cause than those with lower concentrations (e.g. Q4 vs. Q1, HR: 2.05, 95% CI: 1.51-2.77, $p<0.001$) after adjustment for sociodemographic, lifestyle and health variables. Women with elevated plasma vitamin B12 concentrations (>500 pmol/L) had increased risk of all-cause mortality (HR: 1.70, 95% CI: 1.13-2.56, $p=0.011$) compared with those with concentrations 148-500 pmol/L. Higher concentrations of tHcy and plasma vitamin B12 were associated with increased risk of all-cause and CVD mortality in the very old. This confirms findings for tHcy in younger populations but the adverse relationships between elevated plasma vitamin B12 concentrations and mortality in this population are novel and require further investigation.

Chapter 8 is the final chapter of this thesis and will summarise the main findings from the 5 experimental chapters (Chapter 3: Macronutrient intake and food sources; Chapter 4: Micronutrient intake and food sources; Chapter 5: Intakes of folate and vitamin B12 and biomarkers of status; Chapter 6: One-carbon metabolism biomarkers and cognitive decline; and Chapter 7: One-carbon metabolism biomarkers and all-cause and cardiovascular mortality in the very old). It will also discuss the chapters separately, compare them with current relevant literature, state each chapter's strengths and limitations, explore the potential public health implications of the findings and provide recommendations for future research.

CHAPTER EIGHT

8. Discussion and Conclusion

8.1. General findings

There is a dearth of data on dietary intake, nutritional status and associations with health trajectories in the very old in the UK and elsewhere. This thesis successfully used a unique cohort of more than 800 very old adults to reach the objectives laid out in *Chapter 1, Introduction - 1.14 Objectives*. The overall objective of providing an accurate snapshot of the dietary habits of the very old and examine health trajectories with respect to 1-C metabolism biomarkers in the Newcastle 85+ Study was broadly achieved. These are the general findings while specific results will be discussed for chapter 3 to 7 in sections 8.2 to 8.6.

- Participants in the Newcastle 85+ Study had a median energy intake of 6.7 MJ per day where 47%, 37% and 16% were from carbohydrate, fat and protein respectively;
- Median folate intake was 208 μg/day and vitamin B12 was 2.9 μg/day;
- Intake of several macro and micronutrients did not meet the dietary reference values (DRV) currently used in the UK but its health significance in the very old is largely unknown;
- Participants who had higher levels of education, higher social class or were more physically active, had more nutrient-dense diets;
- Cereals and cereal products (CCP) were the top contributors for energy, most macronutrients and several micronutrients, including folate and vitamin B12, followed by meat and meat products;

- Median folate and vitamin B12 status was 863 nmol/L and 232 pmol/L, respectively;
- Folate from CCP may be more bioavailable than from other food sources and the relationship between vitamin B12 intake and status was weak;
- Participants with a higher folate status at baseline had better global cognition over 5 years but there was no difference in the rate of cognitive decline;
- Those with lower concentrations of tHcy performed better in global cognition tests but only in cross-sectional analyses;
- Vitamin B12 status was not predictive of cognitive function and rate of cognitive decline;
- Higher concentrations of tHcy were predictive of all-cause mortality and CVD mortality in the very old;
- Women with elevated vitamin B12 concentrations were at higher risk of death from all-causes.

8.2. Macronutrient intake and food sources

Main findings

Participants in the Newcastle 85+ Study had a median energy intake of 6.65 MJ where 46.8%, 36.8% and 15.7% were from carbohydrate, fat and protein respectively. Cereals and cereal products (CCP) were the top contributors to most macronutrients, followed by meat and meat products. Only 20% of the cohort had higher energy intakes than the estimated average requirement (EAR). This recommendation was established for those aged ≥ 75 years and its relevance for those aged 85 years is unclear. Most participants did not meet the DRV for non-starch polysaccharide (NSP) and saturated fatty acid (SFA) intake (91% and 72%, respectively). NSP intake was higher in non-institutionalised, more educated, from higher social class and more physically active 85 year olds.

Comparison with other studies

The Newcastle 85+ Study is the first to investigate the dietary intakes of such a large and sociodemographically representative sample of 85 year olds in the UK. There have been two earlier studies with considerable numbers of 85 year olds: the 1994-95 NDNS of people aged 65 years old and over (172 men and 287 women aged 85 and over) [10] and the

European Prospective Investigation into Cancer and Nutrition (EPIC)-Oxford study (411 men and 872 women aged 80 and over at the third follow-up in 2010-2014) [14].

Non-institutionalised (standard and sheltered housing) very old adults (aged 85 and over) in the 1994-95 NDNS had lower median energy (6.99 MJ and 5.60 MJ in men and women, respectively), carbohydrate, fat and protein intakes but higher non-milk extrinsic sugar (NMES), SFA, monounsaturated (MUFA) and polyunsaturated fatty acid (PUFA) intake than the Newcastle 85+ participants [49]. The food groups used in the Newcastle 85+ Study are comparable with those used in the NDNS and our analysis showed that food sources of macronutrients for non-institutionalised very old adults were similar in both studies. However, very old respondents in the NDNS derived more energy (15% vs. 9%) and SFA (29% vs. 19%) from milk and milk products, more carbohydrate (13% vs. 9%) and NMES (44% vs. 32%) from sugar, preserves and confectionery and, more NSP from CCP (50% vs. 42%) than in the Newcastle 85+ Study cohort [49]. While the Newcastle 85+ Study included 85 year olds only, the 1994-95 NDNS included those aged 85 and over. Further, dietary data collection diverged by more than a decade which might reflect not only different dietary habits but also different prevalence of age-related diseases and disabilities [135].

At the third follow-up, EPIC-Oxford participants had higher mean energy intake (9.84 MJ and 9.02 MJ in men and women, respectively), percentage of energy from carbohydrate, NSP intake and P: S ratio than the Newcastle 85+ Study participants but fat and SFA did not contribute to energy intake as much (personal communication from Professor Tim Key and Dr. Paul Appleby). Some notable differences between the two studies include different ages (80 and over vs. 85 year olds), dietary assessment methods (food frequency questionnaire vs. 24hr-MPR), dietary habits (14% of the participants aged 80 and over in the EPIC-Oxford were vegetarians or vegans) and use of different descriptive statistics to report the results (mean vs. median).

It will take several years until the current NDNS rolling programme has similar numbers of very old adults to compare with the Newcastle 85+ Study. Until then, the contemporary dietary data from the Newcastle 85+ Study are likely to be the most reliable for this age group. However, younger counterparts in the NDNS (191 men and 237 women aged 65 and over - weighted) had similar macronutrient intakes to those of the Newcastle 85+ Study cohort except for the percentage of energy from protein and NSP intake, which were higher in the NDNS [74,177]. The top food group contributors did not greatly differ but sugar, preserves and confectionery contributed less to NMES intake and CCP and oils and fat

spreads contributed less to total fat intake in the NDNS current rolling programme' older adults than in the Newcastle 85+ Study [74,177]. Differences between studies might reflect age-specific dietary habits.

Significance and explanations

A European Society for Clinical Nutrition and Metabolism (ESPEN) consensus statement defines unintentional weight-loss as >5% over 3 months or >10% of habitual weight (irrespective of time) as one of the criteria for diagnosing malnutrition [204]. The majority of the Newcastle 85+ Study participants did not meet the current energy EAR at baseline which might lead to unintended weight-loss and account for the increased malnutrition risk in this age group. Follow-up analysis of weight change may help to confirm this finding.

Failure to meet the NSP intake DRV of 18g per day might be a cause for concern in very old adults since it can contribute to the high prevalence of constipation [205]. Non-compliance with the NSP's DRV was significantly higher in women than in men, partly because the recommendations do not differ between sexes [69]. The Scientific Advisory Committee on Nutrition (SACN) recommended that the DRV of dietary fibre be increased to 30g per day as measured by international methods (an increase from 18g/day to 23g/day of NSP by the Englyst method) [206]. In comparison with the proposed new reference values, only 16 participants (eleven men and five women) or 2% would meet the DRV for dietary fibre. CCP (half from bread) and vegetables (half from cruciferous vegetables and peas) together account for 64.3% of the NSP intake. Efforts to reduce the burden of constipation and increase NSP intake should focus on these food sources. The use of laxatives (bulk forming, stimulant or osmotic) was collected by the Newcastle 85+ Study and will be explored in a future publication.

Median NMES intake in the Newcastle 85+ Study exceeded the DRV for NMES of less than 11% of dietary energy [69] and would exceed the SACN DRV for free sugars (or NMES) of 5% of total energy intake [206]. This is a consequence of 89% of men and 86% of women who would exceed the new DRV. These findings may be a concern because high NMES intake is strongly associated with dental caries in older people [23].

A plethora of socioeconomic, biological and lifestyle characteristics change with advancing age which may place very old adults at increased risk of poor nutrition. However, the lack of robustly-based dietary recommendations for very old people limits our ability to

interpret the dietary intakes of the Newcastle 85+ Study participants by reference to age-appropriate DRVs. Given the projected increase in the numbers of very old people in the UK [7], filling this evidence gap should have high priority [207].

Malnutrition is prevalent among institutionalised older adults in the UK [208]. In our study, participants living in institutional housing had higher intakes of energy and percentage of energy from NMES while NSP intake and percentage of energy from MUFA and PUFA was lower than for those living in standard housing. Despite the numerous challenges of dietary assessment in an institutional setting, a higher prevalence of disabilities in institutionalised than in non-institutionalised very old adults may explain these differences.

SES is frequently associated with diet quality and this was also seen in the Newcastle 85+ Study [209]. Participants who were more educated or from higher social class (NS-SEC) had higher NSP intakes than those less educated and from a lower social class. Healthy behavioural habits tend to cluster together (healthier diet, higher physical activity, non-smoking, moderate or low alcohol intake) [210]. In the Newcastle 85+ Study, physically active 85 year olds had a higher intake of NSP, MUFA, PUFA and protein than those with low physical activity.

Strengths and weaknesses

The challenges of dietary assessment in this age group were discussed in *Chapter 2, General Methods - 2.2 Dietary intake assessment and food group allocation.* General strengths and weakness of the Newcastle 85+ Study were discussed in *Chapter 2, General Methods - 2.1 The Newcastle 85+ Study.* Thirty-five percent of the 24hr-recalls were performed during summer (June-August) while the rest were evenly distributed throughout the other three seasons. Seasonality is known to influence nutrient intake but the slight bias towards summer is unlikely to have changed the results. For practical reasons, 24hr-MPRs were not conducted during the weekend, therefore food and drink eaten on Fridays and Saturdays was not recalled. Misreporting was estimated to be 26% [211]. Twenty-seven percent (n=214) of the participants were classified as cognitively impaired at baseline (SMMSE ≤25) which was associated with misreporting (OR 1·61; 95% CI 1·11, 2·33, P =0·012). Since most of the estimated misreporting is underreporting (22%)[211], dietary intakes may have been underestimated.

8.3. Micronutrient intake and food sources

Main findings

Median vitamin D, magnesium, potassium and selenium intake were 2.0 (IQR:1.2-6.5) µg/day, 215 (IQR:166-266) mg/day, 2477 (IQR: 1890-3023) mg/day and 39.0 (IQR:27.3-55.5) µg/day, respectively. Median folate intake was 208 (157-264) µg/day and vitamin B12 was 2.9 (1.9-4.4) µg/day. Participants who spent more full-time years in education, were from higher social class and were more physically active had more nutrient-dense diets in several vitamins and minerals. The most notable finding is that 20% or more of the participants in the Newcastle 85+ Study had intakes below the LRNI for magnesium, potassium and selenium and that more than 95% of participants were below the RNI of 10 µg/day of vitamin D.

Comparison with other studies

Since the 1994-95 NDNS of people aged 65 and over, which included 172 men and 287 women aged 85 and over (all non-institutionalised), no study has described micronutrient intakes and food sources in a large sample of very old adults in the UK. Most vitamin and mineral intakes were similar between the 1994-95 NDNS and the Newcastle 85+ Study except for β-carotene (1141 vs. 1516 µg/day), vitamin C (41.4 vs. 56.5 mg/day) and calcium (644 vs. 731 mg/day) which were higher in the latter (intakes from food sources only)[49]. In the 1994-95 NDNS, less vitamin A (34% vs. 40%) and vitamin B12 (43% vs. 53%) were derived from meat and meat products and less potassium from non-alcoholic drinks (10% vs. 19%). However, more vitamin B12 (29% vs. 13%), calcium (54% vs. 31%) and potassium (20% vs. 9%) came from milk and milk products in the 1994-95 NDNS than in the Newcastle 85+ Study. The food sources of vitamin D were considerably different between the studies with fish and fish dishes making a lower contribution to intake (17% vs. 34%) while fat spreads made a higher contribution (23% vs. 8%) in the 1994-95 NDNS than in our study [49]. The observed differences are unlikely to be due to fortification policies. The Newcastle 85+ Study included 85 year olds only while the 1994-95 NDNS included those aged 85 and over. Other possible reasons include different dietary assessments (4-d weighted diet record vs. 2x24hr-MPR) that diverged by more than a decade.

The European Prospective Investigation into Cancer and Nutrition (EPIC)-Oxford third follow-up questionnaire in 2010-2014 included 411 men and 872 women aged 80 and over [14]. Intakes of all vitamins and minerals were at least 20% higher in the EPIC-Oxford than in the Newcastle 85+ Study participants (personal communication with Professor Tim Key and Dr. Paul Appleby). Different descriptive statistics and dietary assessment methods used, different ages (≥80 vs. 85 year olds) and characteristics of the participants (14% of EPIC-Oxford participants were vegetarians) are potential explanations for the wide differences observed in micronutrient intake.

The current NDNS rolling programme (from 2008/2009 to 2011/2012 or years 1 to 4) does not yet have enough very old adults for comparison with our results. Nonetheless, it included 428 adults (191 men and 237 women) aged ≥65 [74]. Although energy intakes were similar between both studies, vitamin and mineral intakes (without supplements) were slightly higher in the NDNS than in the Newcastle 85+ Study (except for sodium where intakes were 1947 and 2383 mg/day, respectively). More than 10% of the participants had intakes for magnesium, potassium and selenium below the LRNI [74]. Similarly, >20% of the Newcastle 85+ Study participants were also below the LRNI for these minerals.

Significance and explanations

In this chapter, men had higher energy intakes than women therefore, it was expected that intakes of most micronutrients by men were also higher. However, when vitamin and mineral intakes were expressed per 1 MJ, vitamin A, C and calcium were higher in women than in men. Conversely, men's diets were more nutrient-dense in vitamin B12, iron and selenium than women's. Higher meat and meat products consumption by men was the main driver for these differences.

Several micronutrient intakes were lower than the current DRVs. Twenty percent or more of the participants were below the LRNI for magnesium, potassium and selenium while 95.3% were below the RNI for vitamin D [the Scientific Advisory committee set the same RNI as the Committee on Medical Aspects of Food and Nutrition Policy [212]]. This is of concern because magnesium is associated with physical performance [213], systemic inflammation, endothelial function [214] and bone mineral density in older adults [215]; inadequate selenium has been linked with anaemia [216], cancer and all-cause mortality [217]; and low vitamin D intake has consistently been associated with musculoskeletal [67] and extra-skeletal outcomes including cognitive impairment and mortality [29,30]. However, the major

"inadequacy" in vitamin D intake may not reflect vitamin D status since circulating concentrations of 25-hydroxyvitamin D depend largely on sun exposure [67]. Higher potassium intakes are a known protective factor for hypertension [218] whereas excessive sodium intake is a risk factor for hypertension in older adults [219]. In this chapter, only a fifth of the participants were below the RNI of 1600 mg per day of sodium but half met the recommendation of less than 2400 mg per day. Sodium intake reduction and increased potassium intake might help reduce the prevalence of stroke and fatal coronary heart disease in this population [220].

More than 10% of participants had vitamin A intakes below the LRNI but, interestingly, 5% had intakes above the upper level (UL) of 3000 µg-RE per day set by the European Food Safety Authority (EFSA) [221]. This classic paradox may not be the result of habitual intake, but the result of consuming high vitamin A content foods (e.g. liver and liver dishes) on one or more of the non-consecutive 24h recalls of the 24hr-MPR [222]. In fact, 35 out of the 36 participants who had vitamin A intakes above the UL of 3000 µg-RE ate liver and liver products on at least one of the 24hr-MPR.

Assessing micronutrient intake inadequacies in this age group has several methodological limitations. Further, due to a scarcity of nutrition data in this age group, most DRVs were extrapolated from younger populations. This leads to uncertainty regarding the health significance of inadequacies in the very old.

In line with previous studies [209] and a recent review on socioeconomic determinants of micronutrient intakes in older adults [223], participants with more education and from a higher social class had overall higher micronutrient intakes. Similarly, perhaps because healthy habits cluster together, those who were more physically active had more nutrient dense diets. It has been argued that nutrient-dense foods are more expensive than less healthy foods in the UK and United States of America (USA) [224,225] and this price differential might explain the difference in nutrient density between lower and higher socio-economic (SES) groups. However, others have challenged the view that healthier foods or dietary patterns are more expensive than unhealthy ones and e.g. price differentials are dependent on the unit of comparison (e.g. per calorie, per mass) [226,227]. Physical proximity to (and/or means to access) fresh-produce stores has been proposed as an explanation for higher micronutrient intakes in high SES groups [228] but this is somewhat debatable in the UK and North-East England [229]. Inaccessibility to fresh produce, higher cost of nutrient-dense foods in the UK and poorer food choices [230] are some of the potential causes that mediate the

diet quality gradient between SES groups. In this age group, with more disabilities and lower income, these issues might be exacerbated.

Strengths and weaknesses

Although vitamins and minerals are not abundantly present in commonly underreported foods, such as sweets and snacks, the inherent retrospective nature of the 24hr-MPR might have proved challenging for some individuals in this age group. The challenges of dietary assessment in this age group were discussed in *Chapter 2, General Methods - 2.2 Dietary intake assessment and food group allocation*. To reduce patient and interviewer burden, only qualitative data on supplement use were collected. Therefore, the frequency of supplement use had to be estimated based on the manufacturer's recommendations. Data on sodium derived from table salt and salt used in cooking was not recorded which might have underestimated sodium intake in the Newcastle 85+ Study. General limitations and strengths of the Newcastle 85+ Study were discussed in *Chapter 2, General Methods - 2.1 The Newcastle 85+ Study*.

8.4. Intakes of folate and vitamin B12 and biomarkers of status

Main findings

This study found that, in the very old, folate intakes from all food sources and from cereals and cereal products were significantly associated with RBC folate. Individuals with higher total folate intake or intake from cereals and cereal products were less likely to have low concentrations of RBC folate. The association between vitamin B12 intakes and plasma vitamin B12 was weak. Individuals with vitamin B12 intakes from all food sources and from meat and meat products were also less likely to be deficient for plasma vitamin B12. In addition, higher concentrations of RBC folate were found in participants with the *MTHFR* (rs1801133) AA genotype compared with those with A/G or GG genotypes. Women homozygous for the *FUT2* (rs492602) G allele also had higher concentrations of plasma vitamin B12 than those with other *FUT2* genotypes.

Folate and vitamin B12 intake and status "inadequacies"

The median (IQR) RBC folate and plasma vitamin B12 concentration was 863 (451-1287) nmol/L and 232 (170-324) pmol/L, respectively. In the Newcastle 85+ Study there was a relatively low prevalence of "inadequate" folate intake (3.1%) and status (3.6%). The NDNS

rolling programme estimated that 1% of older adults (aged 65 and over) were below the UK LRNI for folate but that 7.3% of men and 10.8% of women had RBC folate concentrations <340 nmol/L. In the Newcastle 85+ Study, plasma vitamin B12 deficiency (<148 pmol/L) was present in 17.1% of participants and 9.2% were below the UK LRNI (1 µg/d) for vitamin B12 intake whilst the NDNS estimated that 5.9% had serum vitamin B12 concentrations <150 pmol/L but only 1% were below the LRNI for vitamin B12 [74]. Age, dietary assessment method (4-d weighted diet record vs. 2x24hr-MPR) and other methodological differences are likely explanations for these observed discrepancies. Specifically, the novel method used to assess RBC folate in the NDNS (whole blood folate by a microbiological assay, serum total folate by LC-MS/MS and hematocrit) is likely to give higher estimates of folate "inadequacy" than the one used in this chapter. The NDNS used a similar method to the Newcastle 85+ Study to assess plasma vitamin B12 (competitive immunoassay with direct chemiluminescence (ADVIA Centaur B12 assay)).

In the post-fortification period in the U.S., less than 1% of older adults had deficient folate status (RBC folate <340 nmol/L) [231]. In the National Health and Nutrition Examination Survey (NHANES) 2003-2006, it was estimated that 9% of men and 24% of women > 70 years old were below the North American EAR for folate [320 DFE/d [232]] [233]. In the same NHANES edition, 19% of older adults had plasma vitamin B12 concentrations below 221 pmol/L [234]. Moreover, less than 1% of men and 6% of women >70 years were below the EAR for vitamin B12 (2 µg/d) [233]. Almost 30% (n=235) of the Newcastle 85+ Study participants were below the same EAR for vitamin B12 intake.

Association between folate intake and status

Folate intake from total diets and from cereals and cereal products but not from vegetables or fruit and fruit juice were associated with RBC folate concentrations in the very old. Vegetables and fruit and fruit juice contributed to 25% (16% and 9%, respectively) to folate intake and the relatively lower contribution might explain the lack of associations. Further, folate bioavailability is dependent on the food matrix, stability of labile folates, presence of vitamin C and folate-binding proteins and folate pool sizes [89,93,94]. Nonetheless, there is a consensus that folic acid is better absorbed than dietary folate. Evidence also shows that that folic acid intake is a stronger predictor of RBC folate concentration than total folate intake [95,235]. The US Institute of Medicine estimated that the absorption efficiency of folic acid in supplements or fortified food was 85% taken with food or 100% from

supplements taken on an empty stomach [89] whilst dietary folate absorption efficiency was 50% [89,94]. Breakfast cereals (grouped under cereals and cereal products in this chapter) have frequently been the target of voluntary fortification in the UK and elsewhere which might explain the stronger association between folate intake from cereals and cereal products and RBC folate concentrations. Cereals and cereal products were also the top contributors to folate intake (32%), suggesting that this is an important source of folate/folic acid in the very old. On the other hand, the incomplete release of dietary folate from plant foods cellular structures may explain a weaker association between folate from vegetables and fruit, and RBC folate.

Association between vitamin B12 intake and status

Total vitamin B12 was weakly associated with plasma B12 in the very old (p=0.054) and seemed to saturate at intakes ≈ 10 µg. The relatively weak association might be due to the low vitamin B12 intakes in relation to the large liver stores (1 µg/g of liver) so that intakes only slowly influence plasma concentrations [112]. Further, vitamin B12 absorption is complex. Bound to protein in food, vitamin B12 has to be released by pepsin and hydrochloric acid in the stomach. The ensuing free form of vitamin B12 binds to haptocorrin, forming a B12-haptocorrin complex. This complex is later broken down in the small intestine by pancreatic proteases which enable vitamin B12 to bind to the glycoprotein IF, be recognized (by cubilin) and absorbed by endocytosis in the enterocytes of the distal ileum. These steps present a problem to older adults as 10-30% have atrophic gastritis and therefore, reduced gastric acid secretion which is essential to vitamin B12 release from food proteins [64]. The bioavailability of vitamin B12 in any form or dose is estimated to be 40% in healthy adults with intact IF secretion [112]. Meat and meat products, milk and milk products and, fish and fish products were the top sources of vitamin B12 in the Newcastle 85+ Study [188]. However, unlike some findings [236,237] but in agreement with others [238], vitamin B12 intake from meat and meat products was not associated significantly with plasma vitamin B12. Meat and meat products, especially liver [beef liver can reach to as much as 83 µg per 100 g [178]] and ruminant meat have very high concentrations of vitamin B12 and it is reported that ileal receptors saturate with intakes of 1.5-2.5 µg of vitamin B12 per meal [239]. Only 50% and 5% of vitamin B12 are absorbed with intakes of ~1 and 25 µg, respectively [112]. Others have found that vitamin B12 from dairy products is very bioavailable [240] but also that vitamin B12 in yogurt and cheese is not as bioavailable as that in milk in older individuals

[238]. This could explain why vitamin B12 intake from dairy products (that includes yogurt and cheese) was not associated with plasma B12 in this chapter.

MTHFR and RBC folate, and FUT2 and plasma vitamin B12

Interestingly, and in contrast to most previous findings [241], participants heterozygous for the A allele of the *MTHFR* gene had higher concentrations of RBC folate than those homozygous for the G allele. This was not a reflection of higher folate or folic acid containing supplements intake. However, Molloy AM *et al*. showed that there might be important discrepancies in serum folate or RBC folate concentration by *MTHFR* genotype whether protein-binding assays (such as the chemiluminescence immunoassay used in the Newcastle 85+ Study) or microbiological assays (generally regarded as the "gold standard") were used [99]. Molloy AM *et al*. proposed that a degradation product or a form of folate would accumulate in individuals homozygous for the A allele (where the MTHFR reductase's activity is decreased) and bind in the protein-binding assay. Therefore, the resulting measured folate concentration would appear active but not be microbiologically detectable [99].

Similar to previous findings [117,118,241], women with the *FUT2* GG genotype had higher concentrations of plasma vitamin B12. *FUT2* encodes galactoside 2-alpha-L-fucosyltransferase 2 (EC:2.4.1.69) which is involved in the regulation of the H antigen and is a precursor of the ABO(H) antigens [118]. *FUT2* variants (from the allele A) are proposed to be protective against *Helicobacter pylori* infection or to increase IF production [117]. Both proposed explanations would explain the higher plasma vitamin B12 concentrations in those homozygous for the G allele.

Strengths and weaknesses

As dietary intake assessment consisted of a 24hr-MPR applied on two non-consecutive days, the possibility of unusually high or low vitamin B12/ folate intakes cannot be excluded. Even though the food groups used in the analysis contributed to most of the folate and vitamin B12 intake, other food sources might explain the remaining intake. Intakes of dietary folate equivalents (DFE) and of the crystalline form of vitamin B12 could not be determined because supplement use was collected qualitatively (type and brand but not frequency) and it was not certain which specific foods had been fortified during the dietary collection period (2006/2007). A general limitation of most dietary surveys including

ours is that assessment of supplement usage may not be accurate by dietary intake records, dietary recalls and other questionnaires [73]. Furthermore, the irregular use of supplements by survey participants including the alteration of usual patterns of supplement use during the period of dietary data collection further adds bias to the estimation of true supplement use [73]. It is worth mentioning that the choice of vitamin B12 form used in supplements and fortified foods should also be taken into consideration because of concerns associated with cyanide/thiocyanate from cyanocobalamin [242].

Holotranscobalamin measures the vitamin's active form and because it might better reflect vitamin B12 status than plasma vitamin B12, its use might have yielded different results. There is currently no consensus on the biochemical threshold to use to define folate or vitamin B12 "inadequacy", especially in this population. Therefore, results from the binary logistic models might be different if different thresholds were used. Furthermore, atrophic gastritis impairs folate and vitamin B12 absorption. If available, the incidence of atrophic gastritis or a proxy measure such as *Helicobacter pylori* infection could have been used as an adjusting factor or to conduct a sensitivity analysis. The list of SNPs used is not exhaustive and other polymorphisms, such as some SNPs in the *TCN2* gene (e.g. rs731991) may influence the folate and vitamin B12 intake-status relationship [243]. General limitations and strengths of the Newcastle 85+ Study were discussed in *Chapter 2, General Methods - 2.1 The Newcastle 85+ Study*.

8.5. One-carbon metabolism biomarkers and cognitive decline

Main findings

This study showed that very old adults in higher quartiles of RBC folate (e.g. Q4 vs Q1, β=+1.02, SE=0.43, p=0.02) and lower quartiles of tHcy concentration (Q4 vs Q1, β=-1.05, SE=0.46, p=0.02) measured at baseline were associated with better global cognition (SMMSE) over 5 years. In contrast, plasma vitamin B12 was not associated with cognition (global and attention) at any time points. RBC folate, plasma vitamin B12 and tHcy concentration were not predictive of the rate of decline in SMMSE and attention, except for higher tHcy concentration, which was associated with slower decline in focused attention (PoA) over 3 years.

Comparison with other studies

Results of folate and global cognition and/or specific cognitive domains in prospective cohort studies are frequently in disagreement. This study confirmed findings from studies which showed that folate, as measured by serum folate or RBC folate, was associated with global cognition or specific cognitive domains [81,82,143,244] while others did not find these relationships [107,144,146]. However, apart from Leiden 85-Plus, these studies focused on younger populations.

Voluntary folic acid fortification of cereal grains products introduced in 1996 and changed into mandatory fortification in 1998 might partly explain the lack of associations between folate and cognitive performance in some studies as it is difficult to find overt folate deficiency in the U.S. [146]. The median (IQR) RBC folate concentration in our cohort was 863 (IQR:451-1287) nmol/L and only 4% (n=26) had concentrations <340 nmol/L. With reference to cut-offs derived from younger populations, this RBC folate concentration might be considered replete which could also explain the lack of associations with cognitive decline. The choice of biomarkers to assess folate status also deserves some attention as studies that did not find an association [107,144,146] used serum folate and not RBC folate, which more closely reflects long term status.

In contrast with our findings, some studies have reported that holotranscobalamin or plasma vitamin B12 concentration was predictive of cognitive decline [107,143,245-247] while others failed to find an association [81,82,144,146,244,245]. As with this chapter, some studies [81,82,107,123,143] but not all [144,244,246] have reported that tHcy concentration was associated with global cognition or specific cognitive domains measured over time. The Leiden 85-Plus Study had a very similar design to the Newcastle 85+ Study and included 599 adults aged 85 years old at baseline. Participants were followed for 4 years and global cognitive function was assessed by the MMSE while attention was measured by the Stroop test. Mooijjart *et al* reported similar findings to our study. Low serum folate and high tHcy, but not serum vitamin B12, were associated with cognitive impairment cross-sectionally, but these biomarkers did not predict cognitive decline or rate of cognitive decline over 4 years in the Leiden 85-Plus [144]. In the same study, tHcy concentration was associated with attention at baseline but not folate or vitamin B12 and not longitudinally [144].

The lack of specificity of the assay used for plasma vitamin B12 may explain the lack of associations between cognitive decline and vitamin B12 in the Newcastle 85+ Study and the Leiden 85-Plus. The assay assesses not only the vitamin's active form,

holotranscobalamin which makes up 20-30% of plasma vitamin B12, but also the other 70-80% bound to haptocorrin and considered inert [100]. The use of more recent and more robust markers of vitamin status, such as holotranscobalamin and methylmalonic acid (MMA), might have yielded different results. In fact, a review of longitudinal cohort studies of vitamin B12 status and cognitive decline found that, in all studies where holotranscobalamin and MMA had been used, associations between vitamin B12 status and cognitive decline, dementia or Alzheimer's disease were present [248]. Those with obvious dementia usually show no improvement when treated with vitamin B12 [249]. In the Newcastle 85+ Study, 9% of the participants had been diagnosed with dementia/ Alzheimer's disease at baseline, which might have skewed the results and partly explained the lack of association. However, in sensitivity analyses, when we excluded participants with dementia from the models, the lack of association between plasma vitamin B12 and cognitive performance at baseline and at follow-up remained. Very high plasma vitamin B12 concentrations have been found in patients with inflammatory, liver and kidney diseases which could underestimate the association with cognitive performance [160]. Therefore, models were re-run excluding those with plasma vitamin B12 concentrations > 1000 pmol/L but the conclusion remained. Perhaps the most convincing data so far from RCTs on the association between B vitamins and cognition comes from the VITACOG studies [250]. This RCT included 168 adults >70y with mild cognitive impairment and these were either assigned to a treatment arm (daily dose of 0.8 mg of folic acid, 0.5 mg of vitamin B12 and 20 mg of vitamin B6) or a placebo and followed for 2y. Smith *et al*. found that brain atrophy was 53% slower in those treated with B-vitamins than with placebo but only in those with tHcy concentrations >13 µmol/L at baseline [250].

In the Newcastle 85+ Study, the rate of decline in focused attention (PoA) and sustained attention (CoA) was slower in higher quartiles of tHcy. This is counter intuitive as lower concentrations of tHcy are associated with better health outcomes, including slower rate of cognitive decline [250]. However, this is likely an effect of terminal decline as homocysteine is a strong predictor of mortality in the very old [251,252] which may have selected more cognitively robust survivors in higher quartiles of tHcy. The rate of decline in focused (PoA) and sustained attention (CoA) was no longer associated with tHcy if individuals diagnosed at baseline with dementia or Alzheimer's disease were excluded.

Strengths and weaknesses

In the Newcastle 85+ Study, SMMSE and attention specific CDR System tests were applied at 3 different time points but only over the period of 5 and 3 years, respectively. Such follow-up periods may be too short to detect relationships with 1-C metabolism biomarkers [248]. However, due to the advanced age of the participants, it is likely that cognitive decline was far more rapid than in younger populations and the SMMSE as well as the CDR System would be able to detect this decline over 5 and 3 years, respectively. Although there is no gold standard on how to evaluate cognitive performance, the SMMSE has a well-known ceiling effect of 30 points and a cognitive decline from above 30 points would not be captured [253]. Also for this reason, it has been suggested that the SMMSE might not be sensitive enough to detect subtle cognitive changes typical of most nutritional interventions [254]. Further, there is a well-known learning or practice effect with the SMMSE (but not for CDR), which might have underestimated the rate of cognitive decline and diluted the results [255]. Cognitive function is a strong predictor of mortality. Therefore, because participants with cognitive impairment were more likely to be lost to follow-up due to death, this could potentially dilute the cognitive decline results.

In the Newcastle 85+ Study, 28% of the participants were cognitively impaired (SMMSE ≤25) at baseline. Reverse causality cannot be fully excluded for any cross-sectional associations between homocysteine, folate and cognitive performance. Finally, RBC folate, plasma vitamin B12 and tHcy measurements were available at baseline only which may not reflect their status at other time points and lead to underestimation of their association with cognitive function [107]. General limitations and strengths of the Newcastle 85+ Study were discussed in *Chapter 2, General Methods - 2.1 The Newcastle 85+ Study*.

8.6. One-carbon metabolism biomarkers and cardiovascular and all-cause mortality

Main findings

In this chapter, very old adults with higher baseline tHcy concentrations were at higher risk of death from all-causes (e.g. Q4 vs. Q1, HR: 2.05, 95% CI: 1.51-2.77, p<0.001) and from CVD (e.g. Q4 vs Q1, HR: 2.29, 95% CI: 1.51-3.48, p<0.001) after adjustment for sociodemographic, lifestyle and health variables over 9 years. Elevated plasma vitamin B12 concentrations were associated with increased all-cause mortality (e.g. >500 pmol/L vs 148-

500 pmol/L, HR: 1.70, 95% CI: 1.13-2.56, p=0.011). This effect seemed to be restricted to women and associations were stronger for deaths within the first year.

Comparison with other studies

Evidence from observational studies shows that there might be an association between high concentrations of tHcy and CVD and/or mortality [256,257]. Folate donates methyl groups for homocysteine transmethylation to form methionine and, typically, folic acid supplementation lowers homocysteine concentrations by 13-25% [124]. Although it is still debatable whether homocysteine is a causal factor or a marker of CVD and mortality [258], others have shown that supplementation with folic acid decreased the risk of CVD, especially stroke [149]. In accordance with previous findings [153,156,159,256,259], we found that higher concentrations of tHcy were associated with increased cardiovascular and all-cause mortality. There was no significant association between RBC folate concentration and all-cause and cardiovascular-specific mortality – a finding, similar to some previous studies [151,197-201] but not with others [84,149,260,261]. To the best of our knowledge, the effect of folate on all-cause and cardiovascular-specific mortality has not been investigated in a similar age group. It is possible that this was an age group-specific effect as all the participants in the Newcastle 85+ Study were 85 and over. The relatively low baseline tHcy concentration or high folate concentration is frequently pointed out as a limiting factor to detect an effect. The SEARCH collaborative group conducted a RCT to determine the effects of supplementation with 2 mg of folic acid + 1 mg of vitamin B12 on vascular and non-vascular outcomes in more than 12000 survivors of myocardial infarction [198]. The study found that supplementation with these B vitamins had no effect on vascular and non-vascular deaths during a follow-up of 6.7 years [198]. In the SEARCH trial only 10% of the participants had tHcy concentrations >15 µmol/L [198]. However, 57% of the participants in the Newcastle 85+ Study had hyperhomocysteinemia and still no effect was observed [169].

The raised hazard ratio for mortality for those with higher concentrations of plasma vitamin B12 confirms findings from others in which very high vitamin B12 intake or status was associated with greater all-cause mortality [152,153] and cancer diagnosis/ mortality [154,155]. Arendt *et al*. [154] identified 333667 people from Danish medical registries who were not receiving supplemental vitamin B12 and with plasma vitamin B12 concentrations >200 pmol/L and found that cancer incidence increased with higher vitamin B12 concentrations. The effects were more pronounced for haematological, smoking and alcohol-related cancers,

and during the first year of follow-up [154]. In a retrospective case-control study of 25017 patients with cancer diagnosis and plasma vitamin B12 concentrations >200 pmol/L measured before diagnosis in a retrospective case-control study, the same research team reported that patients with a cancer diagnosis who had vitamin B12 concentrations greater than 600 pmol/L had higher risk of mortality than those with concentrations of 200-600 pmol/L [155].

In addition to the excess intake of vitamin B12 (often parenteral), elevated plasma vitamin B12 concentration has been found in patients with liver, renal, autoimmune, cancers and infectious diseases [160]. Excluding reverse causality, such diseases may disrupt the uptake of vitamin B12 by the liver, the biggest reservoir of vitamin B12 in the body, or alternatively, increased hepatocyte turnover/ damage may lead to greater leakage of vitamin B12 from the liver [160,262,263]. High plasma vitamin B12 has also been associated with haematological malignancies [160]. It is believed that an increase in the number of leukocytes increases haptocorrins's production and release into the circulation [160]. Functional deficiency of vitamin B12 can also happen concomitantly with very high plasma concentrations of vitamin B12 [263]. An increase in haptocorrin and subsequent binding of vitamin B12 could lead to reduced holotranscobalamin binding and therefore less cellular uptake of vitamin B12 [263].

Sensitivity analyses

To determine if elevated plasma vitamin B12 was due to liver damage models we further adjusted the models for alanine aminotransferase (ALT), alkaline phosphatase and bilirubin; and to determine if it was due to other non-liver related conditions, models were adjusted for hs-CRP. Findings were not significantly different in either of the models. We have previously shown a U-shaped relationship between sex-specific quartiles of serum vitamin D and mortality in women in this cohort [29]. This suggests that associations between blood-based biomarkers and health outcomes (i.e. survival) in young-old may not always be the same as in very old adults.

Strengths and weaknesses

One of the biggest strengths of this study is that data was from the Newcastle 85+ Study, a single age cohort of the very old with a large number of participants and extensive health assessment. The study population was socio-demographically representative of the

UK for this birth cohort but the lack of ethnic diversity warrants caution when generalizing to non-white populations. Given that all participants were 85 at recruitment, an age where mortality rates are high, it is impossible to exclude completely any survival or length bias that arose due to selective survivability. The rapid processing of blood samples after venepuncture is another strength of this study. The assay used in this study measures the total circulating vitamin B12. The use of holotranscobalamin, which measures only the metabolically active fraction, might have yielded different associations between vitamin B12 status and mortality. General limitations and strengths of the Newcastle 85+ Study were discussed in *Chapter 2, General Methods - 2.1 The Newcastle 85+ Study*.

8.7. General discussion and public health implications

Very old adults are at increased risk of nutritional deficiencies, which contributes to disability, frailty and loss of physical function [68]. This thesis showed that very old adults in the UK may be at risk of certain macro and micronutrient deficiencies, such as dietary fiber, vitamin D, magnesium, potassium and selenium. These findings draw attention to the risk of nutritional deficiencies in the very old but also the lack of age-specific DRVs. DRVs are generally derived from the average (median) nutrient intake necessary to meet the physiological requirement, as defined by a set of criteria for adequacy for that nutrient (for example vitamin B12 and the maintenance of haematological status) [264]. If not enough data is available, the DRV can be derived from the average nutrient intake of a group of apparently healthy people that is assumed to be adequate (adequate intake) [264]. Despite the extreme importance of establishing age-specific DRVs, this was not an initial objective for this thesis but could be explored in the future. The lack of robustly-based dietary recommendations for very old people limits our ability to interpret the dietary intakes of the Newcastle 85+ Study participants by reference to age-appropriate DRVs.

For example, 72% of the participants did not meet the DRV for SFA and 87% exceeded the SACN DRV for free sugars (or NMES) of 5% of total energy intake [206]. As of 2014, there were 22 countries in Europe with a life expectancy at age 65 higher than 20 years [5]. Nutrition education has shown to be beneficial in changing dietary patterns in this age group [265]. It is fundamental that the very old but also their carers/ family members realize that it is not too late for lifestyle changes to have benefits and whenever necessary, they should be encouraged to do so. However, there are barriers such as the importance of

fat for food palatability which makes it difficult to reduce consumption of food products high in fat without the cooperation of the food industry [266]. Therefore, context should be as important as nutritional knowledge and personal motivation. The food industry could and should play a major role in reducing the sugar and fat content in foods, especially in CCP as these, along with non-alcoholic beverages, are the most widely consumed food group in the Newcastle 85+ Study. If food fortification is deemed necessary, CCP and especially flour would be good candidates.

In line with the SACN recommendations, we agree that there should be consistency on how to determine the dietary fibre content of foods across the UK and Europe to allow for inter-country comparisons and to avoid generating confusion. For this, the Englyst method to measure NSP should be gradually abandoned and the AOAC method adopted [206].

In chapter 4, *Micronutrient intake and food sources in the very old* a fifth of the participants had sodium intakes below the RNI of 1600 mg per day and half met the recommendation of less than 2400 mg per day. Data on sodium derived from table salt and salt used in cooking was not recorded which might have underestimated sodium intake in the Newcastle 85+ Study and further increased the number of participants who did not meet the recommendation. Sodium intake reduction and increased potassium intake might help reduce the prevalence of hypertension, stroke and fatal coronary heart disease in this population [220]. Hypertension is also associated with dementia and Alzheimer's disease [267]. Hypertension is a risk factor for CVD and hence for vascular dementia but it may promote alterations in brain structure and function beyond it. Hypertension may lead to cerebral vessel wall remodelling and endothelial dysfunction, which can lead to reductions in cerebral perfusion and in the brain's capability to clear β-amyloid proteins [267]. It is clear that reduction in CVD risk factors, such as hypertension, will reduce the incidence of dementia and Alzheimer's disease [268].

Although it would be difficult because of time constraints, it would be interesting to have dietary assessment (ideally 2x24hr-MPR) on at least one more time point to allow for direct comparison with baseline dietary intake data to determine how dietary patterns progress (and potentially deteriorate) in this age group. Misreporting was estimated to be ~26% in this cohort [211]. We recommend that misreporting and the importance of accurate records is properly emphasized to the participants prior to the start of the dietary assessment, especially in obese individuals who are more prone to underreport [269]. It is very

difficult to be certain that these 26% were definite misreporters. It would have been useful to confirm these findings by using the doubly labelled water method in a subset of the cohort. A food propensity questionnaire would allow to exclude unusually high or low intakes and confirm that the food recorded was part of the person's habitual dietary pattern. For example, 5% (n=36) of participants had vitamin A intakes above the UL of 3000 µg-RE and 35 of the 36 ate liver and liver products on one of the 24hr-MPR [211].

Greater effort should be placed in harmonising the national food composition food tables, such as the McCance and Widdowson's, so that these can be adequately compared between countries. For example, analysing food folate with HPLC yields, on average, 27% lower concentrations of folate than if measured by microbiological assay [270].

Since energy requirements are largely dependent on energy expenditure, the decrease in energy needs in later life mirrors the age-dependent fall in physical activity. However, the physiological basis for age-dependent changes in vitamin and mineral requirements (if any) is poorly understood. In the absence of such evidence, it may be appropriate that nutritional education for very old adults focuses on healthy food choices, on increasing nutrient density and recommending the use of supplements in specific situations [271]. Increased consumption of fruits, vegetables, fish, lean meats, nuts, whole grains and low-fat dairy products should be encouraged for the general population but also for very old adults. Specific nutritional recommendation in this age group should account for the higher prevalence of disabilities (such as difficulties chewing food, buying ingredients and cooking a hot meal) and chronic diseases, polymedication and financial restraints [56]. Recommendations should also pay attention to possible physiological changes such as loss of lean mass and bone density, fluid and electrolyte dysregulation, decline in taste sensitivity and malabsorption [57]. In order to achieve an optimum intake of vitamins and minerals, dietary manipulation should always be the preferred method but if it proves to be difficult, food fortification and dietary supplementation can be a practical solution to shorten the gap between intakes and recommendations [272].

It is very difficult to exclude reverse causality in cross-sectional associations between nutrition and cognitive function. That is because the cognitive decline process is commonly accompanied by frailty and increased number of disabilities, such as difficulties shopping by yourself, cooking a hot meal or even feeding without the help of others which will lead to decreased or inadequate dietary intake [138]. Furthermore, because malnutrition is strongly associated with disease progression [273] and is a modifiable risk factor, we emphasize the

ESPEN recommendations that malnutrition should be screened in every person with dementia [138].

Several different methods are used to assess/screen cognitive function but the SMMSE is undoubtedly the most widely used. The SMMSE offers several advantages as it is cheap, relatively fast (takes ~5-10 minutes to complete), has easily understandable cut-offs and is comparable to other studies. However, the SMMSE may lack sensitivity to detect subtle cognitive changes typical of most nutritional interventions [254]. Furthermore, the SMMSE has a well-known ceiling effect of 30 points and a cognitive decline from above 30 points would not be captured [253]. More recent tools are available and have performed better at detecting cognitive impairment and dementia than the SMMSE [253]. In summary, there is a need for a universally used tool, such as the SMMSE, but without its shortcomings.

Dementia/ Alzheimer's disease has received growing attention by policy makers and funding bodies but its priority is not yet similar to other chronic diseases such as cancer [274]. The organisation for economic co-operation and development (OECD) estimated that member countries allocated less than 3% of their health budget to dementia prevention [275], an area that has the largest effect at reducing dementia incidence and related disabilities [274]. In summary, policy makers should devote more attention to this area.

Hughes CF *et al.* showed that lower vitamin B6 status and intake was associated with >3.5 times higher risk of accelerated cognitive decline in older adults over 4 years {Hughes, 2017 #850}. Pyridoxic acid (PA) and pyridoxal phosphate (PLP) are routinely used to assess vitamin B6 status and have been measured at baseline in the Newcastle 85+ Study. It would be worthwhile to repeat the analyses of chapter 6, *one-carbon metabolism biomarkers and cognitive decline in the very old* factoring in the vitamin B6 status of the participants. Although chapter 6 of this thesis focused on folate and vitamin B12, it is unlikely that a comprehensive prevention of cognitive decline is achieved by only one nutrient but instead by a dietary pattern that encompasses adequate supply of nutrients such as folate and vitamin B12. It is important to adopt a healthy lifestyle as early as possible as it will benefit cognitive health in later life. In chapter 7, *One-carbon metabolism biomarkers and cardiovascular and all-cause mortality in the very old,* we have shown that elevated plasma vitamin B12 concentrations were associated with increased all-cause mortality. Clinicians are trained to look for vitamin B12 deficiency, commonly defined as a concentration <148 pmol/L, but frequently disregard high plasma vitamin B12 (>500, 600 or 700 pmol/L) and consider it clinically irrelevant [160]. Clinicians and researchers alike need to

be aware of the meaning and prognostic significance of elevated vitamin B12 concentrations. An elevated plasma vitamin B12 value should prompt the clinician or researcher to explore aetiologies, such as inflammatory conditions, hepatic and renal diseases, haematological malignancies and solid neoplasms [160]. Further, functional vitamin B12 deficiency can co-exist with high plasma concentrations and both tHcy and MMA should be measured [263].

1-C metabolism biomarkers in the Newcastle 85+ Study were only measured at baseline (phase 1) (tHcy was measured at phase 3 but not included in the analyses). This has been extremely useful to conduct cross-sectional analyses and predict health outcomes based on baseline 1-C metabolism biomarkers but it assumes that concentrations remain constant throughout follow-up. Measuring 1-C metabolism biomarkers prospectively at further phases (phase 2, 3 and 4) would allow us to confirm our findings and provide associations with exposures in closer temporal proximity to the outcome of interest. RBC folate is closely related to liver concentrations and is routinely used as a marker of long-term status (half-life of 56 to 120 days), arguably being considered the gold-standard for observational studies [89]. Plasma vitamin B12 concentrations reflect short-term vitamin B12 status (half-life of 6 days) and include the metabolically active holotranscobalamin (20-30%) as well as the inactive fraction bound to haptocorrin (70-80%). Total circulating plasma vitamin B12 assays might lack specificity do detect associations with health outcomes such as dementia/ Alzheimer's disease. Measurement of MMA and holotranscobalamin, at baseline and prospectively, would provide more accurate findings [100,112,276].

In summary, very old adults are at increased risk of nutritional deficiencies, which contributes to disability, frailty and loss of physical function [68]. More data on the dietary intake, nutritional status and associations with health trajectories in the very old are urgently needed but until then, dietary changes should focus on increasing nutrient density. Increased consumption of fruits, vegetables, fish, lean meats, nuts, whole grains and low-fat dairy products should be encouraged. However, variation in number and severity of disabilities, physiological needs and context is arguably greater than in younger age groups and nutritional recommendations have to take it into account. Food fortification and dietary supplements can be a practical solution to shorten the gap between intakes and recommendations on specific situations [272]. It would be premature at the moment and over-simplistic to recommend folic acid and vitamin B12 supplements to be taken by everyone on a daily basis, especially with concerns around supplementation and very high

micronutrient concentrations where a U-shaped relationship may exist with health outcomes. However, it seems advisable to maintain an optimal folate and vitamin B12 status through dietary changes. An "optimal" folate and vitamin B12 status in the very old may not be the same as in younger populations from which most biochemical cut-offs are extrapolated from but in the absence of better evidence, these should still be regarded as the target for dietary changes' interventions.

8.8. Recommendations for future research

There is a lack of nutritional data in this age group and given the projected increase in the numbers of very old people in the UK [7], filling this evidence gap should have high priority [207].

Public Health England released a summary of reviews which identified a number of facilitators and barriers for dietary changes [277]. Facilitators: 1) Knowledge of the importance of diet for healthy ageing/ becoming fitter; 2) Dislike of processed food and preference for fresh ingredients; 3) Not wanting to let others down; and 4) Having clear objectives and being supported while achieving them. Barriers: 1) Changing food habits, lack of self-control and insecurity; 2) Misconceptions about their own diet and what a healthy diet is; 3) Self-perception of being too old to change; 4) Lack of clear nutritional advice; 5) Poor access to supermarkets and reduced mobility; and 6) Dislike of some components of a healthy diet (e.g. vegetables) [277]. Nutrition education has shown to be beneficial in changing and maintaining dietary patterns in older adults [265,277] but more behavioural studies are necessary to explore and confirm these barriers and facilitators.

All participants in the Newcastle 85+ Study were 85 at baseline (2006/2007) and born around 1921. This cohort lived through the Great Depression (1929-1932) (which hit Northeast England and its heavy industry especially hard), WW2 (1939-1945), mass availability of antibiotics (1945-Present) and the creation of the NHS (1948-Present) [278]. They would have been ~18 when World War 2 (WW2) broke out and rationing was enforced. During and after WW2 (1940-1954), the Ministry of Food instituted rationing on almost all foods (for example bacon, butter and sugar) except vegetables and bread [279]. It would be interesting to understand how this period influenced their current dietary patterns and understand if there is a cohort effect in the Newcastle 85+ Study.

In chapter 5, *Intakes of folate and vitamin B12 and biomarkers of status in the very old,* we could not provide an accurate value for bioavailability of folate and vitamin B12 per se because of the observational nature of the study design. A well designed controlled feeding study to determine the bioavailability of these vitamins from all sources, from top contributing food groups to total intake of folate and vitamin B12 and from supplements alone would be extremely useful as most of the studies on bioavailability have not been conducted in this population [89,112].

Epigenetic mechanisms could provide a partial explanation to some observed findings in this thesis, such as the higher concentrations of RBC folate in individuals heterozygous for the A allele (T on the opposite strand) than those homozygous for the G allele (C on the opposite strand) of the *MTHFR* gene (rs1801133, chromosome 1, position 11796321). DNA methylation is one of several epigenetic mechanisms recognized to regulate gene expression in mammals. Folate and vitamin B12 are essential to the methionine cycle within the 1-C metabolism, being indirectly involved in the production of SAM. SAM is considered a universal methyl donor and largely responsible for the methylation of cytosine [280]. Folate and vitamin B12, as indirect sources of methyl groups [281,282], may affect methylation patterns in replicating cells [283]. Hypermethylation of CpG islands is frequently associated with gene silencing [283]. The relationship between the *MTHFR* C677T polymorphism and folate status may be different in very old populations than in their younger counterparts [241]. We hypothesise that with ageing, the decreased activity of the MTHFR enzyme (EC: 1.5.1.20) may be compensated by an increase in the binding affinity for the flavin adenine dinucleotide (FAD) mediated by altered DNA methylation patterns. DNA methylation of promoter associated CpG islands [75-100% of methylation occurs on CpG islands[280]] for five loci (EPHA10, HAND2, HOXD4, TUSC3, TWIST2 - chosen from a panel of 15 candidate genes because they were shown to be the most extensively and variably methylated in this population) and long interspersed nucleotide element-1 (LINE-1) repetitive elements (17% of human genome and surrogate marker for global DNA methylation) are available for a subset of the Newcastle 85+ Study participants at baseline (n=480).

It would be interesting to repeat the longitudinal associations between 1-C metabolism biomarkers and; cognition, all-cause mortality and CVD mortality using a Mendelian randomization approach. This approach uses common genetic polymorphisms (normally single nucleotide polymorphisms), associated with the exposure (considered

robust proxies for biomarker concentrations) to make causal inferences between modifiable risk factors and health-related outcomes [110]. Mendelian randomization is less prone to reverse causality and less susceptible to confounding by behavioural or environmental exposures [284]. However, it can still be influenced by genetic pleiotropy and population stratification [285]. For example, individuals homozygous for T allele (A for opposite strand) of the *MTHFR* gene (rs1801133, chromosome 1, position 11796321) and heterozygous for the T allele have ~70% and ~35%, respectively reduced MTHFR enzyme activity (EC: 1.5.1.20) than those with the common genotype (CC or GG). Although *MTHFR* gene has several effects on the 1-C metabolism, this polymorphism is reflected in markedly lower folate and higher tHcy concentrations [286].

RCTs with enough follow-up time and sufficiently powered to detect cognitive decline are needed to confirm our findings that tHcy and RBC folate, but not plasma vitamin B12, concentrations are predictive of better global cognition in the very old but not rate of cognitive decline.

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Appendices

A. List of papers included in this thesis

Mendonça N *et al.* (2016) Macronutrient intake and food sources in the very old: Analysis of the Newcastle 85+ Study. ***Br J Nutr***. 115(12):2170-80.

Mendonça N *et al.* (2016) Micronutrient intake and food sources in the very old: Analysis of the Newcastle 85+ Study. ***Br J Nutr***. 116(4):751-61.

Mendonça N *et al.* (2016) Intakes of folate and vitamin B12 and biomarkers of status in the very old: The Newcastle 85+ Study. ***Nutrients***. 8(10), 604.

Mendonça N *et al.* (2017) One-carbon metabolism biomarkers and cognitive decline in the very old: the Newcastle 85+ Study. ***J Am Med Dir Assoc.*** 18(9): 806. .

Mendonça N et al. (2017) Elevated total homocysteine and plasma vitamin B12 concentrations are associated with all-cause and cardiovascular mortality in the very old: The Newcastle 85+ Study. ***J Gerontol A Biol Sci Med Sci.*** (in review).

B. Published abstracts included in this thesis

Mendonça N *et al.* (2016) Intakes of folate and vitamin B12 from total diets and from specific food groups and biomarkers of status in the very old. ***Proc Nutr Soc****.* 75 (OCE3).

Mendonça N *et al.* (2015) Adequacy of nutrient intake in the very old: Analysis of the Newcastle 85+ Study. ***Ann Nutr Metab****.* 67 (Suppl 1).

Mendonça N *et al.* (2015) Contribution of animal products to dietary intake in the very old. ***Proc Nutr Soc***. 74, doi: 10.1017/S0029665115003699.

Mendonça N *et al.* (2015) Dietary intake and food sources in the very old. ***Proc Nutr Soc****.* 74, doi: 10.1017/S0029665115002736.

Mendonça N *et al.* (2015) Micronutrient intake and food sources in the very old. ***Proc Nutr Soc****.* 74, doi: 10.1017/S0029665115002748.

C. List of papers not included in this thesis

Hill T, Mendonça N *et al.* (2016) What do we know about the nutritional status of the very old? Insights from three cohorts of advanced age from the UK and New Zealand. ***Proc Nutr Soc***. 75(3):420-30.

Madeira T *et al.* (2016) National Survey of the Portuguese Elderly Nutritional Status: Study Protocol. ***BMC Geriatr.*** 16,139.

Mendonça N *et al.* (2017) Prevalence and predictors of low protein intake in very old adults: Insights from the Newcastle 85+ Study. ***Eur J Nutr.*** doi:10.1007/s00394-017-1537-5 .

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D. Tertiary food groups used in the Newcastle 85+ Study

1st level food group	2nd level food group	3rd level food group
Cereals and cereal products (grains)	Pasta, rice and other miscellaneous cereals	Pasta (manufactured products and ready meals)
		Pasta (other, including homemade dishes)
		Pizza
		Rice (manufactured products and ready meals)
		Rice (other, including homemade dishes)
		Other cereals
	Bread	White bread (not high fibre, not multiseed bread)
		Wholemeal bread
		Brown, granary and wheatgerm bread
		High fibre white bread
		Other bread
	Breakfast cereals	High fibre breakfast cereals- high sugar
		High fibre breakfast cereals- low sugar
		Other breakfast cereals (not high fibre) high sugar
		Other breakfast cereals (not high fibre) low sugar
	Biscuits	Biscuits (homemade) savoury
		Biscuits (manufactured/retail) savoury
		Biscuits (homemade) sweet
		Biscuits (manufactured/retail) sweet
	Buns, cakes and pastries	Buns cakes and pastries (homemade)
		Buns cakes and pastries (manufactured)
		Fruit pies (homemade)
		Fruit pies (manufactured)
	Puddings	Cereal based milk puddings (homemade)
		Cereal based milk puddings (manufactured)
		Sponge puddings (homemade)
		Sponge puddings (manufactured)
		Other cereal based puddings (homemade)
		Other cereal based puddings (manufactured)
Milk and milk products	Milk	Whole milk
		Semi-skimmed milk
		Skimmed milk
		1% milk
		Other milk
	Other milk and cream	Cream (including imitation cream)
		Infant formula
	Cheese	Other cheese
		Cottage cheese
	Yoghurt, fromage frais and other dairy desserts	Yogurt- low fat
		Dairy desserts (homemade)
		Fromage frais and other dairy desserts (manufactured)
		Yogurt- other
		Yogurt- full fat
		Ice cream
Eggs and egg dishes	Eggs and egg dishes	Eggs
		Manufactured egg products including ready meals
		Other eggs and egg dishes including homemade

1st level food group	2nd level food group	3rd level food group
Oils and fat spreads	Butter	Butter
	Polyunsaturated margarine and oils	Olive oil
		Polyunsaturated margarine
		Polyunsaturated oils
		Olive oil-based margarine
	Low fat spread	Low fat spread not polyunsaturated
		Olive oil-based
		Polyunsaturated low fat spread
	Margarine and other cooking fats and oils not polyunsaturated	Block margarine
		Other cooking fats and oils not polyunsaturated
		Soft margarine not polyunsaturated
	Reduced fat spread	Reduced fat spread (not polyunsaturated)
		Reduced fat spread (polyunsaturated)
Meat and meat products	Bacon and ham	Other bacon and ham (including homemade dishes)
		Ready meals/meal centres based on bacon and ham
	Beef, veal and dishes	Manufactured beef products (including ready meals)
		Other beef and veal (including homemade recipe dishes)
	Lamb and dishes	Manufactured lamb products (including ready meals)
		Other lamb (including homemade recipe dishes)
	Pork and pork dishes	Manufactured pork products (including ready meals)
		Other pork (including homemade recipe dishes)
	Coated chicken and turkey manufactured	Manufactured coated chicken/ turkey products
	Chicken and turkey dishes	Manufactured chicken products (including ready meals)
		Other chicken/turkey (including homemade recipe dishes)
	Liver, product and dishes	Liver and dishes
	Burgers and kebabs	Burgers and kebabs purchased
	Sausages	Other sausages (including homemade dishes)
		Ready meals based on sausages
	Meat pies and pastries	Meat pies and pastries (homemade)
		Meat pies and pastries (manufactured)
	Other meat and meat products	Other meat (including homemade recipe dishes)
		Other meat products (manufactured including ready meals)
Fish and fish dishes	White fish coated or fried including fish fingers	White fish coated or fried
	Other white fish, shellfish and fish dishes	Manufactured canned tuna products (including ready meals)
		Manufactured shellfish products (including ready meals)
		Manufactured white fish products (including ready meals)
		Other canned tuna (including homemade dishes)
		Other shellfish (including homemade dishes)
		Other white fish (including homemade dishes)

1st level food group	2nd level food group	3rd level food group
	Oily fish	Manufactured oily fish products (including ready meals)
		Other oily fish (including homemade dishes)
Vegetables	Salad and other raw vegetables	Carrots (raw)
		Salad and other raw vegetables
		Tomatoes raw
	Vegetables (not raw)	Baked beans
		Beans and pulses (including ready meal and homemade dishes)
		Carrots not raw
		Cruciferous vegetable
		Green beans not raw
		Leafy green vegetables not raw
		Meat alternatives (including ready meals and homemade dishes)
		Other manufactured vegetable products (including ready meals)
		Other vegetables (including homemade dishes)
		Peas not raw
		Tomatoes not raw
Potatoes	Chips, fried and roast potatoes and potato products	Fried chips purchased including takeaway
		Other manufactured potato product fried/baked
		Other fried/roast potatoes
		Oven chips purchased
	Other potatoes, potato salads and dishes	Other potato products and dishes (manufactured)
		Other potatoes (including homemade dishes)
Savoury snacks	Crisps and savoury snacks	Non-potato based snacks
		Potato and mixed cereal snacks
		Potato based snacks
Nuts and seeds	Nuts and seeds	Nuts and seeds
Fruit	Fruit	Apples and pears not canned
		Bananas
		Canned fruit in juice
		Canned fruit in syrup
		Citrus fruit not canned
		Dried fruit
		Other fruit not canned
Sugar, preserves and confectionery	Sugars, preserves and sweet spreads	Preserves
		Sugar
		Sweet spreads fillings and icing
	Confectionary	Sugar confectionery
		Chocolate confectionery
Non-alcoholic beverages	Fruit juice	Fruit juice
	Tea, coffee and water	Bottled water still or carbonated
		Coffee (made up weight)
		Tea (made up)
		Herbal tea (made up)
		Tap water only
	Soft drinks, not diet	Soft drinks not low calorie carbonated
		Soft drinks not low calorie concentrated
		Soft drinks not low calorie, ready to drink, still

1st level food group	2nd level food group	3rd level food group
	Soft drinks, diet	Soft drinks low calorie carbonated
		Soft drinks low calorie concentrated
		Soft drinks low calorie, ready to drink, still
Alcoholic beverages	Spirits and liqueurs	Liqueurs
		Spirits
	Wine	Fortified wine
		Low alcohol and alcohol free wine
		Red wine
		White wine including rose
	Beer lager cider and perry	Alcoholic soft drinks
		Beers and lagers
		Cider and perry
		Low alcohol and alcohol free beer and lager
Miscellaneous	Miscellaneous	Beverages dry weight
		Nutrition powders and drinks
		Savoury sauces, pickles, gravies and condiments
		Soup (meat) (homemade)
		Soup (non-meat) (homemade)
		Soup (non-meat) (manufactured/retail)
		Soup (meat) (manufactured/retail)

E. Overview of variables collected in the Newcastle 85+ Study from baseline to phase 4

Variables	Ph1	Ph2	Ph3	Ph4	Comment
Questionnaires					
Aids/appliances and household modifications	Yes	No	No	No	
Alcohol	Yes	No	No	No	
Chest pain (cardiac)	Yes	Yes	Yes	No	Phase 2 cardiac subset only
Cough, sputum, wheeze	Yes	Yes	Yes	No	Questions on recent chest infection, use of antibiotics, use of steroids in phases 2 and 3 only
Dietary assessment	Yes	No	No	No	
Disability and help received	Yes	Yes	Yes	Yes	In phase 1 also coded up to 4 causes of difficulty for each activity participant could not do
Education and work	Yes	No	No	No	
Ethnic origin	Yes	No	No	No	
Exhaustion	No	No	No	Yes	
Eyesight	Yes	Yes	Yes	Yes	
Falls	Yes	Yes	Yes	Yes	In phase 1 extended questions for falls and dizziness
Family data	Yes	No	No	No	
Finances	Yes	No	No	Yes	
Formal health and social care	Yes	Yes	Yes	Yes	
Fractures	Yes	No	No	No	
Geriatric depression scale	Yes	Yes	Yes	Yes	
Hearing	Yes	Yes	Yes	Yes	
How are you feeling today?	Yes	Yes	Yes	Yes	
Incontinence	Yes	Yes	Yes	Yes	
Joint pain	Yes	No	No	No	
Key events since previous phase	No	Yes	Yes	Yes	
Living arrangements	Yes	Yes	Yes	Yes	
Loneliness	Yes	Yes	Yes	Yes	
Long standing illness/disability/infirmity	Yes	Yes	Yes	Yes	
Medication, non-prescribed	Yes	No	No	No	
Medication, prescribed	No	Yes	No	No	Phase 2 cardiac subset only from GP records at phase 1 and phase 3
Occupational exposure to lung toxins	Yes	Yes	Yes	No	
Oral health	Yes	No	Yes	No	
Pain	Yes	Yes	Yes	Yes	Phase 1 included body map for pain locations
Physical activity	Yes	Yes	Yes	Yes	
Self-rated health	Yes	Yes	Yes	Yes	
Shortness of breath	Yes	Yes	Yes	Yes	Phase 2 cardiac subset only
Sleep	No	No	Yes	No	

Variables	Ph1	Ph2	Ph3	Ph4	Comment
Smoking	Yes	No	No	No	
Social participation	Yes	Yes	Yes	Yes	
Social support	Yes	Yes	Yes	Yes	
Measurements and function tests					
Demi-span	Yes	No	No	No	
Weight	Yes	Yes	Yes	Yes	
Bioimpedance	Yes	No	Yes	No	
Waist-hip circumference	Yes	No	No	No	
CDR (Computerised cognitive assessment)	Yes	Yes	Yes	No	
SMMSE (Standardised mini-mental state examination)	Yes	No	Yes	Yes	
Hand-grip strength	Yes	Yes	Yes	Yes	
Chair stand test	No	Yes	Yes	No	
Timed up and go test	Yes	Yes	Yes	Yes	
7 day activity monitoring (accelerometer)	No	Yes	Yes	No	
Blood pressure	Yes	Yes	Yes	No	
Spirometry	Yes	Yes	Yes	No	
Oximetry	Yes	Yes	Yes	No	
ECG (Electrocardiogram)	Yes	Yes	Yes	No	Phase 2 cardiac subset only
Tooth count	Yes	No	Yes	No	
Cardiac echocardiogram	No	Yes	Yes	No	Cardiac subset only in phase 2 or phase 3
Carotid intima media thickness	No	Yes	Yes	No	Cardiac subset only in phase 2 or phase 3
Vascular resistance (sphygmocor and vicorder)	No	Yes	Yes	No	Cardiac subset only in phase 2 or phase 3
Blood assays (no bloods in phase 4)					
Creatinine, urea, electrolytes	Yes	Yes	Yes	No	
Urate	Yes	No	No	No	
Liver function test/bone panel	Yes	No	Yes	No	
Full blood cell count	Yes	Yes	Yes	No	
HbA1c	Yes	Yes	Yes	No	
Fasting glucose	Yes	No	Yes	No	
Fasting lipid profile (Cholesterol, Triglycerides, HDL, LDL)	Yes	No	Yes	No	
Apolipoproteins	Yes	No	Yes	No	
C-reactive protein (hs-CRP)	Yes	Yes	Yes	No	
Cortisol	Yes	No	Yes	No	
Thyroid function: TSH, FT4, T3 and ATPO	Yes (rev T3)	No	Yes (not ATPO)		
Rheumatoid factor	Yes	No	No	No	Cardiac subset only in phase 2 or phase 3
N-terminal pro-brain natriuretic peptide (NT-PRO BNP)	Yes	Yes	Yes	No	Cardiac subset only in phase 2 or phase 3
Neuregulin 1 (NRGB-1)	Yes	Yes	Yes	No	Cardiac subset only in phase 2 or phase 3
Growth differentiation factor 15 (GDF-15)	Yes	Yes	Yes	No	Cardiac subset only in phase 2 or phase 3
ST-2	Yes	Yes	Yes	No	Cardiac subset only in

Variables	Ph1	Ph2	Ph3	Ph4	Comment
					phase 2 or phase 3
Endoglin	No	Yes	Yes	No	
DNA damage and repair	Yes	Yes	Yes	No	
Telomere length	Yes	Yes	Yes	No	
Telomerase	Yes	No	No	No	
Isoprostanes	Yes	Yes	Yes	No	
4-colour flow cytometry analysis of lymphocyte subpopulations	Yes	Yes	Yes	No	Fresh samples
10-colour flow cytometry analysis of lymphocyte subpopulations	No	Yes	No	No	Frozen samples
LPS-stimulated cytokine (IL-6 and TNF-alpha) production	Yes	Yes	Yes	No	
CMV (Cytomegalovirus)	Yes	Yes	No	No	Measured on all participants in phase 2 and those not measured in phase 2 were measured in phase 1
Vitamin B12	Yes	No	No	No	
Red blood cell folate	Yes	No	No	No	
Ferritin	Yes	No	No	No	
Total homocysteine	Yes	No	Yes	No	
Vitamin B2 (EGRac)	Yes	No	No	No	
Vitamin B6 (PLP and PA)	Yes	No	No	No	
Vitamin C	No	Yes	No	Yes	
Vitamin D	Yes	No	No	No	
Mitochondrial haplotype	Yes	No	No	No	
Mitochondrial DNA sequencing	Yes	No	No	No	
Reactive oxygen production by mitochondria	No	No	Yes	No	
Genotyping	Yes	No	No	No	
GP record review (not done on phase 2)					
Diseases-ever diagnoses, pre-specified list of conditions for each category					
Cardiovascular/cerebrovascular	Yes	No	Yes	Yes	
Cancer	Yes	No	Yes	Yes	
Endocrine	Yes	No	Yes	Yes	
Eye	Yes	No	Yes	Yes	
Liver	Yes	No	Yes	Yes	
Musculoskeletal	Yes	No	Yes	Yes	
Neurological	Yes	No	Yes	Yes	
Psychiatric	Yes	No	Yes	Yes	
Respiratory	Yes	No	Yes	Yes	
Key events in last 6-12 months					
Blood pressure check (12m)	Yes	No	Yes	Yes	
Influenza vaccination (12m)	Yes	No	Yes	Yes	
Depression (12m)	Yes	No	Yes	Yes	
Infections (12m)	Yes	No	Yes	Yes	
Medication check (6 months)	Yes	No	Yes	Yes	
Prescribed medication	Yes	No	Yes	Yes	
Consultations with primary care team members in previous 12 months	Yes	No	Yes	Yes (GP/Other)	
Hospital admissions	No	No	Yes	No	

F. Cognitive drug research (CDR) attention tests and outcome scores

CDR Task	Description	Outcome Variables
Simple reaction time	The participant is instructed to press "YES" as quickly as possible every time the word "YES" is presented on the screen. In total, 30 "YES" stimuli are presented with varying inter-stimulus interval.	Mean reaction time (milliseconds, ms)
Choice reaction time	Either the word "YES" or "NO" is presented on the screen and the participant is instructed to press the corresponding button as quickly as possible. There are 30 trials for each stimulus word, which is chosen randomly with equal probability, with varying inter-stimulus interval.	Mean reaction time (milliseconds, ms) Choice reaction time accuracy (# errors)
Digit vigilance task	A target digit is randomly selected and constantly displayed to the right of the screen. A series of digits (0-9) are presented in the centre of the screen at the rate of 150 per minute. The participant is required to press the "YES" button as quickly as possible every time the digit in the series matches the target digit. There are 300 digits in the series and the task lasts for 2 minutes.	Mean reaction time (milliseconds, ms) Targets Detected (%) Number of false alarms (# errors)
Power of attention	A composite score calculated by summing simple reaction time (SRT), choice reaction time (CRT) and digit vigilance task (DVT) mean reaction times (ms). Lower scores represent better performance.	Intensity of concentration (milliseconds, ms)
Continuity of attention	A composite score calculated by combining the accuracy scores from the CDR and DVT (CRT accurate responses*0.30 + DVT accurate responses*0.30 –DVT false alarms). Higher scores represent better performance.	Ability to sustain attention (milliseconds, ms)
Reaction time variability	A composite score calculated by summing up the coefficients of variance from SRT, CRT and DVT mean reaction times. Lower scores represent better performance.	Fluctuation in attention and consistency in responding to correct target stimuli (coefficient of variance)

G. Conferences and workshops attended

Conference/ Training	Theme/ Notes	Start Date	Days	Location
Year 1				
Safety Course	NA	05 May 2014	1	Newcastle, UK
Induction for 1st Year PGRs	NA	08 May 2014	1	Newcastle, UK
Writing your PhD project proposal	NA	12 May 2014	1	Newcastle, UK
Design of Experiment Statistics	NA	19 May 2014	1	Newcastle, UK
AFRD PGR Conference 2014	Miscellaneous	21 May 2014	2	Newcastle, UK
MRes. Module Clinical Epidemiology	Until 08 January 2015	02 October 2014	16	Newcastle, UK
HNRC 20th Anniversary Research Day	Nutrition/ Poster	15 October 2014	1	Newcastle, UK
Northeast Post-Graduate Conference 2014	Biomedical Sciences	31 October 2014	1	Newcastle, UK
Advanced Medline Workshop	NA	10 November 2014	1	Newcastle, UK
Basic Stats Workshop	NA	19 November 2014	1	Newcastle, UK
SPSS Beginners Workshop	NA	21 November 2014	1	Newcastle, UK
SPSS Advanced Workshop	NA	27 November 2014	1	Newcastle, UK
PGR Who Teach Workshop	Na	29 January 2015	1	Newcastle, UK
NUIA PGR Day	Miscellaneous/ Poster	10 March 2015	1	Newcastle, UK
Steam A Multi-Variate Techniques Workshop	NA	14 April 2015	4	Newcastle, UK
Year 2				
AFRD PGR Conference 2015	Miscellaneous/ Poster	21 May 2015	2	Newcastle, UK
Nutrition Society Irish Section Meeting	Nutrition in the critical stages of the life cycle/ 2xPoster	17 June 2015	3	Cork, Ireland
Data Handling and Spreadsheet Skills Workshop	NA	18 June 2015	1	Newcastle, UK
Nutrition Society Summer Meeting	The future of animal products in the diet: health and environmental concerns/ Poster	15 July 2015	3	Nottingham, UK
Phlebotomy Course	NA	25 September 2015	1	Newcastle, UK
Northeast Postgraduate Conference 10th Anniversary	Biomedical Sciences/ Talk	12 October 2015	1	Newcastle, UK
12th FENS Conference	Nutrition and health throughout the life-cycle/ Poster	20 October 2015	4	Berlin, Germany
HNRC Research Day 2015	Nutrition/ 2xPoster + Talk	28 October 2015	1	Newcastle, UK

Conference/ Training	Theme/ Notes	Start Date	Days	Location
Transition to Post-Doc Workshop	NA	30 November 2015	1	Newcastle, UK
Practical Statistics I (Incorporating Introduction to R) Workshop	NA	16 December 2015	2	Newcastle, UK
Year 3				
Analysis of Longitudinal Datasets	NA	21 April 2016	1	Newcastle, UK
MIA Summer School	Biology of Ageing	25 April 2016	5	Algor, Portugal
AFRD postgraduate 2016	The Future of Food/ Talk	23 May 2016	2	Newcastle, UK
Practical Statistics II	NA	02 June 2016	1	Newcastle, UK
Practical Statistics III	NA	26 June 2016	2	Newcastle, UK
Nutrition Society Summer Meeting 2016	New technology in nutrition research and practice/ Poster	11 July 2016	4	Dublin, Ireland
MRes. Module Comparative Cognition	Until 20 December 2016	04 October 2016	12	Newcastle, UK
HNRC Research Day 2016	Nutrition/ Poster	28 October 2016	1	Newcastle, UK
FQH meeting	Talk	30 November 2016	1	Newcastle, UK
ARUK 2017	Alzheimer/ Poster	14 March 2017	2	Aberdeen, UK
Rank Prize Funds Symposium	Role of wheat in diet/ Talk	24 April 2017	4	Lake District, UK
XVI Congresso de Nutrição e Alimentação	Food sustainability/ Talk	4 May 2017	2	Lisbon, Portugal
ICHOCM 2017	1-C metabolism/ Poster	14 May 2017	5	Aarhus, Denmark
VIU Summer Institute on Ageing	Global ageing	5 June 2017	6	Venice, Italy
PROMISS consortium meeting	Protein intake and ageing	19 September 2017	2	Nice, France
EUGMS	Gerontology/ Talk	20 September 2017	3	Nice, France

H. Initial PhD gantt chart