

Genetic and Dietary Determinants of Homocysteine in Relation to Bone Health

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Genetic and Dietary Determinants of Homocysteine in Relation to Bone Health

**Genetische en dieetgerelateerde determinanten van
homocysteïne in relatie tot de conditie van het bot**

Proefschrift

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All we know is still infinitely less than all
that remains unknown.

William Harvey (1578-1657)

*To my beloved mother,
and to the loving memory of my father*

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Publications and manuscripts based on the studies described in this thesis

Chapter 2

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Chapter 3

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Chapter 4.2

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Chapter 6

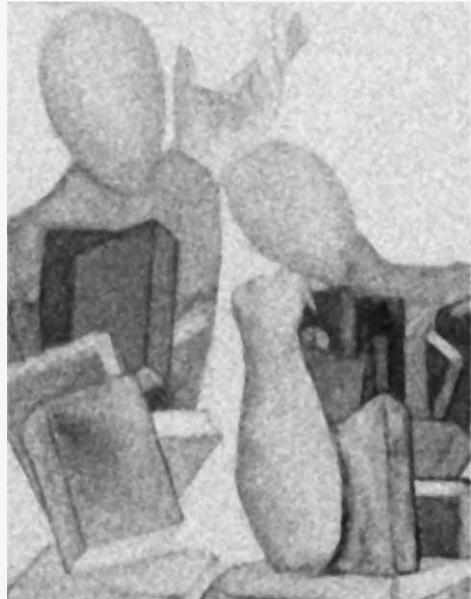
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G. Uitterlinden. The -1997 G/T and Sp1 polymorphisms in the collagen type I alpha1 (COL1A1) gene in relation to changes in femoral neck bone mineral density and the risk of fracture in the elderly: the Rotterdam study.

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CHAPTER 1

General Introduction



Osteoporosis is characterized by low bone mineral density (BMD), and deterioration of the bone microarchitecture leading to an increased risk of fracture ¹. Osteoporotic fractures are a major health problem in the western society; they are associated with increased morbidity and mortality and with substantial economic costs. In the coming years, the number of fractures will increase throughout the world as the population ages, so prevention of fractures with inexpensive and well-established interventions is becoming increasingly important.

Determinants of osteoporosis

Several studies have investigated risk factors for incident fractures, with most of the studies focusing on hip fractures ². One of the most important risk factors for incident fractures is a low BMD ³. With decreasing BMD, the risk of an incident fracture increases rapidly, both in men and women. Besides BMD, the most important risk factors for fractures are age, female gender, low body weight, a personal history of prior fractures and a family history of fractures.

Evidence from twin and family studies indicates that genetic factors play an important role in the regulation of BMD and other skeletal phenotypes relevant to the pathogenesis of fragility fractures. For example, the heritability of BMD at the spine and hip has been estimated to lie between 70 and 85% ^{4,6}. Many gene polymorphisms have been proposed to be involved in BMD ⁷. One of the most prominent candidate genes, which has been associated with osteoporosis in different populations is the collagen type I Alpha 1 gene ⁸, which encodes the alpha chain of collagen type I. COL1A1 is the most abundant structural protein in the bone matrix. One of the most widely studied polymorphisms in the COL1A1 gene is the Sp1 binding site polymorphism. This polymorphism has been examined for its association with BMD and osteoporotic fracture in several studies, but the results have been conflicting. This polymorphism was also studied in the Genetic Markers for Osteoporosis ⁹ Study (GENOMOS), which is Eu-sponsored collaborative effort to identify genetic variants for osteoporosis. In a total sample of 20,786 subjects, GENOMOS found a very modest association between the Sp1 polymorphism and incident risk of vertebral fractures mainly in the TT homozygotes ⁹. In the work presented in this thesis, we have attempted to clarify the association of this polymorphism in relation to another nearby polymorphism with BMD and osteoporotic fracture in the Rotterdam Study ¹⁰.

Recently, a new and potentially modifiable biochemical risk factor for osteoporotic fracture was identified: a mildly increased circulating homocysteine level ^{11,12}. These observational studies showed that a relatively high Hcy level predicts a higher frac-

ture risk, independent of other known risk factors for fracture. Since this finding, numerous studies have investigated different nutritional determinants of Hcy, i.e., the vitamins B11 (folate), B6 (pyridoxine), B12 (cobalamin), and/or genetic polymorphisms involved in the Hcy metabolism. The overall results from these studies are inconclusive. In Chapter 2 we performed a systematic review and meta-analysis on studies that investigated the association of Hcy and related B vitamins with osteoporosis.

Homocysteine metabolism

Homocysteine is a highly reactive amino-acid, which is produced as an intermediate in the metabolism of methionine. The biochemistry of Hcy and related pathways, also referred to as the one-carbon metabolism, is illustrated in Figure 1. As shown, the control of the methionine metabolism is complex, as a result of the very basic and important need to satisfy the requirements of the individual for protein synthesis, DNA synthesis and methylation of DNA, RNA and proteins.

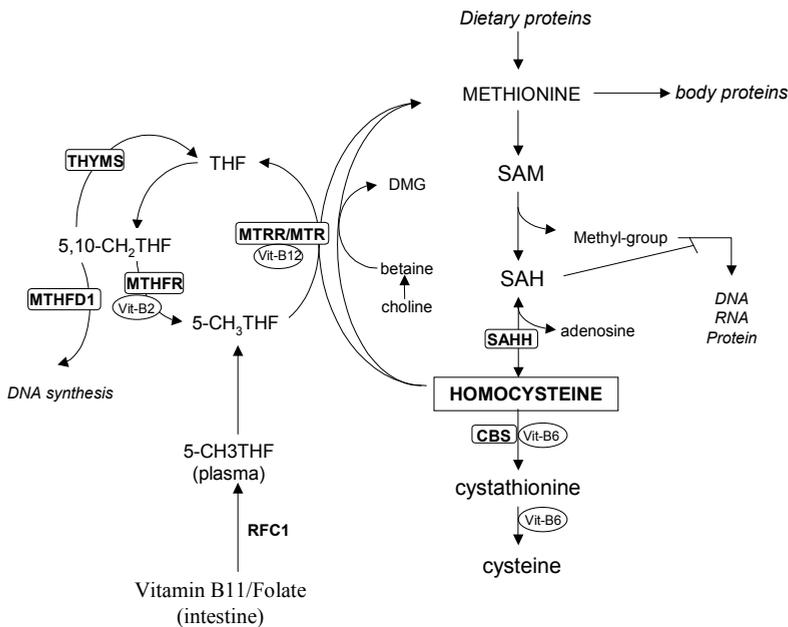


Figure 1: Summary of homocysteine metabolism and relevant enzymes (boxed) and cofactors (circled) SAHH:s-adenosylhomocysteine hydrolase, CBS:cystathionine β-synthase, MTR: methionine synthase; MTRR: methionine synthase reductase, MTHFR:methylenetetrahydro folate reductase, MTHFD: methylenetetrahydro folate dehydrogenase, THYMS: thymidylate synthase, RFC1: reduced folate carrier, THF: tetrahydrofolate

Table 1. B vitamins involved in homocysteine metabolism

<i>Name</i>	<i>Code</i>	<i>Present in</i>
Riboflavin	B2	Milk and other dairy products, enriched bread, cereal and other grain products; eggs, meat, green leafy vegetables, nuts, liver, kidney, and heart
pyridoxine	B6	chicken, fish, pork, liver, kidney, whole grains, nuts and legumes
Folate	B11	leafy vegetables, orange juice and some fruits, legumes, liver, yeast breads, wheat germ and some fortified cereals
Cobalamin	B12	Animal products and some fortified foods

Methionine is first converted to its active form S-adenosylmethionine (SAM). This converts to S-adenosyl-homocysteine (SAH) by donating a methyl group for cellular methylation, and is then hydrolysed to Hcy. Further metabolism of Hcy occurs through two pathways: transsulfuration to cysteine, or remethylation to methionine. Several B vitamins are important for Hcy metabolism (Table 1): transsulfuration is dependent on vitamin B6 (pyridoxine), while remethylation is dependent on vitamin B11 (folate), vitamin B2 (riboflavin) and B12 (cobalamin). Remethylation of Hcy is performed by methionine synthase (MTR), and methionine synthase reductase (MTRR) is necessary to keep MS in its activation form. Cystathionine β synthase (CBS) is essential for transsulfuration of Hcy.

Deficiencies in a number of essential enzymes in the one-carbon metabolism causes the rare (frequency:1/344000) classical metabolic disorder homocysteinuria which is characterized by very high Hcy levels ($>200 \mu\text{mol/l}$)¹³. In addition to mental retardation and thromboembolic events, this metabolic disorder is characterized by symptoms related to connective tissue such as subluxation of the ocular lens, genu valgum, pes cavus, long extremities and early osteoporosis. If untreated, 80% of the patients will develop osteoporosis by the age of 30 years¹³.

Determinants of homocysteine level

Determinants of plasma Hcy in the normal population include genetic, nutritional, and lifestyle factors, but also different disease conditions and certain drugs can affect the Hcy metabolism¹⁴⁻¹⁸. Many of these factors cause a change in Hcy concentrations thereby influencing enzyme activity or modifying the function or blood concentrations of the B vitamins involved in the Hcy metabolism.

Suboptimal nutritional status is acknowledged to result in moderately elevated Hcy levels (hyperhomocysteinemia) which is prevalent in approximately 19% of people older than 60 years¹⁹. Elderly are at high risk for developing hyperhomocysteinemia, because especially in this group nutrition is often suboptimal, while absorption of certain nutrients is also impaired. For example, vitamin B12 deficiency is present

in 20-25% of the Dutch elderly population ²⁰ mainly due to malabsorption. Dietary determinants of Hcy levels include the B vitamins (B2, 6, 12, and folate), but also betaine, choline and protein intakes.

Hcy levels are strongly determined by genetic variation, and studies have suggested a high heritability (47-57%) for Hcy levels ^{21,22}. Therefore, Hcy concentrations are determined by environmental and genetic factors and the interaction between the two. In healthy well-nourished individuals the Hcy metabolism is well regulated and the plasma concentration is usually less than 12 μ M. However, genetic variants and/ or nutritional deficiencies lead to elevation of the levels of Hcy.

Genetic determinants of homocysteine

Table 2 shows the best characterized genetic variants in the Hcy metabolism at the time the work on this thesis was started. The most widely studied polymorphism in the Hcy metabolism is the MTHFR C677T (Ala222Val), which has been identified as an important determinant of Hcy levels ²³. It is established that both vitamin B2 and folate play a critical role in modulating the Hcy level of 222 ValVal homozygotes ^{24,25}. A second variant in this gene, the A1298C polymorphism (Glu429Ala) ²⁶, was found associated with decreased enzyme activity together with the MTHFR 222ValVal homozygous ²⁷. No effect of this variant alone on Hcy was observed ^{28,29}, except for one study which reported increased Hcy in 429AlaAla compared to 429Glu carriers individuals ³⁰.

Table 2. Genetic variants studied for their effect on plasma homocysteine levels

<i>Gene name</i>	<i>Chromosomal location</i>	<i>Variant</i>	<i>Amino acid substitution</i>	<i>Abbreviation</i>	<i>Risk allele frequency</i>	<i>Effect on Hcy level</i>
Methylenetetrahydrofolate reductase	1p36.3	C677T	Ala222Val	MTHFR	T=0.33	Increased ^{81,82}
Methylenetetrahydrofolate reductase	1p36.3	A1298C	Glu429Ala	MTHFR	C=0.32	No effect ^{28,29}
Methionine synthase	1q43	A2756G	Asp919Gly	MTR	G=0.17	Decreased- no effect ^{32,33,81,83}
Methionine synthase reductase	5p15.31	A66G	Ile22Met	MTRR	G=0.43	Increased- no effect ^{35,36}
Reduced folate carrier	21q22.3	G80A	Arg27His	RFC1	A=0.40	Decreased- no effect ³⁸
Transcobalamin	22q12.2	C776G	Pro259Arg	TCN	G=0.45	Increased- no effect ^{39,40}
Thymidylate Synthase	18p11.32	28bp	28 bp repeat3R/2R	THYMS	3R=0.52	No effect, interaction with MTHFR C677T
cystathionine β -synthase	21q22.3	844ins68	68-bp insertion	CBS	2=0.10	Increased- no effect ⁴⁴

Both methionine synthase (MTR) and methionine synthase reductase (MTRR) enzymes are involved in Hcy remethylation, and the common MTR A2756G (Asp919Gly) and MTRR A66G (Ile22Met) variants were found to be associated with Hcy. Several studies reported that the MTR 919GlyGly genotype was associated with higher Hcy levels³¹⁻³³. The MTRR A66G variant was first described by Leclerc *et al.*³⁴. There are also several studies that could not show this, so results are inconclusive. *In vitro* studies showed that this transition mildly decreases enzyme activity³⁵, but only one study showed the MTRR A66G polymorphism to be a determinant of Hcy in the general population³⁶.

The reduced folate carrier RFC1 protein is involved in cellular uptake of reduced folate. The variant G80A (Arg27His) is thought to affect carrier function³⁷. Chango *et al.* found a trend towards higher Hcy in 27ArgArg individuals, which increased and inversely affected RBC folate in the MTHFR 222ValVal individuals³⁸.

Transcobalamin (TCN) is essential in the cellular uptake of vitamin B12. Vitamin B12 binds to TCN which accounts for 10-20% of the total cellular B12 uptake. Several polymorphisms have been identified in the TCN gene, including a C776G (Pro259Arg) transition which results in an altered binding of vitamin B12³⁹. Heterozygosity for this variant was associated with significantly higher Hcy compared with both homozygous genotypes⁴⁰.

Thymidine synthase (THYMS) catalyzes the reductive methylation and competes with MTHFR for the one-carbon unit of 5,10-methylenTHF. One of the polymorphisms in this enzyme is a 28-bp repeat in the 5' UTR (resulting in the common genotypes 2/2, 2/3, 3/3)⁴¹. In a Chinese population, reduced plasma folate and increased Hcy levels were found in 3/3 individuals; this effect became more pronounced in MTHFR 222ValVal individuals⁴².

Homocysteine is irreversibly converted by CBS to cystathionine, which is the first step in the transulfuration of Hcy. The CBS gene harbors many polymorphisms, including a common 68 bp insertion (844ins68)⁴³. De Stefano *et al.* reported that this polymorphism, although by itself having no effect on Hcy level, did seem to abolish the Hcy raising effect of homozygosity for the MTHFR 222ValVal individuals⁴⁴.

Most of the described polymorphisms, (except for the MTHFR Ala222Val variant), have been studied sporadically and have yielded inconclusive results. This could partly be due to the small sample size of most of the studies, while many studies also ignore gene-gene and gene-environment interaction. Since we expect relatively modest effect sizes for each individual polymorphism (according to the common variant – common disease hypothesis), large sample sizes are needed to study this properly.

Homocysteine and disease associations

Increased Hcy levels have been linked to the risk for a wide range of adverse health conditions throughout life, from birth defects to cardiovascular disease, cancer and cognitive dysfunction in the elderly. The cause of these associations is not yet known. Several mechanisms have been proposed to explain how Hcy may lead to disease. Regarding cardiovascular disease these include impaired (nitric oxide-mediated) vasodilatation due to endothelial dysfunction⁴⁵ and oxidative stress⁴⁶. A more general mechanism was recently reviewed by Jacobsen *et al.* who suggest that molecular targeting of proteins by Hcy (called homocysteinylation) may disrupt protein function and contribute to the pathogenesis of cardiovascular disease⁴⁷.

Although hyperhomocysteinemia has been associated with several diseases, the mechanism of homocysteine induced deleterious effects is not fully elucidated. Prominent among the various mechanisms proposed for the harmful effects of Hcy is its ability to modulate the expression of certain genes that may either directly or indirectly lead to several pathological conditions⁴⁸. Homocysteine-induced modulation of gene expression may be due to altered methylation status, because the levels of intracellular S-adenosylhomocysteine (SAH), an inhibitor of many S-adenosylmethionine-dependent (SAM) methyl transferases (Mtase), are elevated during hyperhomocysteinemic conditions⁴⁹.

Homocysteine and osteoporosis

A few years ago a mildly elevated Hcy was identified as a risk factor for osteoporotic fracture in both men and women^{11,12}. Hcy level in the highest 25% of the population resulted in an increased risk for fracture of approximately two-fold. In addition, a randomized, double-blinded study showed that folate and cobalamin supplementation is effective in preventing hip fracture in Japanese stroke patients^{50,51}. However, this observation needs to be replicated in other (larger) intervention studies. In order to prove a causal relationship we need intervention studies directed at lowering Hcy levels. Nevertheless, it is possible that the higher risk of fracture is not caused by higher Hcy levels, but by other factors related to a higher Hcy level.

Mendelian randomization

Mendelian randomization is the term that has been given to studies that use genetic variants in observational epidemiology to make causal inferences about modifiable (i.e. Hcy levels) risk factors for disease (i.e. osteoporosis)^{52,53}. Such studies exploit what is known as Mendel's second law or the law of independent assortment:

The independent distribution of alleles (or blocks of alleles in linkage disequilibrium) from parents to their offspring means that a study relating health outcomes in the offspring to genetic variation transmitted from the parents will not suffer from confounding. This holds true for full siblings who are not monozygotic twins. Despite the actual random allocation of groups of alleles being at the level of parents to their offspring yoke, at a population level—when relating genetic variants to disease outcome—alleles are generally unrelated to those confounding factors (in particular, socio-economic position and lifestyle factors) that distort the interpretations of findings from observational epidemiology.

Furthermore, genetic variants that are related to a modifiable exposure will generally be related to it throughout life from birth to adulthood and therefore their use in causal inference can also avoid attenuation by errors (regression dilution bias). Mendelian randomization studies are therefore defined as any study that uses genetic variation that serves as a robust proxy for an environmentally modifiable exposure in order to make causal inferences about the outcomes of the modifiable exposure. Therefore, we have utilized the Mendelian randomization approach to determine the magnitude of causal relationships between elevated Hcy levels and fracture risk. Mendelian randomization provides a method to assess the causal nature of some environmental exposures, by studying an association between a disease and a DNA polymorphism that mimics the biological link between a proposed exposure (such as increasing Hcy levels) and disease (such as fracture risk)⁵⁴. This approach was applied to explore the association between Hcy and stroke. A consistent increased risk of stroke was found in the 222ValVal homozygotes of the MTHFR Ala222Val polymorphism⁵⁵.

Observational studies have shown that riboflavin and folate modify the association between the MTHFR Ala222Val polymorphism and disease. It has been shown that 222ValVal individuals had significantly lower BMD than the AlaVal or AlaAla genotype group, but only when dietary riboflavin and/or folate intake was low⁵⁶. In order to be active, MTHFR needs to bind to a cofactor, flavin adenine dinucleotide (FAD), a derivative of riboflavin. The 222Val allele encodes the valine (Val) amino acid instead of alanine (Ala) amino acid and this slightly changes the binding site for FAD, resulting in a lower affinity for FAD than the 222Ala allele^{57,58}. Yet, this binding can be stabilized by the addition of folate^{57,58}. Therefore, low riboflavin or folate status may reduce MTHFR activity, especially in individuals with the 222ValVal genotype, which results in increased Hcy levels. Indeed, riboflavin and folate levels were shown to be predictors of Hcy levels in individuals homozygous for the MTHFR T677, and it is thought that higher riboflavin and folate intake are required in 222Val-homozygous individuals to maintain low Hcy levels^{24,57,58}.

Mechanisms explaining the relationship between Hcy and bone

A possible biological mechanism underlying the deleterious effect of Hcy on bone quality might involve the inhibition of collagen cross-linking by high Hcy concentrations^{5,59-62}. This hypothesis is based on observations in homocystenuria patients. *In vivo* evidence for this hypothesis is limited, and it remains to be determined whether collagen cross-linking is disturbed when Hcy levels are only mildly elevated. Recent evidence does not corroborate this hypothesis, since higher levels of Hcy were found to be correlated with excretion of higher levels of collagen cross-links⁶³. In a pilot study of 100 individuals, we also found that high Hcy levels are not associated with lower levels of collagen cross-links per excreted collagen molecule; instead we found a tendency towards higher number of collagen cross-links (Table 3). However, these markers are related mainly to resorbed bone, and one expects the Hcy effect (if it exists) to be on the quality of the newly formed bone, something that is not monitored effectively by any of the markers studied so far. In addition, the exact local concentration of Hcy at the tissue level of the active osteoid, and whether it is sufficient to interfere with collagen cross-links is not known. Interestingly, Paschalis *et al.* observed altered cross-links in fragile bone of osteoporotic patients specifically in the microenvironment of the bone-forming surfaces, which would support this hypothesis⁶⁴.

The association between Hcy and fracture could also be explained by other factors accompanying higher Hcy and not caused by Hcy itself. Higher Hcy could reflect a poor vitamin B status, which could directly affect bone metabolism. Patients with vitamin B12 deficiency have a higher risk for fracture⁶⁵, while recent population-based studies suggest that vitamin B12 status is important for maintenance of BMD^{63,66}.

Table 3. Collage cross link markers across the highest and lowest quartile of homocysteine levels

<i>Collagen cross link markers</i>	<i>Low homocysteine</i>	<i>High homocysteine</i>	<i>p-value*</i>
Number	N=50	N=50	
HP/Creat	51.6 ± 20.1	49.5 ± 15.5	0.51
LP/Creat	14.8 ± 5.9	14.5 ± 5.9	0.75
Hyp/Creat	24.7 ± 8.2	20.7 ± 8.2	0.02
Pro/Creat	49.9 ± 12.8	50.6 ± 12.8	0.89
HP/Col	0.68 ± 0.28	0.79 ± 0.29	0.08
LP/Col	0.19 ± 0.09	0.23 ± 0.09	0.09
Pro/Hyp	2.16 ± 0.73	2.63 ± 0.73	0.003

* p-values were calculated with ANCOVA, measures were adjusted for age, quartiles are sex adjusted. Urine measurements (HPLC):

HydroxylysylPyridinoline (HP) cross-links

LysylPyridinoline (LP) crosslinks

Hydroxyproline (Hyp, total amount of collagen)

Proline (Pro, total amount of protein).

Vitamin B12 has also been found to affect osteoblast activity and bone formation⁶⁷. Furthermore, Hcy or related B vitamins could affect the bone resorbing cells directly, as suggested by Herrmann *et al.*, who observed direct effects of Hcy on osteoclast activity⁶⁸.

Alternatively, Hcy is produced as a result of methylation reactions, higher Hcy levels could reflect the methylation status of the individual as reviewed above. Recent studies on DNA have revealed that DNA methylation is one of the age-associated changes. Chronic elevation in Hcy levels results in parallel increases in intracellular SAH, which is a potent inhibitor of DNA methyltransferases. It was shown that higher SAH levels lead to decreased methylation of lymphocyte DNA^{69,70}. In addition, folate treatment could restore DNA methylation to normal levels and correct the patterns of abnormal gene expression both in animal models⁷¹ and well as in humans⁷².

These results suggest that elevated Hcy levels may be an indirect indicator of elevated intracellular SAH and compromised cellular methylation capacity. In this light, this hypothesis was supported by studying a gene, PASG (Proliferation Associated SNF2-like Gene). They demonstrated that the disruption of a protein that facilitates DNA methylation (PASG), causes global hypomethylation, developmental growth retardation and a premature aging phenotype, including osteoporosis^{73,74}.

Thus, the decreased methylation associated with high Hcy levels may alter gene regulation, which could eventually lead to disease. Such “epigenetic” influence on disease has been studied intensely, in particular in cancer and arteriosclerosis⁷⁵⁻⁷⁹. Studies also indicate that the relation between Hcy and dementia might in part be explained by a lower methylation level in the brain leading to a lower activity of key methyltransferases, which is important in neurotransmitter metabolism⁸⁰.

Scope of this thesis

The general aim of this thesis is to study the association between Hcy and osteoporosis by examining genetic and dietary determinants of Hcy status on bone health. All the studies were performed within the Rotterdam Study, a population-based cohort study among 7,983 individuals aged 55 years and older.

In **chapter 2** we performed a systematic review and meta-analysis to gain insight in the current state of knowledge on the association between B vitamins related to Hcy metabolism and bone health.

In **chapter 3** we examined an effect of dietary B vitamins on BMD and risk of fracture in elderly men and women in the Rotterdam Study.

In **chapter 4** we examined the association between polymorphisms involved in homocysteine remethylation with bone phenotypes.

In the first part of this chapter, we aimed to examine the influence of riboflavin and/ or folate status on the relation between the MTHFR Ala222Val variant and Hcy levels, BMD, bone loss and risk of fracture.

In the second part of this chapter, we evaluated an association between the MTR Arg919Gly variant with BMD, bone loss and fracture risk. Furthermore, we also examined whether the dietary cobalamin intake influences the relationship of MTR Arg919Gly variant with bone end points.

In **chapter 5** we examined a possible association of Hcy levels with eight polymorphisms involved in Hcy metabolism. Moreover, we studied gene-nutrient interactions to address all genetic and dietary determinants of Hcy levels in one analysis.

In **chapter 6** we investigated the influence of both COLIA1 polymorphisms independently and in the form of haplotypes in relation to baseline femoral neck BMD, change in BMD with follow-up, and risk of vertebral and non-vertebral fractures.

Finally in the general discussion (**chapter 7**) the main findings of this thesis are addressed and suggestions are made for further research.

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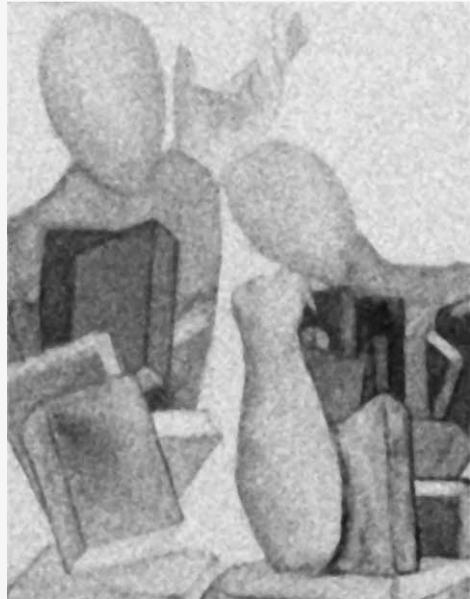
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CHAPTER 2

The association between homocysteine, related B vitamins and bone health: a systematic review and meta-analysis



Abstract

Many studies have examined the relation between Homocysteine (Hcy) level and B-vitamins in relation to bone metabolism, bone quality and fracture risk. This systematic review of observational studies investigates the association between Hcy or B vitamin status and bone health, defined as fracture risk or bone mineral density (BMD), in a comprehensive way.

A literature search was conducted in PubMed and Web of science up to 27 July 2007. We included longitudinal cohorts and cross-sectional studies of Hcy and related B vitamins in relation to BMD and fracture risk. The quality and heterogeneity of the study outcomes were assessed and graded.

Studies varied in the method of analysis precluding statistically pooling the data, therefore we used a best-evidence synthesis. Eight studies (all prospective cohorts), with a sample size ranging from 199 to 4766 subjects (four graded as good and four graded as fair) evaluated the association between Hcy levels and fracture risk. These studies show strong evidence for such an association between elevated Hcy levels and fracture risk, 75% of the studies reported this effect. A meta-analysis on studies which examined an association between Hcy levels and fracture shows 1.78 times an increased risk of fracture (95% CI: 1.43-2.23, $p < 0.0001$). Thirteen studies (all cross-sectional in design), examined the effect of Hcy levels on BMD; but with conflicting evidence. Sixteen studies (14 cross-sectional design, and two with a prospective design), evaluated the association between B vitamins and BMD. There is moderate evidence for an association between folate and vitamin B12 and BMD reporting on the effect of folate and one large good-quality study that reports on the effect of vitamin B12. Seven prospective cohort studies tested an association between B12 and/ or folate and fracture risk; but with conflicting evidence.

In conclusion, the heterogeneity in assessment methodology, and a lack of standardized threshold levels for categorizing low B vitamin status precluded definite conclusions to be drawn for relating B vitamins and bone health. However, there is convincing data to support a positive association between Hcy levels and fracture risk, while the data also suggest a positive association between BMD and vitamin B12 and folate status.

Introduction

Osteoporosis is a skeletal disorder characterized by compromised bone strength, which predisposes a person to increased risk of fracture¹. Osteoporotic fractures are a major health problem in the western society; they are associated with increased morbidity, mortality, and substantial economic costs. In the coming years, the number of fractures will increase throughout the world as the population ages, so prevention of fractures with inexpensive and well-established interventions is becoming increasingly important.

Homocysteine (Hcy) is a sulfur amino acid produced as an intermediate in the metabolism of methionine. Recent studies suggest that elevated Hcy may affect bone metabolism and bone quality, as well as fracture risk in humans^{2,3}. This suggests that circulating Hcy may be used as a modifiable risk indicator for osteoporosis, but a cause and effect relationship remains to be proven^{2,3}.

Since the first description many studies have investigated the relation between Hcy levels or nutritional determinants of Hcy levels and bone mineral density (BMD) and/or fracture risk³⁻¹⁴. The results of these studies however, are not consistent and a complete and systematic overview on the present level of knowledge on this relationship is lacking. More information on the association between high Hcy concentrations and/or the associated B vitamins that contribute to the risk of osteoporosis is needed.

Therefore, the aim of this systematic review is to gain insight into the current state of knowledge on the association between Hcy levels and bone health.

Materials and methods

Study Eligibility

The articles were identified by systematically searching Web of Science and PubMed, up to 27 July 2007. Osteoporosis, fracture, BMD, B vitamins (limited to B2, B6, B12 and folate), Hcy levels and equivalents of these words were used as keywords (see Appendix I).

A study was included when all of the following criteria were fulfilled. First, the article presents original data on a human study population. Second, the trait of interest is fracture, BMD at femoral neck (FN) and/ or lumbar spine (LS), or bone loss. We covered studies which examined either Hcy levels or one of the four B vitamins involved in Hcy metabolism (riboflavin, pyridoxine, folate and cobalamin) in relation to the above mentioned traits of interest. Third, the report was written in English.

Two independent researchers checked the abstracts regarding the above-mentioned criteria. We divided the studies into those dealing with Hcy associated with BMD or fracture, and those exploring Hcy-related B vitamins associated with BMD or fracture. Studies were excluded if the measured relevant data (results of the analysis) were unavailable or incomplete.

Methodological quality

A three-category grading system (good, fair, and poor) was used to denote the quality of each study included in our review. Each study was graded by two reviewers, and disagreements were resolved through consensus.

In the good grade, results were valid without obvious major bias. The study provided a clear description of the population, settings, B vitamin measurement technique and status, and the appropriate outcome measurements used (e.g., diagnosis of osteoporosis) applying validated methods for BMD, fracture and B vitamins assessment criteria, appropriate statistical and analytical methods (including adjustment for other risk factors such as gender, age and BMI), had less than 20% dropout, and clearly reported reasons for dropouts.

In the fair grade, the study had some deficiencies or was susceptible to some bias but not major bias. The study might be missing information, making assessment of the limitations and potential problems difficult, but omissions were not sufficient to invalidate the results. This category included studies that did not meet all the criteria in the good grade.

In the poor grade, a bias was present that could invalidate results. The study had serious errors in design, analysis, or reporting, or had large amounts of missing information or discrepancies in reporting.

Evidence synthesis

In homogeneous studies data were pooled and a meta-analysis was performed. For the meta-analysis we applied the Cochrane Review Manager (RevMan) version 4.2.10 program, the Cochrane Collaboration, Oxford, UK). The association between elevated Hcy levels and fracture risk was assessed by defining highest quartile of Hcy levels versus the three remaining quartile within each study with 95% confidence intervals (95% CI). In contrast to all the other studies, that examined the upper quartile against the rest, the Hordaland study only presented cut-off point of greater than 15 μM . Heterogeneity between the studies was calculated by means of X^2 – distributed Q statistic (with p -value considered significant at a level of 0.10)¹⁵, and the inconsistency index I^2 (suggesting inconsistency among the studies' results with values of 50%, or higher¹⁶ estimated by RevMan program. Hazard ratios (HRs) with 95% CI were estimated by random effects model. Random effects assume that there may be substantial diversity

among different study groups, and assesses both within-study sampling error and between-study variance. Random effects are preferable in the presence of significant between-study heterogeneity.

In case of heterogeneity, we refrained from statistically pooling the data and performed a best-evidence synthesis. The studies were ranked according to the methodological scores described above, and then were graded using the levels of evidence shown in (Appendix II) ¹⁷.

Data extraction

One reviewer performed data extractions of each accepted article, and a second reviewer independently verified the data. Discrepancies were resolved through consensus. The characteristics covered the study design, study population, year, design, location, follow-up, baseline characteristics, mean age, total number of subjects, metric outcome, and the outcome of the studies.

Results

The search of Web of Science and PubMed yielded 409 abstracts and articles on human studies that examined the association between Hcy levels, B vitamins and bone health. Among those, 73 abstracts met the inclusion criteria by reading the titles. Of these, 30 studies, including 24 articles and 6 abstracts met the eligibility criteria (see figure 1).

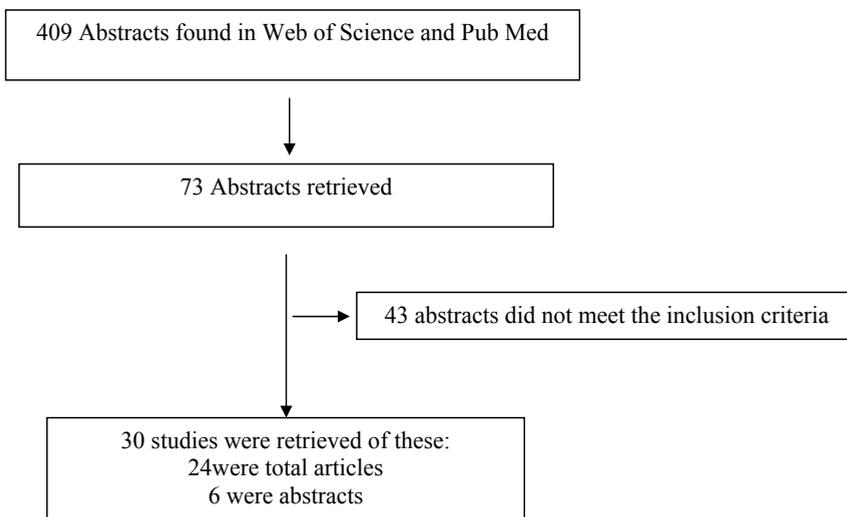


Figure 1. Flow diagram of the study selection process for this systematic review.

Table 1. Summary of observational studies evaluating the association between Hcy, B vitamins and bone health

<i>Studied association</i>	<i>No. of Studies</i>	<i>No. of participants</i>	<i>No. of fracture</i>	<i>Quality-level</i>			<i>Reported association</i>			<i>Level of evidence</i>
				<i>Good</i>	<i>Fair</i>	<i>Poor</i>	<i>Abstract</i>	<i>Poor</i>	<i>Fair</i>	
<i>Association between Hcy and fracture</i>										
	8									
		10505	1231	4	4				Association	Strong
<i>Association between Hcy and BMD</i>										
	13									Conflicting
	5	4249		1	1	1	1	1	Hcy associated	
	8	6559		1	6	1			Hcy not associated	
<i>Association between B vitamin and fracture</i>										
	7									Conflicting
<i>B12</i>										
	2									
	1	1210	55		1				B12 associated	
	1	904	91				1		B12 not associated	
<i>B12, Folate</i>										
	4									Conflicting
	2	5468	318	1	1				Folate associated	
	2	3012	627	1	1				Not Folate, not B12	
<i>B6, B12, B2, Folate</i>										
	1	5304		1					B6 associated	Limited
<i>Association between B vitamin and BMD</i>										
	16									
	3	2853		1	2		1		B12 associated	Moderate
	1	93					1		Folate not associated	Insufficient

<i>Studied association</i>	<i>No. of Studies</i>	<i>No. of participants</i>	<i>No. of fracture</i>	<i>Quality-level</i>			<i>Reported association</i>		<i>Level of evidence</i>
				<i>Good</i>	<i>Fair</i>	<i>Poor</i>	<i>Abstract</i>		
B6	1	1135				1	B6 associated	Insufficient	
B12, Folate	7								
	6	4293		2	2	1	Folate associated, Not B12	Strong	
	1	1550		1			B12 associated, Not Folate	Moderate	
B12, Folate, B6	2	447						Insufficient	
		119				1	B6, B12 and Folate associated		
		328				1	Folate associated, B12, B6 Not		
B6, B12, B2, Folate	2							Limited	
		5304		1			B2 and B6 associated		
		1241			1		B12 associated		

Meta-analysis of the associations between Hcy, B vitamins and BMD or B vitamins and fracture risk could not be performed due to heterogeneity in Hcy and B vitamin thresholds and data analysis. Additional sources of heterogeneity in these studies included differences in biological sample (plasma/serum) sample type (fasting, non-fasting), and differences in determination of B vitamin status (circulating levels, dietary intake). All the findings of our systematic review are summarized in table 1.

Association between Hcy levels and fracture risk

Eight studies (all prospective cohorts), of which four were of good quality and four were graded fair, evaluated the association between Hcy levels and fracture risk^{2,3,7,11-13,18,19} (Table 2). Sample sizes ranged from 199 to 4766 subjects, and the number of fracture cases ranged from 44 to 348 per study. All eight studies investigated fracture risks among subjects with high Hcy levels compared to low levels, and the threshold for defining high Hcy levels ranged from 15 to 26.4 $\mu\text{mol/l}$. Three of these studies reported increased fracture risk per 1 SD increasing Hcy levels^{2,11,19}. Six of the eight studies reported an association between elevated Hcy levels and risk of fracture^{2,3,7,11-13}. In one study this association was diminished after correcting for age¹⁹. In another study the association of Hcy levels with risk of fracture was partly dependent on folate levels¹¹. One study did not observe an association between elevated Hcy levels and risk of fracture¹⁸. Four studies observed that Hcy was associated with fracture risk independent of BMD^{2,13,18,19}, while the remaining studies did not study this aspect.

There is strong evidence to support a positive association between Hcy levels and fracture risk. There is heterogeneity in the thresholds used to define elevated Hcy

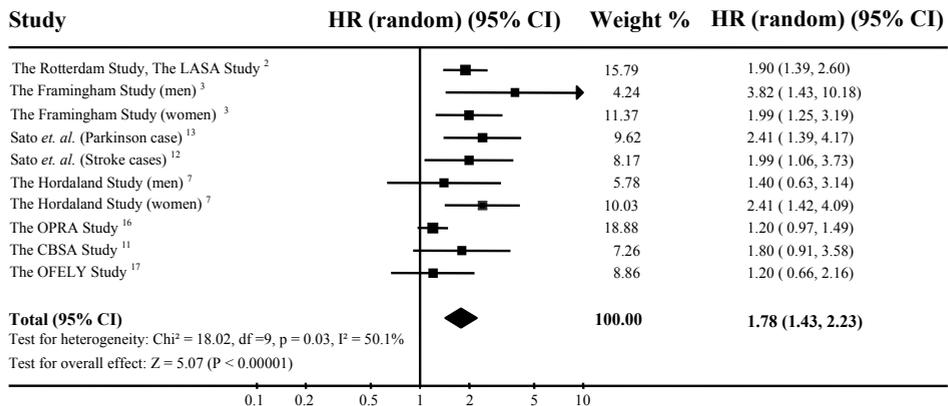


Figure 2. Meta-analysis of relationship between Hcy levels and fracture risk. Point estimates and 95% confidence intervals (CI) are shown for the hazards ratios (HR) in each study group. Summary estimates of HR and its 95% CI (diamonds), test for heterogeneity and effect are given by random effects model.

Table 2. Characteristics of the observational studies evaluating the association between Hcy and fracture

Authors, Year Design, country Source	Mean age (range) years	Total sample No. (W%)	Follow-up (years)	No. of fractures	Effect measure	Results	Reported association	Study quality	Additional Adjustments	
									B vitamins	BMD
van Meurs <i>et al.</i> ² , 2004 PC, The Netherlands <i>The Rotterdam Study</i> <i>The LASA Study</i>	75 (>55)	2406 (54)	3-8	180	HR	1.3 (1.1-1.5) per SD 1.9 (1.4-2.6) Q4 (range) vs rest	Yes	G	B12, Folate, B6 dietary intake (no effect)	Yes (no effect)
McLean <i>et al.</i> ³ , 2004 PC, USA <i>The Framingham study</i>	75 (59-91)	1999 (70)	14	M: 41 W: 146	HR	M: 3.8 (1.4-10.7) Q4 vs Q1 (>20.8 vs <8.5) W: 1.9 (1.2-3.1) Q4 vs Q1 (>18.6 vs <7.6)	Yes	G	No	No
Sato <i>et al.</i> ¹³ , 2005 PC, Japan <i>Parkinson cases</i>	71 (>65)	199 (100)	5	66	HR	2.4 (1.2-3.6) Q4 vs Q1 (>26.4 vs <7.1)	Yes	F	No	Yes (no effect)
Sato <i>et al.</i> ¹² , 2005 PC, Japan <i>Stroke cases</i>	75 (>65)	433 (53)	10	M: 33 W: 46	HR	2.0 (1.1-3.8) Q4 vs Q1 (>21.6 vs <10.4)	Yes	F	No	Yes (no effect)
Gjesdal <i>et al.</i> ⁷ , 2007 PC, Norway <i>The Hordaland Study</i>	66 (65-67)	4766 (55)	13	M: 90 W: 184	HR	M: 1.4 (0.6-3.0) > 15 vs < 9 W: 2.4 (1.4-4.1) > 15 vs < 9	Yes	G	B12, Folate levels (No effect)	No
Gerdtzen <i>et al.</i> ¹⁸ , 2007 PC, Sweden <i>The OPRA Study</i>	75 (75)	996 (100)	7	Inc.: 267 Prev.: 81	HR OR	Incident 1.2 (0.9-1.4) Q4 (range: 17.4-109.0) vs rest Prevalent 1.8 (1.1-3.0) Q4 (range: 17.4-109.0) vs rest	No	G	No	Yes (no effect)
Ravaglia <i>et al.</i> ¹¹ , 2007 PC, Italy <i>The CESA Study</i>	80 (65-94)	702 (53)	4	44	OR	1.3 (1.0-1.7) per SD 1.8 (0.9-3.6) >15 vs <15	Yes	F	B12, Folate: slightly reduced OR	No
Perier <i>et al.</i> ¹⁹ , 2007 PC, France <i>The OFELY Study</i>	62 (31-89)	671 (100)	10	134	HR	1.1 (0.9-1.3) Per 1 SD 1.2 (0.7-2.3) Q4 vs Q1 > 15.3	No	F	No	Yes (no effect)

HR=hazard ratio, OR= odds ratio, PC=prospective cohort, W=women, M=men, N.A= not available, Inc.=incident, Prev.=prevalent
Circulating levels of B vitamins have been implemented unless it has been described as dietary B vitamin intake

levels. There is moderate evidence that the increased fracture risk is independent of BMD.

A meta-analysis on studies which examined an association between elevated Hcy levels and fracture shows 1.78 times an increased risk of fractures (95% CI: 1.43-2.23, $p < 0.0001$) (Figure 2). There was a significant heterogeneity between the studies examining risk of fractures and Hcy levels across studies $I^2 = 50.1\%$, $p = 0.03$. However Hordaland Study ⁷ considered a cut-off point of greater than 15 μM and not highest quartile of Hcy levels against the rest. Therefore, there is a need to perform this meta-analysis with including details of quartile analysis of Hcy levels especially for Hordaland Study in a more homogeneity manner.

Association between Hcy levels and BMD

Thirteen studies (all with a cross-sectional design) of variable quality investigated the relationship between Hcy levels and BMD ^{2,4,6,8,10,12,18-23} (Table 3). For two studies only an abstract was available, so we were unable to evaluate the quality. Sample sizes ranged from 143 to 5329 subjects. Across the studies, six different sites of BMD measures were examined. Five studies, found a significant association between Hcy levels and BMD ^{6,8,20,21,23}. Among those five, in two studies the association disappeared after correction for folate and vitamin B12 ^{8,20}, while the other three studies did not adjust for B vitamin status. Eight studies, (one good, six fair, and one poor) with a sample size ranging from 143 to 2406 subjects found no association between Hcy levels and BMD ^{2,4,10,12,13,22,18,19}. Taken together evidence for the association between Hcy and BMD appears conflicting. In addition, large heterogeneity exists across these studies in terms of BMD outcome (site of BMD) and statistical analysis, which makes it difficult to support any definite conclusion regarding the relationship between BMD and Hcy levels.

Association between B vitamin and fracture risk

Seven prospective cohort studies evaluated an association between B vitamin status and fracture risk (Table 4). Among the studies the examined B vitamin(s) varied, as well as the method of determination of B vitamin status (serum vs dietary intake). Two studies, (one graded fair and one abstract) examined only vitamin B12 in relation to risk of fracture, ^{5,24}. One study (sample size of 1210 subjects and 55 fracture cases), observed a negative effect of vitamin B12 on fracture risk ⁵, while the other study (sample size 904 subjects, 91 fracture cases) found a similar relationship that failed to reach significance.

Four studies, examined both folate and B12 in relation to risk of fracture ^{7,11,18,25}. Two studies with a total sample size of 5468 subjects (318 fracture cases), reported a negative association between folate and risk of fracture, independent of Hcy and

Table 3. Characteristics of observational studies evaluating the association between Hcy and BMD

Authors, Year Design Location Source	Mean age (range) years	Total sample No. (W%)	Effect measure	Results	Reported association	Study quality	Adjustments Age Other B vitamins
Van Meurs <i>et al.</i> ² , 2004 PC, Netherlands The Rotterdam Study The LASA Study	73 (>55)	2406 (54)	Mean \pm SD	FN-BMD: No difference across quartiles of Hcy	No	G	Yes B2, B6, Folate, B12 dietary intake
Golbahar <i>et al.</i> ⁸ , 2004 CS, Iran	61 (58-63)	271 (100)	Correlation coefficient	FN-BMD: $r = -0.18$, $p = 0.003$ LS-BMD: $r = -0.16$, $p = 0.01$	Yes	F	Yes Folate, B12
Ardawi <i>et al.</i> ²⁰ , 2005 CS, Saudi-Arabia	60	278 (100)	Correlation coefficient	FN-BMD: $r = 0.25$, $p < 0.001$ LS-BMD: $r = 0.23$, $p < 0.01$	Yes	Abstract	Yes Folate, B12
Sato <i>et al.</i> ¹² , 2005 CS, Japan Stroke cases	75 (>65)	433 (53)	N.A.	Metacarpal BMD: No association	No	P	Yes -
Sato <i>et al.</i> ¹⁵ , 2005 CS, Japan Parkinson Cases	71 (>65)	199 (100)	Mean \pm SD	Metacarpal BMD: No difference across quartiles of Hcy	No	F	Yes Folate, B12
Morris <i>et al.</i> ¹⁰ , 2005 CS, US NHANES	68 >55	1550 (53)	Mean \pm SD	Total hip BMD: No Difference across quartiles of Hcy	No	F	Yes -
Gjesdal <i>et al.</i> ⁶ , 2006 PC, Norway The Hordaland Study	47-50 71-75	3094 (60) 2235 (54)	Regression coefficient	Hip-BMD W: $\beta = 0.004$, $p < 0.001$ M: No association	W: Yes M: No	G	No Folate, B12
Miyao <i>et al.</i> ²³ , 2000 CS, Japan	61	273 (100)	Correlation coefficient	LS-BMD: $r = 0.012$, $p < 0.01$	Yes	Abstract	Yes -
Herrman <i>et al.</i> ²² , 2005 CS, Switzerland	67 (57-75)	143 (100)	Correlation coefficient	Hip BMD: $r = 0.104$, $p = 0.23$ LS-BMD: $r = 0.157$, $p = 0.07$	No	F	Yes -
Cagnacci <i>et al.</i> ⁴ , 2003 CS, Italy	53 (50-57)	161 (100)	Correlation coefficient	No association LS-BMD	No	F	Yes Folate, B12
Gerdhem <i>et al.</i> ¹⁸ , 2007 PC, Sweden The OPRA Study	75 (75)	996 (100)	Mean \pm SD	FN-BMD: Difference between Q4 vs rest: 0.03 LS-BMD: no association	No	F	Yes -
Perier <i>et al.</i> ¹⁹ , 2007 PC, France The OFELY Study	62 (39-89)	671 (100)	Correlation coefficient	FN-BMD: no association LS-BMD: no association	No	F	Yes -
Baines <i>et al.</i> ²¹ , 2007 CC UK	67.5 (40-86)	328 (100)	Correlation coefficient	Os calcis BMD: $r = -0.130$, $p = 0.03$	Yes	P	Yes -

FN=femoral neck, LS=lumber spine, PC=prospective cohort, W=women, M=men, N.A= not available
Circulating levels of B vitamins have been implemented unless it has been described as dietary B vitamin intake

Table 4. Characteristics of observational studies evaluating the association between B vitamins and fracture

Authors, Year Design Location Source	Mean age (range) years	Total sample No.(W%)	Mean follow-up (years)	No. of fracture	B-vitamin measure	Effect measure	Results	Reported association	Study quality	Adjustments	
										BMD	Hcy/ B vitamins
Vitamin B12											
Dionukshe-Rutten <i>et al.</i> ⁵ , 2005 PC, The Netherlands <i>The LASA Study</i>	76 (73-78)	1210 (53)	3	M: 21 W: 34	Serum B12	RR	B12: W: 2.2 (1.1-4.3) M: 0.7(0.3-1.8) < 200 pM vs the rest	B12:W: Yes M: No	F	No	No
McLean <i>et al.</i> ²⁴ , 2005 PC, USA <i>The Framingham Study</i>	76 (59-91)	904 (59)	9	91	Plasma B12	HR	B12: 1.4 (0.9-2.1) < 258 pM vs the rest	B12: No	Abstract	No	Hcy
Vitamins B12, Folate											
Ravaglia <i>et al.</i> ¹¹ , 2005 PC, Italy <i>The CERSA Study</i>	80 (77-83)	702 (53)	4	44	Serum Folate and B12	OR	Folate: 2.4 (1.2-4.6) Q1 vs rest B12: No association	Folate: Yes B12: No	F	Yes	Hcy, B12
Gjesdal <i>et al.</i> ⁷ , 2007 PC, Norway <i>The Hordaland Study</i>	66 (63-69)	4766 (55)	13	W: 184 M: 90	Plasma Folate and B12	HR	Folate: W: 2.4 (1.5-3.8) <2.9 vs ≥ 6.6mM M: 1.4 (0.6-3.0) <2.9 vs ≥6.6mM B12: No association	Folate: W: Yes M: No B12: No	G	No	Hcy, B12:
Rejmanek <i>et al.</i> ²⁵ , 2007 PC, Denmark <i>DOFS Study</i>	N.A	2016	10	360	Folate and B12 intake	OR	N.A	Folate: No B12: No	Abstract	N.A	N.A
Gerdhem <i>et al.</i> ¹⁸ , 2007 PC, Sweden <i>The OPRA study</i>	75 (75)	996 (100)	7	267	Serum Folate and B12	N.A.	N.A	Folate: No B12: No	F	No	Hcy
Vitamins B12, Folate, B6, B2											
Yazdampah <i>et al.</i> ²⁶ , 2007 PC, The Netherlands <i>The Rotterdam Study</i>	68 (65-71)	5304 (59)	7	Non- vertebral n=744 fragility n=279	B2, B6, B12 and Folate dietary intake	HR	B6: 0.8 (0.7-0.9) Q4 vs rest > 2.0 mg/day (B6) vs <2.0 mg/day	B6 Yes Folate, B12: No	G	Yes	Hcy

HR=hazard ratio, OR= odds ratio, PC=prospective cohort. W=women, M=men. N.A= not available

vitamin B12 status ^{7,11}. Two studies (one fair and one abstract) with a sample size of 3012 subjects (627 fracture cases) found no association between either vitamin B12 or folate with risk of fracture ^{18,25}. There is conflicting evidence regarding the negative relationship between folate status and fracture risk. Only a limited number of studies is available and there was large heterogeneity across the studies regarding measurement of folate status and statistical analysis.

Only one study, (with a sample size of 5304 subjects) explored dietary vitamin B2, B6, B12 and folate intake, and observed an effect of dietary vitamin B6 intake with risk of fracture independent of Hcy levels and other dietary B vitamin intake ²⁶. However, because this is a single study, there is limited evidence to support this association.

Association between B vitamins and BMD

Sixteen studies (14 cross-sectional, ^{4,6,8-10,20,21,25-31}, and two prospective ^{14,25} (sample size ranging from 83 to 5304 subjects) evaluated the association between B vitamins and BMD (Table 5). Three studies examining an effect of vitamin B12 on BMD, (one good and two fair) found a positive association between vitamin B12 and BMD ^{14,27,31}. One of the three found this association independent of Hcy levels ³¹ while the others did not adjust for Hcy values. Taken together, there is moderate evidence for a positive association between vitamin B12 and BMD.

A single study, examined vitamin B6 in relation to BMD and reported a positive association between vitamin B6 and BMD at baseline, and during four years of follow-up ³². In conclusion, studying only vitamin B6, there is insufficient evidence for an association between vitamin B6 and BMD.

Seven studies with a sample size ranging from 161 to 5329 subjects, examined a possible effect of both vitamin B12 and folate on BMD ^{4,6,8,10,20,25,28}. Six of the seven studies observed a positive effect of folate on BMD ^{4,6,8,20,25,28}, but did not observe any association between vitamin B12 and BMD. In contrast, one study from the USA with a sample size of 1550 subjects and of good quality, reported an association between vitamin B12 and BMD ¹⁰. Overall, from the studies on vitamins B12 and folate, there is strong evidence for a positive association between folate and BMD.

In the studies examining vitamins B6, B12 and folate, there is insufficient evidence to draw any conclusion from the reported results because there were only two studies: one of which had insufficient data and the other was of poor quality.

Finally, two studies, (one fair and one good) assessed the effect of dietary intake of all four B vitamins in relation to BMD ^{9,26}. One study found a positive association between vitamin B12 and BMD ⁹, but did not correct for other related B vitamins or Hcy levels, and the other study (with a sample size of 5304 subjects) found a positive association between vitamin B2 and B6, independently of each other with

Table 5. Characteristics of observational studies evaluating the association between B vitamins and BMD

Authors, Year Design Location Source	Mean age (range), years	Total sample No. (W%)	B-vitamin Measure	Effect measure	Results	Reported association	Adjustments	
							Study quality	Age Others
Vitamin B12								
Dhonushe-Rutten <i>et al.</i> 7 2003 CS, The Netherlands	78 (75-81)	194 (74)	Plasma B12, Plasma MMA	Regression coefficient	$\beta = 0.12, p < 0.05$	B12: Yes MMA: No	F	Yes Weight
Stone <i>et al.</i> ¹⁴ , 2004 PC, USA The SOF Study	71 (69-74)	83 (100)	Serum B12	Mean difference in bone loss	1.6% difference, p=0.003 < 207.2 vs > 207.2 pMol/l	B12: Yes	F	Yes Weight
Tucker <i>et al.</i> ³¹ , 2005 CS, USA The Framingham Study	58.9 (56-62)	2576 (56)	Plasma B12	Mean \pm SD	FN-BMD: 6% difference LS-BMD: 6% difference > 259 vs \leq 148 pMol/l	B12: Yes	G	Yes Hcy, BMI
Folate								
Sheng <i>et al.</i> ³⁰ , 2007 CS, China	N.A	93 (100)	Serum N.A	Correlation coefficient	Hip-BMD: r=0.005, p>0.05 FN-BMD r=0.021, p>0.05	Folate: No	Abstract	N.A N.A
Vitamin B6								
McLean <i>et al.</i> ³² , 2006 PC, USA The Framingham Study	75.1 M 75.6 W (73-78)	1135 (50.2)	Plasma B6	Mean \pm SD	W: 0.032 g/cm lower BMD in < 20 vs \geq 30 (pM/l) M: 4% higher bone loss in < 20 vs \geq 30 M loss no	B6: M-BMD; bone loss Yes W: BMD Yes, bone loss no	Abstract	Yes Height, Weight

Table 5. Characteristics of observational studies evaluating the association between B vitamins and BMD (cont.)

Authors, Year Design Location Source	Mean age (range), years	Total sample No. (W%)	B-vitamin Measure	Effect measure	Results	Reported association	Study quality	Adjustments	
								Age	Others
Folate and vitamin B12									
Cagnacci <i>et al.</i> ⁴ , 2003 CS, Italy	53.5 (50-57)	161 (100)	Serum B12 Serum Folate	Correlation coefficient	r=0.371, p<0.0001	Folate: Yes B12: No	F	Yes	BMI
Golbahar <i>et al.</i> ⁸ , 2004 CS, Iran	60.8 (58-63)	271 (100)	Serum B2 Folate	Regression coefficient	FN-BMD: $\beta = 0.12$, p=0.001 LS-BMD: $\beta = 0.12$, p=0.05	Folate: Yes B12: No	F	Yes	BMI
Golbahar <i>et al.</i> ²⁸ , 2005 CS, Iran	60.8 (58-63)	366 (100)	Plasma B12 RBC- MTHF 5-MTHF	Correlation coefficient	FN-BMD: r =0.19, p =0.004 LS-BMD: r =0.21, p=0.001	Folate: Yes B12: No	P	Yes	BMI, B12, Hcy
Morris <i>et al.</i> ¹⁰ , 2005 CS, USA NHANES	68.0 >55	1550 (52)	Serum MMA(n=725), B12 Serum Folate, RBC Folate	Mean \pm SD	MMA:0.05 g/cm lower BMD in Q4 vs Q1, p=0.003 B12: lower BMD, p=0.01 <220 vs >220 (pM/l)	MMA, B12: Yes Folate, RBC Folate: No	G	Yes	Sex, BMI
Gjesdal <i>et al.</i> ⁶ , 2006 CS, Norway The Hordaland Study	47-50 71-75	3094 (60) 2235 (54)	Plasma Folate Plasma B12	Regression coefficient	W: $\beta = 0.001$, p=0.003	Folate: Yes B12: No	G	Yes	BMI, Hcy
Rejnmark <i>et al.</i> ²⁵ , 2007 CS, PC, Danish The DOPS Study	N.A	2016 (100)	Dietary intake (μ g/day) B12 Folate	Regression coefficient	Folate: BMD: $\beta = 0.027$, p=0.005	Folate: Yes BMD No association with bone -loss B12: No association	Abstract	N.A	N.A

Table 5. Characteristics of observational studies evaluating the association between B vitamins and BMD (cont.)

Authors, Year Design Location Source	Mean age (range), years	Total sample No. (W%)	B-vitamin Measure	Effect measure	Results	Reported association	Study quality	Adjustments	
								Age	Others
Ardawi <i>et al.</i> ²⁰ , 2005 CC, Saudi-Arabia	5-60	278 (100)	Plasma N.A.	Correlation coefficient	Folate: FN-BMD: $r=0.18$, $p<0.01$ LS-BMD: $r=0.15$, $p<0.02$	Folate: Yes B12: No	Abstract	Yes	Hcy, B12
Folate, vitamin B12, B6									
Martinez <i>et al.</i> ²⁹ , 2006 CS, Arizona	60	119	Serum B12 Serum Folate Serum B6 Dietary B12, B6, Folate	Beta coefficient	Folate W $\beta=0.031$, $p=0.07$	B6, B12, Folate Yes	Abstract	N.A.	N.A.
Baines <i>et al.</i> ²¹ , 2007 CS, UK	68 (40-86)	328 (100)	Serum B12 Serum Folate Plasma B6 (PDPP)	Correlation coefficient	Folate: $r=0.14$, $p=0.02$	Folate Yes B12, B6: No	P	Yes	Weight, Height
Folate, vitamin B12, B2, B6									
Macdonald <i>et al.</i> ⁹ , 2004, PC, CS, UK APOSS Study	48 (45-51)	1241 (100)	Dietary intake B2, B6, B12, Folate	Correlation coefficient	B12: LS-BMD: $r=0.064$, $p<0.05$	B12: Yes B2, B6, Folate: No	F	Yes	Height, Weight
Yazdanpanah <i>et al.</i> ²⁶ , 2007 PC, CS ²⁶ The Netherlands The Rotterdam Study	68 (65-71)	5304 (59)	Dietary intake B2, B6, Folate, B12	Regression coefficient	$\beta=0.09$, $p=1*10^{-8}$ B6 $\beta=0.06$, $p=0.002$ B2	B2, B6: Yes B12, Folate: No	G	Yes	Sex, B12, Folate

HR=hazard ratio, OR= odds ratio, PC=prospective cohort, CS=cross sectional. W=women, M=men. N.A.= not available

BMD²⁶. In summary, studies examining dietary intake of all four B vitamins (B2, B6, B12 and folate), produced conflicting results. This could be partly due to the heterogeneity in the statistical approach applied in these two studies while studying the same outcome.

Discussion

Our study highlights a substantial degree of heterogeneity in the literature evaluating the association of Hcy and related B vitamins with bone health. Notably, among the studies that examined the relationship between Hcy and bone health, different cut-off points for higher Hcy levels were used. Association studies on serum levels of B vitamins consider a variety of cut off points for low or high B vitamins status.

Most of the studies did not examine all four B vitamins (B2, B6, B12 and folate) involved in Hcy metabolism. In addition, although all B vitamins are involved in Hcy metabolism (as co-factors or substrates)³³ there is a lack of studies that explore B vitamins and Hcy levels simultaneously. Furthermore, the studies used different types of Hcy measurements (plasma/serum, or fasting/non-fasting) which may have affected the results. Moreover, there is considerable heterogeneity due to use of different statistical methods to express relationships and adjustments leading to methodological bias, which hinders drawing conclusions from a comparison of the studies.

Despite these limitations, 75% of studies reported a positive association between elevated Hcy levels and fracture risk^{2,3,7,11-13}. This association was consistent for Hcy levels in the highest population-specific quartile. Only two studies used a different cut-off point^{7,11}. Moreover, the result of the meta-analysis confirms the association between elevated Hcy levels and risk of fracture. Nevertheless, we think there is a need to repeat this analysis with having similar cut-off points across all studies. We are now in the process of contacting the authors of the studies which this issue is needed to be addressed. The relationship between Hcy and fracture risk seems to be independent of BMD, because adjustment for BMD did not alter the association. The fracture risks that were observed, generally are about two times higher for the highest quartile versus the rest of the population and, therefore quite large^{2,3,12,13}. Thus, if these risks were caused by low BMD values, the difference between mean BMDs in high versus low Hcy must be relatively large given the fact that one SD difference in BMD will generally result in a two times higher fracture risk. The reports that studied only the relation between Hcy levels and BMD (and not fracture) gave conflicting results. The studies that did observe an association between Hcy and BMD reported a modest effect size (0.2 SD). Taken together, all this suggests that the association

between Hcy and fracture risk is probably not mediated through BMD. Nevertheless, still it could be that Hcy is associated with BMD-change rather than cross-sectional BMD measures. Up to now there is lack of studies addressed this issue, therefore, future studies are needed to clarify.

The evidence from B vitamin studies in relation to BMD suggests that there is a moderate evidence for an association between BMD and circulating levels of folate and vitamin B12. However, the studies used different cut-off points to distinguish low from high levels in their analysis which could have introduced some heterogeneity in the results. Interestingly, studies that examined both folate and vitamin B12 status consistently reported folate to be associated with BMD and not vitamin B12, except one study from the USA which reported the opposite result. This apparent inconsistency can be explained by the mandatory fortification of folate in USA, which results in a better folate status in the American population. This is supported by the observation that vitamin B12, and not folate, is the major determinant of Hcy levels in the American population after folic acid fortification³⁴.

There are some limitations in studying the effects of B vitamins on bone health which require more attention. Since various B vitamins are often present in the same foods (see table 1, chapter1) simultaneous adjustment for the intake of other vitamins is required when studying associations with plasma Hcy concentrations. In addition, circulating B vitamin levels may not be the best marker for B vitamins status³⁵, considering the fact that up to 50% of subjects with low intracellular vitamin B12 status have normal plasma vitamin B12 levels³⁶. There is evidence that metabolites of B vitamins (such as methylmalonic acid for B12, and RBC-folate for folate) are more accurate indicators for assessing B vitamins status rather than studying solely circulating B vitamin levels^{35,37}. Therefore, for accuracy of assessing B vitamins status it might be more appropriate to measure circulating levels of B vitamin metabolites. Also, measuring vitamin levels only once may not reflect the accurate and long-term amount of circulating B vitamin and/or Hcy levels.

Several biological mechanisms could explain how elevated Hcy levels are related to fracture risk, including: collagen cross-linking, bone remodelling and/ or DNA methylation. A mechanistic role of high Hcy concentrations in bone metabolism was first suggested in patients with homocystinuria. This disease is characterised by extremely high Hcy levels and an increased prevalence of early osteoporosis³⁸ and in which a lower amount of collagen cross-links were found in the skin^{39,40}. It was suggested that high Hcy concentrations may interfere with collagen cross-linking, resulting in poor quality of bone and increased susceptibility to fracture⁴⁰. Because collagen cross-links are important for the stability and strength of the collagen network, interference in the cross-linking may lead to higher bone fragility. Interestingly, Paschalis *et al.*⁴¹ observed altered cross-links in fragile bone specifically in

the micro-environment of the bone-forming surfaces, which would support this hypothesis. In addition, Saito *et al.*⁴² recently showed that bone tissue of fracture cases had a lower amount of collagen cross-links and increased Hcy levels as compared to controls.

Alternatively, Hcy may have a direct effect on bone homeostasis by stimulating osteoclast formation and osteoclast activity²². This is corroborated by the fact that higher Hcy levels are associated with higher circulating concentrations of bone turnover markers⁴³. A higher bone turnover could result in bone micro-damage, which in turn leads to more fragile bone⁴⁴.

On the other hand, Hcy is produced as a result of demethylation of methionine. During this reaction, a free methyl group is donated and available to methylate DNA and/or proteins in the cell. Higher Hcy levels could therefore, reflect an impaired methylation status. Elevated Hcy levels may thus be an indirect indicator of elevated intracellular S-adenosylhomocysteine (SAH) and compromised cellular methylation capacity. It was indeed shown that higher circulating SAH levels are associated with decreased methylation of lymphocyte DNA^{45,46}. In addition, folate treatment was shown to lower Hcy levels and simultaneously restore DNA methylation to normal levels and correct the patterns of abnormal gene expression both in animal models⁴⁷ and humans⁴⁸. Thus, the decreased methylation associated with high Hcy levels may alter gene regulation, which could eventually lead to disease. This hypothesis was supported by studying a gene, called Proliferation Associated SNF2-like Gene (PASG), which facilitates DNA methylation. They demonstrated that the disruption of PASG causes global hypomethylation, developmental growth retardation and a premature aging phenotype, including osteoporosis^{49,50}.

In conclusion, there are convincing data to support an association between Hcy levels and fracture risk. There is moderate evidence that suggests a positive association between BMD and vitamin B12 and folate status. It is difficult to draw firm conclusions on the relation between B vitamins and bone health. This is due to the heterogeneity in the assessment methodology, and a lack of standardized threshold levels for categorizing low B vitamins status.

Appendix I. Key words.

#1	(BMD OR "bone mineral" OR" bone density" OR"bone loss" OR Fracture OR osteopor*)
#2	(homocyst* OR folate OR folic acid OR cobalamin OR B11 OR B12 OR riboflavin OR B2 OR "B vitamin" OR pyridoxine OR B6)
#2 AND #3	

*denotes any extension for the key words to cover all the related topics.

Appendix II. Levels of evidence for interpretation of the study results ¹⁷.

<i>Level of evidence</i>	<i>Interpretation</i>
Strong evidence	Consistent findings (>75%) in multiple high-quality studies
Moderate evidence	Consistent findings (>75%) in: - one high-quality study and some other low-quality studies - multiple low-quality studies
Limited evidence	Only one high-quality study
Conflicting evidence	Inconsistent findings in several studies of equal quality
Insufficient evidence	Less than 2 low-quality studies available
No evidence	No studies could be found

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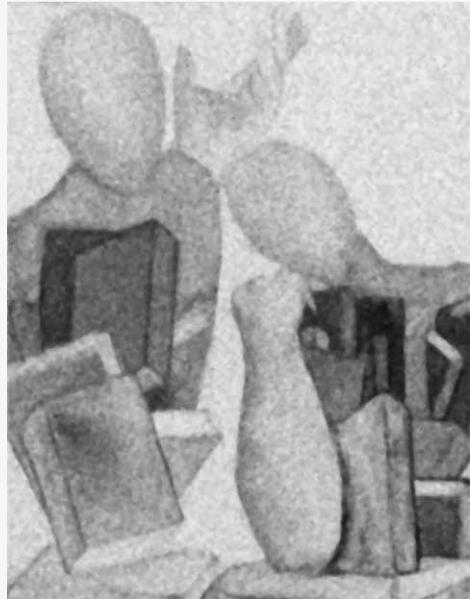
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CHAPTER 3

Effect of dietary B vitamins on BMD and risk of fracture in elderly men and women The Rotterdam Study



Abstract

A mildly elevated homocysteine (Hcy) level is a novel and potentially modifiable risk factor for age-related osteoporotic fractures. Elevated Hcy levels can have a nutritional cause, such as inadequate intake of folate, riboflavin, pyridoxine or cobalamin, which serve as cofactors or substrates for the enzymes involved in the Hcy metabolism. We examined the association between intake of Hcy-related B vitamin (riboflavin, pyridoxine, folate and cobalamin) and femoral neck bone mineral density BMD (FN-BMD) and the risk of fracture in a large population-based cohort of elderly Caucasians.

We studied 5304 individuals aged 55 years and over from the Rotterdam Study. Dietary intake of nutrients was obtained from food frequency questionnaires. Incident non-vertebral fractures were recorded during a mean follow-up period of 7.4 years, and vertebral fractures were assessed by x-rays during a mean follow-up period of 6.4 years. We observed a small but significant positive association between dietary pyridoxine ($\beta=0.09$, $p=1*10^{-8}$) and riboflavin intake ($\beta=0.06$, $p=0.002$) and baseline FN-BMD. In addition, after controlling for gender, age and BMI, pyridoxine intake was inversely correlated to fracture risk. As compared to the three lowest quartiles, individuals in the highest quartile of age and energy-adjusted dietary pyridoxine intake had a decreased risk of non-vertebral fractures (HR=0.77, 95% CI; 0.65-0.92, $p=0.005$), and fragility fractures (HR=0.55, 95% CI; 0.40-0.77, $p=0.0004$). Further adjustments for other dietary B vitamins (riboflavin, folate and cobalamin), dietary intake of calcium, vitamin D, A and K, protein and energy content, smoking and BMD did not essentially modify these results.

We conclude that increased dietary riboflavin and pyridoxine intake was associated with higher FN-BMD. Furthermore, we found a reduction in risk of fracture in relation to dietary pyridoxine intake independent of BMD. These findings highlight the importance of considering nutritional factors in epidemiological studies of osteoporosis and fracture.

Introduction

Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture. Several risk factors have been identified for osteoporotic fracture, such as age, low body mass index (BMI) and low bone mineral density (BMD). More recently, mildly elevated homocysteine (Hcy) concentrations were identified as a novel and potentially modifiable risk factor for age-related osteoporotic fractures^{1,2}.

Hcy is a sulfur-containing amino acid formed from the essential amino acid methionine. Defects in intracellular Hcy metabolism lead to an elevation of plasma Hcy concentrations. These metabolic defects can have a genetic cause, i.e., polymorphisms in genes involved in the Hcy metabolism. On the other hand, defects in the Hcy metabolism can also have a nutritional cause, such as inadequate intake of folate (vitamin B11), riboflavin (vitamin B2), pyridoxine (vitamin B6) or cobalamin (vitamin B12) which serve as cofactors or substrates for the enzymes involved in the Hcy metabolism^{3,4}. It is well established that increased plasma Hcy concentrations are associated with low dietary intake of riboflavin, folate, cobalamin, and pyridoxine⁵⁻⁷.

Several epidemiological studies have shown a positive association between folate and/or cobalamin status and bone end points. Some have found that higher serum concentrations of cobalamin⁸⁻¹¹ or folate¹² are associated with increased BMD, decreased bone loss¹³, and decreased risk of fracture^{9,14}. In addition, a randomized, double-blinded study in Japanese patients, showed that combined serum cobalamin and folate supplementation was effective in preventing hip fracture presumably by decreasing Hcy concentrations¹⁵. This indicates a possible effect of folate and cobalamin on bone strength through effects on the Hcy metabolism. There are limited data available on the effect of Hcy-related B vitamins on bone end points, especially the risk of fracture.

We examined the relation between intake of the Hcy-related B vitamins (riboflavin, pyridoxine, folate and cobalamin) and BMD and risk of fracture in a large population-based cohort of individuals aged 55 years and over.

Materials and methods

Study population

This study was conducted within the framework of the Rotterdam Study, an ongoing prospective population-based cohort study among subjects aged 55 years and over, living in Ommoord, a suburb of Rotterdam, the Netherlands. The rationale and

design of the Rotterdam Study have been described elsewhere ¹⁶. Approval of the Medical Ethics Committee of the Erasmus University Rotterdam was obtained. From all participants written informed consent was acquired. We studied 5304 subjects who had data available on dietary intake.

Anthropometric measurements

Height (cm) and weight (kg) were measured at the initial examination, in standing position wearing indoor clothes without shoes. Body mass index (BMI) was calculated as weight in kilograms divided by height in centimeters squared (kg/cm²).

Dietary intake

Dietary intake of vitamins (including riboflavin, pyridoxine, cobalamin folate, vitamin A, vitamin K, vitamin D and calcium intake) and use of supplements were assessed using validated food intake data obtained from a food frequency questionnaire. A validation study comparing this questionnaire with a 2-week food diary demonstrated reproducible and valid estimates ^{17,18}. For dietary vitamin B intake data were available for 5304 subjects. Dietary vitamin B intake was adjusted for age and energy intake as described elsewhere ¹⁹. Persons who reported taking supplements containing vitamins B2 (riboflavin), B6 (pyridoxine), B12 (cobalamin) or B-complex, as well as multivitamins, were classified as B vitamin supplement users (n=790).

Potential confounders

The presence of type 2 diabetes mellitus was defined by the current use of anti-diabetic medication or by a non-fasting or post-load plasma glucose level above 11.1 mmol per liter. Concentrations of serum creatinine were measured with the use of standard laboratory procedures. Prevalence of myocardial infarction was defined according to the international classification of diseases, 10th revision (ICD-10) ²⁰. Dementia was diagnosed with the use of the mini-mental state examination and the geriatric mental state schedule ²⁰. The number of falls in the preceding year, and current smoking status were assessed with the use of a questionnaire. A lower limb disability index was obtained by calculating the mean score of answers to questions concerning rising, walking, bending, and getting in and out of a car. The index is represented by a continuous score, ranging from 0 to 3, where 0 indicates no impairment and 3 indicates severe impairment ²¹.

Measurement of Hcy levels

Non-fasting blood samples from 738 subjects at baseline were immediately placed on ice and processed within 60 minutes. At baseline, serum samples were kept frozen until Hcy levels were measured. Total Hcy levels were determined as a fluorescence

derivate with the use of high-pressure liquid chromatography and expressed as micro mol per liter ($\mu\text{M/L}$)^{22,23}.

Measurement of bone parameters

BMD (in grams per square centimeter) of the hip and lumbar spine (L2-L4) was measured by dual-energy x-ray absorptiometry (DXA) using a Lunar DPX- densitometry apparatus (DPX-L, Lunar Corp. Madison, WI, USA), under standard protocols. Methods, quality, assurance, accuracy, and precision issues of the DXA measurements have been described previously (n=4891)²⁴. To increase the accuracy of BMD measurements on follow-up, the search and template tools in the comparison mode were used to position the femoral neck region of interest in scans of the same individual. The rate of change in BMD was calculated as the differences between baseline and the second follow-up, with a mean follow-up period of 6.5 ± 0.6 (SD) years (n=2422).

Fracture follow-up

Fracture events were obtained from the computerized records of the general practitioners (GPs) in the research area. Research physicians regularly followed participant information in the GP's records outside the research area, and made an independent review and encoding of all reported events. Subsequently, a medical expert in the field reviewed all coded events for the final classification of diseases, 10th revision (ICD-10)²⁰. Additional information on hip fractures was gathered through the Dutch national hospital registration system. Information on incident non-vertebral fractures has been collected within an average follow-up period of 7.4 ± 3.3 (SD) years. For studying incident fractures, all fractures which were considered not osteoporotic (fractures caused by cancer and all hand, foot, skull, and face fractures) were excluded. We considered separately fragility fractures occurring at the hip, pelvis and proximal humerus²⁵.

Vertebral fracture assessment

Both at baseline (1990-1993) and at the second follow-up visit (between 1997 and 1999) thoracolumbar radiographs of the spine were available for 3469 individuals in a mean follow-up of 6.4 years. All thoracolumbar radiographs of the follow-up visit were scored for the presence of vertebral fracture using the McCloskey/Kanis method, as described previously²⁶. If vertebral fractures were detected, the baseline radiograph was also evaluated. If the vertebral fracture was already present at baseline, it was considered to be a baseline prevalent fracture. If it was not present at baseline, the fracture was defined to be incident.

Statistical analysis

To examine a relation between dietary intake and BMD, a multivariable linear regression analysis was used. In this analysis femoral neck BMD (FN-BMD) and lumbar spine BMD (LS-BMD) were used as dependent variables and gender, age, BMI, dietary B (riboflavin, pyridoxine, folate, cobalamin) vitamins intake, calcium, vitamin D, K, and A, protein and energy content energy and protein intake were used as covariates. We applied a stepwise multiple regression approach to identify the best predictors for baseline BMD. Analysis of variance (ANOVA) was used to examine the associations between baseline general characteristics across quartiles of pyridoxine intake. Analysis of covariance (ANCOVA) was performed to adjust for possible confounders such as BMI, age and important factors related to co-morbidity. Variables were log transformed if they did not meet normality assumptions, this was the case for Hcy levels and dietary intake of folate and cobalamin. Quartiles of dietary pyridoxine intake were created for each gender after adjustment for age and energy by using the residual method¹⁹.

Incidence rates for all non-vertebral fractures were calculated by dividing the number of incident cases by the total number of fracture-free person-years, and 95% confidence intervals (CI) were calculated using the exact Poisson formula. The incidence rate of non-vertebral fractures was calculated for quartiles of dietary pyridoxine intake (age and energy-intake adjusted), taking the quartile with lowest pyridoxine intake as reference for the Cox proportional hazards analysis.

Cox's proportional hazards regression was used to calculate the hazard ratio (HR) and the 95% CI to estimate the relative risk of non-vertebral fractures. Odds ratios (OR) and 95% CI to assess vertebral fracture risk were estimated using logistic regression. Cox proportional hazards analysis was used to evaluate the contribution of dietary pyridoxine intake to mortality, based on a proportional hazards model.

All analyses were adjusted for gender, age and BMI. Subsequently, additional adjustments were made for the following confounders: Dietary B vitamins other than pyridoxine (riboflavin, cobalamin and folate), vitamin A and vitamin K intake, protein intake, current smoking, type 2 diabetes, serum creatinine, prevalence of myocardial infarction at baseline, history of recent falls, lower limb disability, and disability index.

All analyses were done using the SPSS package version 11 (SPSS, Chicago, IL, USA). P-values lower than 0.05 were considered significant.

Results

General characteristics of the study population are presented in Table 1. For each of the four B vitamins we found a significant association with both FN-BMD and LS-BMD after correcting for intake of protein, energy, gender, age and BMI (Table 2). Furthermore, we observed that for baseline FN-BMD among the B-vitamins, riboflavin

Table 1. Baseline characteristics of the study population

Characteristic	Study population
	n=5304
Age (years)	67.66 ± 7.75
Height (cm)	167.20 ± 9.20
Weight (kg)	73.63 ± 11.66
Body mass index (kg/m ²)	26.33 ± 3.66
<i>Dietary intakes</i>	
Riboflavin (mg/day)	1.59 ± 0.56
Pyridoxine (mg/day)	1.63 ± 0.40
Folate (µg/day)	218.60 ± 77.99
Cobalamin (µg/day)	5.26 ± 4.55
Vitamin K (µg/day)	264.7 ± 127.2
Vitamin A (µg/day)	1090.9 ± 781.6
Vitamin D (µg/day)	1.58 ± 1.01
Calcium (mg/day)	1127.0 ± 401.0
Protein intake (g/day)	81.33 ± 19.50
Energy intake (kJ/day)	8253.5 ± 2106.6
Current smoking (%)	23.2
Femoral neck BMD (g/cm ²)	0.87 ± 0.14
Lumbar spine BMD (g/cm ²)	1.09 ± 0.20

Data given as mean ± SD

Table 2. Single linear regression analysis between dietary B vitamin intakes and BMD in 5304 men and women in the Rotterdam Study

Dietary B vitamins intake	Model 1				Model 2			
	FN-BMD		LS-BMD		FN-BMD		LS-BMD	
	β [†]	p-value ^{††}	β	p-value ^{††}	β	p-value ^{††}	β	p-value ^{††}
Riboflavin	0.082	2.6*10 ⁻¹¹	0.058	7*10 ⁻⁶	0.10	7*10 ⁻⁴	0.079	6*10 ⁻⁵
Pyridoxine	0.061	3*10 ⁻⁶	0.041	0.003	0.07	4*10 ⁻⁴	0.069	0.001
Folate	0.043	0.001	0.041	0.002	0.03	0.05	0.04	0.01
Cobalamin	0.039	0.001	0.026	0.049	0.01	0.55	0.01	0.51

Model 1: adjusted for gender, age and BMI.

Model 2: Model 1 plus adjustment for energy intake and protein intake.

† Standardized coefficients beta, FN: femoral neck; LS: lumbar spine.

†† Calculated by using linear regression.

Table 3. Multivariable regression analysis of determinants of femoral neck and lumbar spine BMD in 5304 men and women in the Rotterdam Study

Variable	<i>Starting model</i>				<i>Final Model</i>			
	FN-BMD		LS-BMD		FN-BMD		LS-BMD	
	β †	<i>p</i> -value‡‡	β †	<i>p</i> -value‡‡	β †	<i>p</i> -value‡‡	β †	<i>p</i> -value‡‡
Female	-0.33	10 ⁻¹¹²	-0.35	10 ⁻¹¹⁶	-0.33	10 ⁻¹¹²	-0.35	10 ⁻¹¹⁶
Age (years)	-0.27	10 ⁻⁹⁸	-0.004	0.75	-0.27	10 ⁻⁹⁹	-	-
Body mass index (kg/m ²)	0.28	10 ⁻¹⁰²	0.25	10 ⁻⁷⁴	0.28	10 ⁻¹⁰²	0.25	10 ⁻⁷⁴
Riboflavin (mg/day)	0.11	10 ⁻⁸	0.08	0.0002	0.09	10 ⁻⁸	0.06	10 ⁻⁵
Pyridoxine (mg/day)	0.07	0.002	0.06	0.01	-0.06	0.002	-0.06	0.002
Energy intake (kJ/day)	-0.05	0.01	-0.07	0.002	-0.06	4*10 ⁻⁴	-0.08	10 ⁻⁵
Folate (µg/day)	-0.02	0.46	0.01	0.73	-	-	-	-
Vitamin D	-0.01	0.88	-0.01	0.42	-	-	-	-
Calcium	0.01	0.68	-0.02	0.29	-	-	-	-
Vitamin A	0.003	0.88	0.003	0.90	-	-	-	-
Vitamin K	-0.01	0.53	-0.03	0.07	-	-	-	-
Cobalamin (µg/day)	-0.01	0.50	-0.01	0.51	-	-	-	-
Protein intake (g/day)	-0.03	0.29	-0.03	0.27	-	-	-	-

† Standardized coefficients beta. ‡‡ calculated by using linear regression. FN: femoral neck; LS: lumbar spine. In the multivariable linear regression analysis FN-BMD or LS-BMD was used as the dependent variable and gender, age, BMI, dietary B (riboflavin, pyridoxine, folate, cobalamin) vitamins intake, dietary intakes of: calcium, vitamin D, K, and A, protein and energy content were used as covariates. In the final regression model, only variables significantly and independently associated with FN-BMD (LS-BMD) were selected through a stepwise regression method. Adjusted R² for final model (SE); FN-BMD: (0.25 ± 0.12, *p*<0.001); LS-BMD: (0.17 ± 0.18, *p*<0.001).

was the strongest predictor ($\beta=0.09$, *p*=1*10⁻⁸) and pyridoxine was a good predictor ($\beta=0.06$, *p*=0.002) (Table 3). Gender, age and BMI explained 24% of the variation in FN-BMD. Pyridoxine and riboflavin together explained 1% extra variation (data not shown). For LS- BMD we found similar results for riboflavin ($\beta=0.06$, *p*=1*10⁻⁵) and pyridoxine ($\beta=0.06$, *p*=0.002) (Table 3). At the lumbar spine, gender and BMI explained 16% of the variation in BMD, while pyridoxine and riboflavin together also explained another 1% of the variation (data not shown). Age was not a predictor for LS-BMD. Furthermore, in a separate analysis we additionally adjusted for age at menopause and parity. These adjustments did not affect the results (data not shown).

We investigated whether B vitamin intake was associated with non-vertebral and fragility fractures. Only pyridoxine (as a continuous variable) was inversely associated with non-vertebral, fragility and vertebral fractures (Table 4). Further adjustment for other nutritional factors, (including four dietary B vitamins intake (riboflavin, pyridoxine, cobalamin, folate) and dietary of :vitamin D, calcium, vitamin A and vitamin K intake) and baseline FN-BMD or co-morbidity, did not essentially change the result.

Table 4. Association of dietary pyridoxine intake as a continuous variable with risk of fracture in 5304 men and women in the Rotterdam Study

Types of fracture	No. fracture/ Total no (%)	β †	Relative Risk (95% CI)	p-value††
<i>Non-vertebral</i>				
Model 1	744/5304 (14.0)	-0.25	0.78 (0.60-1.00)	0.06
Model 2		-0.36	0.70 (0.49-1.00)	0.05
Model 3		-0.45	0.64 (0.42-0.98)	0.04
<i>Fragility</i>				
Model 1	279/5304 (5.3)	-0.54	0.58 (0.38-0.90)	0.02
Model 2		-0.85	0.43 (0.24-0.77)	0.004
Model 3		-1.09	0.34 (0.18-0.64)	0.001
<i>Vertebral</i>				
Model 1	328/3003 (6.2)	-0.44	0.64 (0.42-0.98)	0.04‡
Model 2		-0.63	0.46 (0.33-1.20)	0.16 ‡
Model 3		-0.64	0.53 (0.27-1.04)	0.06 ‡

Model 1: Adjustment for gender, age, BMI and energy intake

Model 2: Model 1 plus additional adjustments including: dietary intake of riboflavin, cobalamin, folate, protein, calcium, vitamin D, vitamin K, vitamin A and co-morbidity status including; prevalence of myocardial infarction, type 2 diabetes, dementia, creatinine level, current smoking and recent falling

Model 3: Model 2 plus additional adjustment for FN-BMD for all non-vertebral and fragility fractures, For vertebral fractures adjustment for LS-BMD

† Indicates beta coefficient by unit of milligram per day

††Calculated by Cox's proportional hazards regression, ‡Calculated by logistic regression

Table 5 shows the comparisons of general characteristics of the study population across quartiles of (age and energy adjusted) intake of pyridoxine. There was a significant difference in weight, BMI, dietary intake of riboflavin, folate and cobalamin across quartiles of dietary pyridoxine intake ($p < 10^{-4}$) (Table 5). Supplemental therapy did not differ across quartiles. To avoid confounding by supplement use, we also performed the analyses excluding users of supplements but the results remained unchanged ($n = 790$, 9.9%) (data not shown). FN-BMD and LS-BMD increased within quartiles of dietary pyridoxine intake ($p = 10^{-4}$). Moreover, we observed a significantly reduced bone loss at the femoral neck; the latter result remained unchanged after adjustment for baseline FN-BMD.

Comparison of a number of important factors related to co-morbidity across quartiles of dietary pyridoxine intake are presented in Table 6. Subjects in the highest quartile of dietary pyridoxine intake had the lowest percentage of current smoking. The remainder of the factors related to co-morbidity did not differ across the quartiles. The results were not affected by adjustment for other nutritional factors.

Figure 1 shows the incidence rate of non-vertebral (A) and fragility (B) fractures, and percentage of vertebral fractures (C) by quartiles of dietary pyridoxine intake.

Table 5. General characteristics of age and energy-adjusted pyridoxine quartiles in 5304 men and women in the Rotterdam Study

	<i>Quartile 1</i> n=1326	<i>Quartile 2</i> n=1325	<i>Quartile 3</i> n=1327	<i>Quartile 4</i> n=1326	<i>p-value</i> †
Mean pyridoxine (mg/day)	1.30	1.50	1.67	2.03	
Male (%)	40.8	40.8	40.8	40.8	
Age (year)	67.64 ± 0.21	67.61 ± 0.21	67.96 ± 0.21	67.42 ± 0.21	0.34
Weight (kg)	72.52 ± 0.29	73.60 ± 0.29	74.27 ± 0.29	74.14 ± 0.29	10 ⁻⁷
Height (cm)	167.12 ± 0.17	167.09 ± 0.17	167.42 ± 0.17	167.17 ± 0.17	0.51
BMI (kg/m ²)	25.95 ± 0.10	26.35 ± 0.10	26.50 ± 0.10	26.54 ± 0.10	10 ⁻⁷
Riboflavin (mg/day)	1.41 ± 0.01	1.50 ± 0.01	1.61 ± 0.01	1.83 ± 0.01	10 ⁻⁹⁴
Folate (µg/day) ††	177.83 ± 0.07	196.20 ± 0.07	214.42 ± 0.07	252.93 ± 0.07	10 ⁻²⁵⁶
Cobalamin (µg/day) ††	4.02 ± 0.07	4.27 ± 0.07	4.41 ± 0.07	5.05 ± 0.07	10 ⁻¹⁵
Protein intake (g/day)	74.56 ± 0.48	78.29 ± 0.48	81.94 ± 0.48	90.51 ± 0.48	10 ⁻¹²⁶
Femoral neck BMD (g/cm ²)	0.853 ± 0.004	0.861 ± 0.004	0.880 ± 0.004	0.879 ± 0.004	10 ⁻⁴
Lumbar spine BMD (g/cm ²)	1.074 ± 0.005	1.076 ± 0.005	1.099 ± 0.005	1.106 ± 0.005	10 ⁻⁴
Rate of change FN-BMD (g/cm ² per yr)	-0.0067 ± 0.0004	-0.0064 ± 0.0004	-0.0052 ± 0.0004	-0.0055 ± 0.0004	0.01

Data given as mean ± SE. Adjustment for gender and age.

† Calculated by Analysis of variance (ANCOVA) †† Back log transformed

Table 6. Comparison of co-morbidity markers by quartiles of dietary pyridoxine intake in 5304 men and women in the Rotterdam Study

	<i>Quartile 1</i> n=1326	<i>Quartile 2</i> n=1325	<i>Quartile 3</i> n=1327	<i>Quartile 4</i> n=1326	<i>p-value</i> †
Mean pyridoxine (mg/day)	1.30	1.50	1.67	2.03	
<i>Co-morbidity</i>					
Dementia	3.5 (47)	3.7 (49)	4.2 (56)	3.2 (42)	0.55
Current smoking	31.0 (401)	23.9 (308)	21.0 (273)	17.7 (230)	0.01
Diabetes	9.5 (126)	10.0 (132)	10.8 (143)	12.1 (160)	0.15
Prevalent MI	13.0 (170)	11.6 (150)	12.3 (160)	12.0 (156)	0.69
Recent fall	14.8 (196)	15.0 (199)	13.1 (174)	13.1 (174)	0.32
Disability index ≥ 0.5	26.5 (351)	24.0 (318)	24.3 (322)	23.5 (311)	0.33

Data given as percentage (cases)

† Calculated by Analysis of variance (ANOVA)

The results suggest a possible threshold between the fourth and the three remaining quartiles of dietary pyridoxine intake. There was a decreased risk of fractures in the highest quartile of dietary pyridoxine intake compared with the three remaining quartiles (for incidence of non-vertebral fracture HR=0.77, 95%CI; 0.65-0.92, p=0.005, for fragility fracture HR=0.55, 95%CI; 0.40-0.77, p=4*10⁻⁴ and for vertebral fracture OR=0.86; 95%CI; 0.65-1.13, p=0.27) after controlling for gender, age and BMI. Further adjustment for other dietary B vitamins, protein, energy intake, calcium, vitamin D,

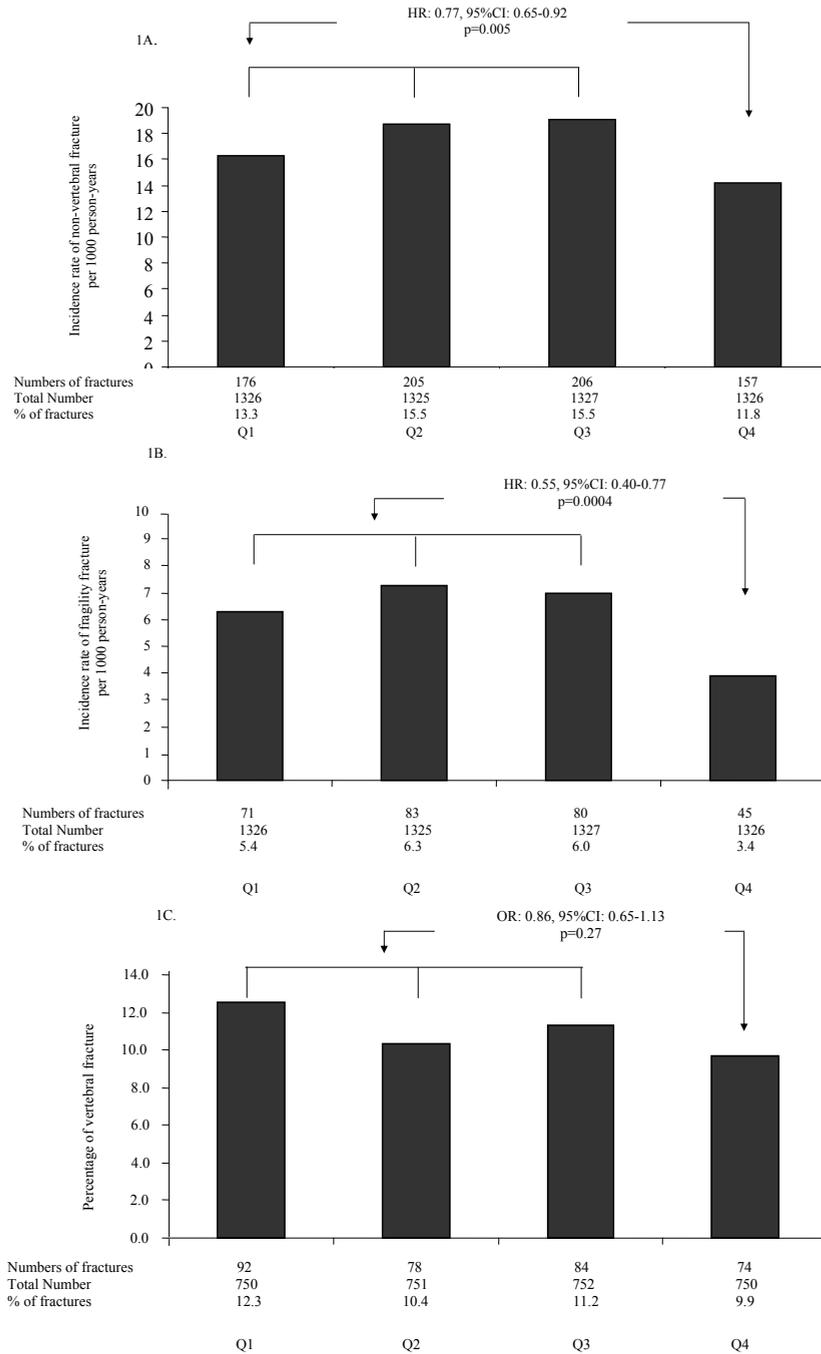


Figure 1. Incidence rate of A. non-vertebral fracture and B. fragility fracture and C. rate of vertebral fracture by quartiles of dietary pyridoxine intake.

HR: hazard ratio, CI: confidence interval, Q: quartile

Quartiles of dietary pyridoxine intake were defined by gender, age and energy intake adjusted.

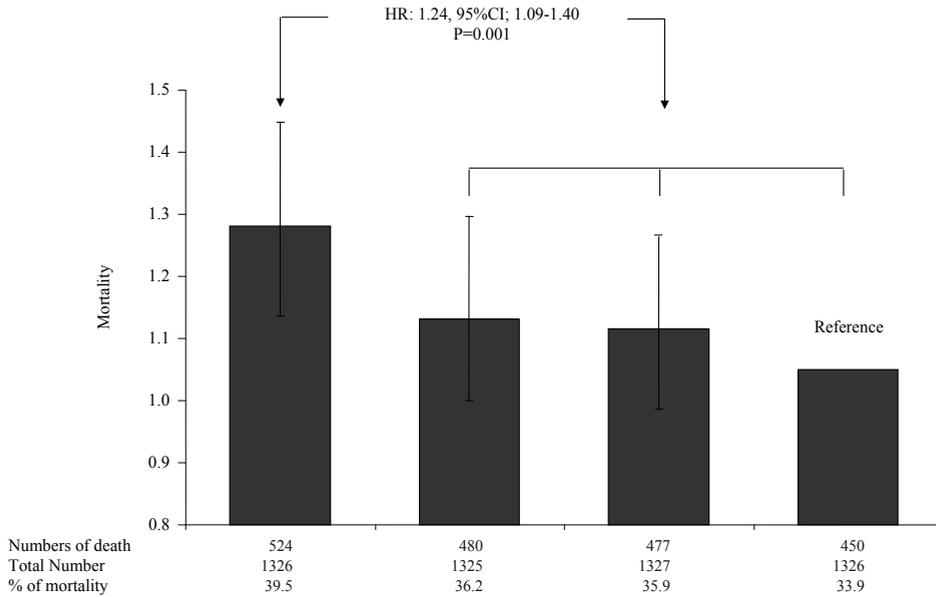


Figure 2. Risk of mortality by quartiles of dietary pyridoxine intake (highest quartile is reference), Gender and age adjusted

Table 7. Association of fragility fracture and dietary pyridoxine intake in a subgroup of 738 subjects with Hcy measurement

Fractures	No.fracture/ Total no (%)	Relative risk	p-value†
<i>Non-vertebral</i>			
Model 1	101/739 (13.7)	0.56 (0.30-1.06)	0.08
Model 2		0.59 (0.31-1.13)	0.11
<i>Fragility</i>			
Model 1	42/739 (5.7)	0.37 (0.13-1.06)	0.06
Model 2		0.40 (0.13-1.16)	0.09
<i>Vertebral</i>			
Model 1	45/428 (6.1)	0.60 (0.24-1.53)	0.29
Model 2		0.71 (0.28-1.79)	0.47

†Calculated by Cox's proportional hazards regression

Model 1: Gender, age and BMI adjusted

Model 2: Model 1 plus adjustment for Hcy measurement

A and K intake, co morbidity, and baseline FN-BMD did not alter the results. For the other three B vitamins we found no association with fractures (data not shown).

Since subjects with low vitamin intake might have increased mortality related to lifestyle or co-morbidity, we investigated the risk of mortality by quartiles of pyridoxine intake (Figure 2). Subjects in the lowest quartile had 1.24 times higher risk of mortality compared with the three remaining quartiles (95% CI; 1.09-1.40, p=0.001).

Table 7 shows the association between fractures and pyridoxine intake in the

subset (n=738) of subjects who had Hcy measurements in this subset. Adjustment for Hcy levels did not alter the association with fractures.

Discussion

In this population-based study in elderly individuals we observed a positive and independent relation between dietary intake of riboflavin and pyridoxine with BMD. Furthermore, high intake of pyridoxine was associated with a significantly decreased risk of fracture. This effect was not modified either by factors related to co-morbidity or by dietary intake of other B vitamins (riboflavin, folate, cobalamin), energy, protein, calcium and vitamin D. In addition, this effect appears to be independent of FN-BMD.

Pyridoxine and certain other B vitamins (riboflavin, folate and cobalamin) function as cofactors for enzymes that maintain low Hcy levels²⁷. We hypothesized that high intakes of riboflavin, folate, pyridoxine and cobalamin might be related to a lower risk of osteoporotic fracture by decreasing Hcy levels. Several biological mechanisms could explain how elevated Hcy levels are related to fracture risk. It has been suggested that Hcy concentrations may interfere with collagen cross-linking, resulting in poor quality of bone and increased susceptibility to fracture²⁸. Alternatively, Hcy or related B-vitamins could affect the bone cells directly, something that has been suggested by recent studies of Herrmann *et al.*, who observed direct effects of Hcy on osteoclasts²⁹.

Among the four studied B vitamins we observed that only higher dietary pyridoxine intake was associated with lower risk of fracture. After correcting for Hcy levels, the increased risk of fracture at low pyridoxine intake remained unchanged, suggesting that the effect of pyridoxine is independent of Hcy levels. However, since Hcy levels were available only for a small subgroup of our population and were measured only once, we cannot fully address the question whether or not the protective effect of dietary pyridoxine on fracture risk is mediated through lowering Hcy or not. The lack of a sizeable population in which both Hcys and B-vitamins are measured also makes it difficult to compare the effect-sizes and/or examine interaction between the two factors with respect to their effect on fracture risk.

The present study confirms earlier findings that gender and BMI are the main determinants of FN-BMD and LS-BMD. Among the four B vitamins, only pyridoxine and riboflavin were independent predictors for BMD. This result is not consistent with previous studies which reported cobalamin^{9,13} or folate¹² to be important determinants of BMD; however, these studies did not examine the status of riboflavin and pyridoxine. In a study by Macdonald *et al.*³⁰ a weak but significant association

was observed between intake of each single dietary B complex vitamins and BMD; however, most of these associations disappeared after adjustment for confounders such as age, height, weight and smoking. Because they did not consider all four B vitamins in a multivariate analysis, this casts doubt on whether the associations of the B vitamins with BMD were independent of each other or not. In contrast, in the present study we examined contributions of all B vitamins in a multivariate approach in order to explain the variation in BMD and found the effects of both pyridoxine and riboflavin to be independent of each other.

Little is known about an effect of pyridoxine on bone. However, some reports suggest a role for this vitamin in maintaining structural integrity of connective tissue. Pyridoxine serves as an essential co-factor for lysyl oxidase, a key enzyme for the formation of enzymatic cross-links in bone³¹. Mice studies showed that pyridoxine deficiency results in a low (25.3%) amount of cross-link intermediates and impaired cross-link formation in bone³². In addition, a correlation was found between decreased circulating pyridoxine concentrations and impaired cross-link formation in bone of human individuals with fracture³³. These observations suggest that pyridoxine deficiency may lead to impaired cross-link formation, resulting in increased bone fragility.

It is known that pyridoxine acts not only as a cofactor for lysyl oxidase but also as cofactor for over 100 enzyme-catalysed reactions in the body, including many involved in the synthesis or catabolism of neurotransmitters including gamma-aminobutyrate (GABA)³⁴. Therefore, pyridoxine deficiency could affect the locomotor system and thus, increase the risk of falling, and thereby increase fracture rates. However, we did not observe an effect of pyridoxine intake on the rate of falls, which makes this a less likely explanation.

Because low dietary intake of vitamins may reflect bad dietary habits or compromised health, we studied mortality rates across quartiles of dietary pyridoxine intake and found an increased risk of mortality in individuals in the lowest quartile. This selective mortality might have reduced the contrast in fracture risk between the lowest quartile and the highest quartile. Thus, subjects in the lowest quartile of dietary pyridoxine intake may have died before a fracture could have occurred. Therefore, the real effect of low pyridoxine intake on fracture risk in the lowest quartile might be even larger than that observed in our study.

The main strengths of the present study are the size of our study population, and the validated dietary assessment. The study also has some limitations. Because we did not have serum levels of B vitamins available to study their relation with BMD and fractures, our findings are likely to be biased by self-report. Furthermore, only baseline dietary intakes were available, and duration of any possible vitamin B deficiency could not be assessed. Nevertheless, in Europe the dietary intakes

are relatively stable over time, especially among the elderly³⁵. The validated food frequency test is therefore a good measure for long-term assessment of nutrient intake. Using supplement therapy might dilute the relation between the quartile of pyridoxine intake with fracture. Nonetheless, our results were unchanged after either excluding supplement users at baseline (9.9%) from the analysis, or after controlling for B vitamin supplement use, suggesting that it is highly unlikely that residual confounding caused by intake of vitamin B supplements influenced our results. Although we adjusted for known confounders (such as important factors related to co-morbidity and using B vitamin supplements and dietary intake of vitamin K, A and, D), we cannot completely exclude that the effect of pyridoxine intake on fracture may be a reflection of residual confounding by unknown factors.

In conclusion, we observed a reduction in the risk of fracture in relation to dietary pyridoxine intake. We cannot conclude whether the association between pyridoxine and fracture risk is causal. Therefore, performing placebo-controlled trials with pyridoxine supplements are needed to elucidate this association. The relative impact of pyridoxine intake and Hcy levels and the mechanisms through which these compounds may affect the risk of fracture should also be further investigated.

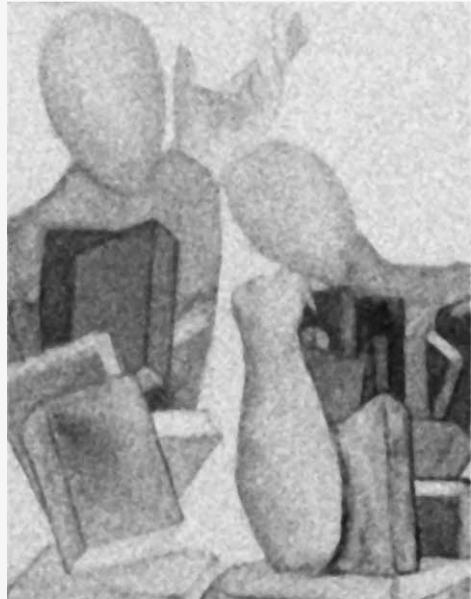
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CHAPTER 4

The association between polymorphisms involved in homocysteine remethylation with bone phenotype



CHAPTER 4.1

**Low dietary riboflavin but not folate
predicts increased fracture risk in
postmenopausal women homozygous for
the MTHFR 677T allele**

Abstract

The MTHFR C677T polymorphism is associated with mildly elevated homocysteine (Hcy) levels in the presence of low folate and/or riboflavin status. A mildly elevated Hcy level was recently identified as a modifiable risk factor for osteoporotic fracture. We investigated whether dietary intake of riboflavin and folate modifies the effects of the MTHFR C677T polymorphism on bone mineral density (BMD) and fracture risk.

We studied 5035 individuals, from the Rotterdam Study aged 55 years and older, who had data available on the MTHFR, nutrient intake and fracture risk. We have performed analysis on Hcy levels in a total of 666 individuals while BMD-data was present for 4646 individuals (2692 women).

In the total population, neither the MTHFR C677T polymorphism nor low riboflavin intake was associated with fracture risk and BMD. However, in the lowest quartile of riboflavin intake female 677-TT homozygotes had a 1.8 (95% CI: 1.1-2.9, $p=0.01$) times higher risk for incident osteoporotic fractures and a 2.6 (95% CI: 1.3-5.1, $p=0.01$) times higher risk for fragility fractures as compared to the 677-CC genotype (p -interaction=0.0002). This effect was not seen for baseline BMD in both men and women. No significant influence was found of dietary folate intake on the association between the MTHFR C677T genotype and fracture risk or BMD. In the lowest quartile of dietary riboflavin intake, T-homozygous individuals (men and women combined) had higher (22.5%) Hcy levels compared with C-homozygotes (mean difference=3.44 $\mu\text{mol/L}$, $p=0.01$) (p -trend=0.02).

In this cohort of elderly Caucasians, the MTHFR C677T variant interacts with dietary riboflavin intake to influence fracture risk in women.

Introduction

Osteoporosis is a condition characterized by low bone mineral density (BMD) and micro-architectural change in bone tissue, which leads to reduced bone strength and increased risk of fracture ¹. Genetic and environmental risk factors interact to influence BMD, bone loss ^{2,3}, and fracture risk ⁴. We and others have described that mildly elevated circulating homocysteine (Hcy) level is a potentially modifiable risk factor for osteoporotic fracture ^{5,6}.

It is not yet clear whether the relationship between homocysteine and fracture risk is causal or not. A small placebo-controlled trial in stroke patients showed reduced fracture risk in response to lowering Hcy levels suggesting a causal link ⁷. However, this trial needs replication in the general elderly population using an appropriate design and sufficient power to detect the differences in fracture risk ⁸. Another way to demonstrate a causal relationship between increased Hcy and fracture risk is by studying genetic variants that determine Hcy levels ⁹. The most well known genetic determinant of homocysteine levels in the general population is the common C677T polymorphism in the methyltetrahydrofolate reductase (MTHFR) gene ¹⁰.

MTHFR is required for the formation of 5, 10 methyltetrahydrofolate, which in turn is necessary to convert Hcy to methionine. The MTHFR C677T variant is associated with increased Hcy levels and has been implicated in increased risk of a wide range of adverse health conditions throughout life, from birth defects ¹¹ to cardiovascular disease ¹², cancer ¹³, pregnancy complications ¹⁴, psychiatry disorders ¹⁵, and osteoporosis ¹⁶ in the elderly.

The MTHFR C677T polymorphism results in an alanine (Ala) to valine (Val) substitution at position 222 of the protein, giving rise to a thermolabile enzyme with reduced activity ¹⁰. In order to be active MTHFR needs to bind to a cofactor, flavin adenine dinucleotide (FAD), a derivative of riboflavin. The Val222 (T677) allele affects the binding site for FAD resulting in a lower affinity for FAD than the C677 allele ^{17,18}. This binding can be stabilized by the addition of folate or riboflavin ^{17,18}. Therefore, low riboflavin or folate status may reduce MTHFR activity, especially in individuals with the TT genotype, which results in increased Hcy levels. Indeed, riboflavin and folate levels were shown to be predictors of Hcy levels in individuals homozygous for the MTHFR T677, and it is thought that higher riboflavin and folate intake are required in T-homozygous individuals to maintain low Hcy levels ^{17,19,20}.

A number of studies found a relationship between this polymorphism and BMD ^{16,21,22} or fracture ¹⁶. Some studies have investigated the association of the MTHFR C677T variant with BMD in relation to the four B-vitamins (riboflavin (B2), pyridoxine (B6), folate (B11), and cobalamin (B12)) involved in the homocysteine metabolism ²²⁻²⁴ but results are inconsistent. These inconsistencies could be due to differences

in nutrient intake across the populations studied, since it is well known that the phenotypic expression of the MTHFR C677T variant is dependent on folate and riboflavin status.

We have examined the influence of riboflavin and/or folate status on the relation between the MTHFR C677T variant and risk of fracture and investigated whether the dietary riboflavin and folate intake influence the relationship of MTHFR C677T variant with BMD, bone loss, and Hcy levels in a large population-based prospective cohort of elderly Caucasian of the Rotterdam Study.

Materials and Methods

Study population

This study was embedded in the Rotterdam Study, a population-based cohort study of men and women in which all residents of the Rotterdam suburb Ommoord aged 55 years and older were invited to take part. The design of the study has been described elsewhere²⁵. Written informed consent was obtained from all participants and the Medical Ethics Committee of the Erasmus Medical Center approved the study. Baseline data collection was conducted between January 1990 until June 1993, while the follow-up assessment of BMD was performed between July 1996 and December 1999. A total of 7983 men and women participated in the study (response rate 78%) and for the present analysis we studied 5035 men and women who were genotyped for the MTHFR C677T variant and had available data on nutrient intake and fracture data. We have performed analysis on Hcy levels in a total of 666 individuals and BMD data was present for 4646 individuals.

Dietary intake

At baseline participants completed a checklist at home that queried foods and drinks they had consumed at least twice a month during the preceding year as well as dietary habits, use of supplements, and prescribed diets. Next, during their visit to the research center, they underwent a standardized interview with a dietician based on the checklist, using a 170-item semi-quantitative food frequency questionnaire. A validation study comparing this questionnaire with a 2-week food diary demonstrated reproducible and valid estimates²⁶. These dietary data were converted to total energy intake and nutrient intake per day with the computerized Dutch Food Composition (*Dutch Food Composition Table* (in Dutch). Rev ed. The Hague, the Netherlands: Voorlichtingsbureau voor de Voeding; 2001). Therefore, dietary habits were assessed using validated food intake data from a food frequency questionnaire

and were available for 5035 men and women who have been genotyped. Dietary vitamin B intake was adjusted for energy intake as described elsewhere²⁷.

Measurement of homocysteine levels

Non-fasting blood samples from 666 subjects at baseline (250 men and 416 women) were immediately placed on ice and processed within 60 minutes. Serum samples were kept frozen until Hcy levels were measured. Total Hcy levels were determined as a fluorescence derivative with the use of high-pressure liquid chromatography and expressed as micro mol per liter ($\mu\text{mol/L}$)^{28,29}.

Fracture follow-up

Fracture events were obtained from the computerized records of the general practitioners (GPs) in the research area. Research physicians regularly followed participant information in the GP's records outside the research area, and made an independent review and encoding of all reported events. Subsequently, a medical expert in the field reviewed all coded events for the final classification of diseases, 10th revision (ICD-10)³⁰. Additional information on hip fractures was gathered through the Dutch National Hospital Registration system. Information on incident osteoporotic fracture was available for an average follow-up period of $7.4 \pm 3.3(\text{SD})$ years ($n=707$ fractures). For studying incident fractures all fractures which were considered not osteoporotic (fractures caused by cancer and all hand, foot, skull, and face fractures) were excluded. We considered separately fragility fractures occurring at the hip, pelvic, proximal humerus ($n=269$ fractures)³¹.

Measurement of BMD

BMD (in grams per square centimeter) of the hip and lumbar spine (L2-L4) (LS) was measured by dual-energy x-ray absorptiometry (DXA) using a Lunar DPX-densitometry apparatus (DPX-L, Lunar Corp. Madison, Wisconsin), under standard protocols. To increase the accuracy of BMD measurements on follow-up, the search and template tools in the comparison mode were used to position the femoral neck region of interest in scans of the same individual using DPX-IQ software. The rate of change in BMD (mg/cm^2 per year) was calculated as the difference between baseline and follow-up BMD divided by the time (in years) elapsed between measurements (and multiplied by a factor of 1000 for scale convenience) (mean $6.5 \pm \text{SD } 0.6$ years). The relative change of BMD from baseline was estimated as the difference in BMD between assessment periods divided by the BMD at baseline.

Anthropometric measurements

Height (cm) and weight (kg) were measured at the initial examination, in standing position wearing indoor clothes without shoes. Body mass index (BMI) was computed as weight in kilogram divided by height in meters squared (kg/m^2).

Possible confounders

Possible confounders were taken from measures made at baseline. The presence of type II diabetes mellitus was defined by the current use of anti-diabetic medication or by a non-fasting or post-load plasma glucose levels above 11.1 mmol per liter³². Dementia was diagnosed with the use of the Mini-Mental State Examination and the Geriatric Mental State Schedule³³. The presence of myocardial infarction was assessed by questionnaires and by analysis the ECG's using the Modular Electrocardiogram Analysis System (MEANS)³⁴. The number of falls in the preceding year, and current smoking status were assessed with the use of a questionnaire.

Genotyping

Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. 1-2 ng genomic DNA was dispensed into 384-well plates using a Caliper Sciclone ALH3000 pipetting robot (Caliper LS, Mountain View, CA, USA). Genotypes were determined using the Taqman allelic discrimination assay. The Assay-by-Design service (www.appliedbio-systems.com) was used to set up a Taqman allelic discrimination assay for the MTHFR C677T polymorphism (Primers Fw: CCTCAAAGAAAAGCTGCGTGATG Rv: GCACTTGAAGGAGAAGGTGTCT Probes FAM-ATGAAATCGACTCCCGC, VIC-ATGAAATCGGCTCCCGC). The PCR reaction mixture included 1-2 ng of genomic DNA in a 2 μl volume and the following reagents: FAM and VIC probes (200 nM), primers (0.9 μM), 2x Taqman PCR master mix (ABgene, Epsom, UK). Reagents were dispensed in a 384-well plate using the Deerac Equator NS808 (Deerac Fluidics, Dublin, Ireland). PCR cycling reaction was performed in 384 well PCR plates in an ABI 9700 PCR system (Applied Biosystems Inc., Foster City, CA, USA) and consisted of initial denaturation for 15 minutes at 95° C, and 40 cycles with denaturation of 15 seconds at 95° C, and annealing and extension for 60 seconds at 60° C. Results were analysed by the ABI Taqman 7900HT using the sequence detection system 2.22 software (Applied Biosystems Inc., Foster City, CA, USA). To confirm the accuracy of genotyping results, 332 (5%) randomly selected samples were re-genotyped with the same method. No inconsistencies were observed.

Statistical analysis

Allele and genotype frequencies of the MTHFR C677T variant were tested for Hardy-Weinberg equilibrium proportions with χ^2 test.

Analysis of variance (ANOVA) was used to examine the associations between the MTHFR C677T variant with Hcy levels and BMD measurements across genotypes and quartiles of vitamin intake. Analysis of covariance (ANCOVA) was performed to adjust for possible confounders such as BMI, age and co-morbidity. In case of a consistent trend, shown as an allele dose effect, we performed a linear regression analysis to quantify the association.

Variables were log transformed if they did not meet normality assumptions, this was the case for homocysteine levels and dietary folate intake. Riboflavin and folate intake was analysed as a categorical variable with quartile cut-off points. Quartiles have been made in a gender specific manner and have been energy adjusted.

To estimate incident fracture risk we used Cox proportional hazard models, thereby taking potential differences in follow-up time into account with adjustment for age. Further adjustments were also made for possible confounders, i.e., type II diabetes mellitus, dementia, current smoking status and recent falls. We investigated possible interaction between dietary riboflavin intake and the MTHFR C677T genotype in relation to fracture by including a product term of the two main effects in a Cox regression model with fracture as the dependent variable. Population attributable risks were calculated with the use of the formula $\{P (RR-1) \div (P (RR-1) + 1)\} * 100$, where P is the percentage of the population exposed and RR is the relative risk of fracture. P-values lower than 0.05 were considered significant. All analyses were done using the SPSS package version 11.

Results

Allele and genotype frequencies were in Hardy Weinberg equilibrium proportions ($p=0.2$), with a T-allele frequency of 33%. The T-allele frequency was similar in men and women and did not change with age. Table 1 shows general characteristics of the study population across MTHFR C677T genotypes. No significant differences in age, height, weight and BMI were seen across the MTHFR C677T genotype groups in both genders (Table 1). Serum plasma Hcy levels were 3.5 $\mu\text{mol/L}$ (23.9%) higher in male TT carriers and 1.1 $\mu\text{mol/L}$ (8.2%) higher in female TT carriers compared to the CC genotype with evidence of an allele dose effect (p for trend <0.001 in males and p for trend $=0.03$ in females respectively, Table 1). Overall, women were significantly older and had significantly lower dietary intake of nutrients compared with men.

Hcy levels

In a subset of individuals with Hcy level measurements, we investigated the relationship between the MTHFR genotype and Hcy levels across quartiles of dietary ribofla-

Table 1. Baseline characteristics by MTHFR genotype (C677T) in 2093 men and 2942 women of the Rotterdam Study

	CC	CT	TT	¶p
	<i>Men</i>			
N * (%)	961 (45.9)	923 (44.1)	209 (10.1)	
Age (y)	67.0 ± 7.4	67.5 ± 7.3	67.4 ± 7.5	0.32
Height (cm)	175.0 ± 6.6	174.8 ± 6.7	175.2 ± 6.5	0.67
Weight (kg)	78.4 ± 10.6	78.7 ± 11.0	77.7 ± 9.9	0.14
Body mass index (kg/m ²)	25.7 ± 2.9	26.0 ± 2.9	25.4 ± 2.9	0.03
Riboflavin (B2) intake (mg/day)	1.68 ± 0.62	1.63 ± 0.57	1.70 ± 0.70	0.09
Folate (B11) intake (µg/day) (min-max) †	224.8 (75.0-1352.5)	218.2 (67.3-707.3)	222.3 (66.9-915.0)	0.08
N (%) **	116 (46.4)	116 (46.4)	18 (7.2)	
Hcy level (µmol/L) (min-max) †	14.77 (9.20-37.10)	15.79 (8.30-27.20)	18.30 (10.60-41.40)	‡<0.001
	<i>Women</i>			
N * (%)	1318 (44.8)	1311 (44.6)	311 (10.6)	
Age (y)	68.2 ± 8.0	67.8 ± 8.0	68.8 ± 8.4	0.12
Height (cm)	161.8 ± 6.4	162.0 ± 6.2	161.4 ± 6.9	0.23
Weight (kg)	69.4 ± 11.4	69.5 ± 11.4	69.6 ± 11.3	0.75
Body mass index (kg/m ²)	26.8 ± 4.0	26.7 ± 4.1	26.7 ± 4.0	1.00
Riboflavin (B2) intake (mg/day)	1.53 ± 0.51	1.54 ± 0.51	1.49 ± 0.49	0.31
Folate (B11) intake (µg/day) (min-max) †	197.8 (45.4-973.7)	199.8 (63.9-1012.7)	196.9 (84.2-753.0)	0.50
N (%) **	196 (47.1)	175 (42.1)	45 (10.8)	
Hcy level (µmol/L) (min-max) †	13.96 (1.30-38.70)	14.44 (6.20-63.70)	15.10 (8.60-54.40)	0.03

Data are given as unadjusted mean ± SD for continuous variables

†Geometric mean (min-max), ‡P for trend after adjusting for age

* Number of individuals with nutrient intake data, ** Number of individuals with Hcy levels data, ¶ p ANOVA

vin and folate intake. In the lowest quartile of dietary riboflavin intake, T-homozygous individuals (men and women combined) had 3.44 µmol/L higher (22.5%) Hcy levels compared with C-homozygotes (p=0.01) (p-interaction=0.05) (Figure 1). For dietary folate intake we found similar results, with Hcy levels being 4.77 µmol/L higher in T-homozygous individuals compared with C-homozygotes (p=0.001), (p-interaction=0.1). The results suggested a possible threshold effect in the lowest quartile of dietary riboflavin or folate intake. When we stratified according to gender, we only observed a significant effect in women, while in men a similar trend was seen, however not reaching significance. Further adjustments for all possible confounders and factors related to co-morbidity did not alter the results.

BMD and BMD-loss

No association was observed in men or women between the MTHFR C677T variant and baseline BMD (femoral neck and lumbar spine), BMD-loss at the femoral neck and risk of fracture (Table 2).

Table 2. Bone parameters by MTHFR genotype (C677T) in 2093 men and 2942 women of the Rotterdam Study

	Men			Women			p-value*
	CC	CT	TT	CC	CT	TT	
<i>BMD</i>							
N (%)	884 (45.2)	871 (44.6)	199 (10.2)	1200 (44.6)	1196 (44.4)	296 (11.0)	
FN BMD (g/cm ²)	0.92 ± 0.14	0.92 ± 0.14	0.92 ± 0.14	0.84 ± 0.13	0.83 ± 0.14	0.82 ± 0.13	0.19
LS BMD (g/cm ²)	1.17 ± 0.19	1.16 ± 0.20	1.17 ± 0.21	1.04 ± 0.18	1.03 ± 0.18	1.03 ± 0.18	0.26
N (%)	448 (50.0)	442 (45.4)	104 (10.7)	574 (43.4)	619 (46.8)	129 (9.8)	
FN BMD-loss (% of baseline year) [†]	-0.42 ± 0.96	-0.44 ± 0.82	-0.36 ± 0.91	-0.84 ± 1.10	-0.80 ± 1.10	-0.96 ± 1.06	0.43
<i>Fracture</i>							
N (%)	961 (45.9)	923 (44.1)	209 (10.0)	1318 (44.8)	1311 (44.6)	313 (10.6)	
No of osteoporotic fractures (%)	72 (7.5)	64 (6.9)	19 (9.1)	241 (18.3)	247 (18.8)	64 (20.4)	
HR (95% CI)	1 (reference)	0.85 (0.63-1.15)	1.06 (0.67-1.69)	1 (reference)	1.07 (0.91-1.25)	1.05 (0.82-1.35)	0.49
No of fragility fractures (%)	31 (3.2)	28 (3.0)	7 (3.3)	95 (7.2)	82 (6.3)	26 (8.3)	
HR (95% CI)	1 (reference)	0.80 (0.51-1.25)	0.81 (0.38-1.71)	1 (reference)	0.96 (0.76-1.23)	0.98 (0.67-1.44)	0.82

Data are given as mean ± SD, FN= femoral neck, LS= lumbar spine, HR=hazard ratio

* p for ANOVA, adjusted for age and BMI, [†] FN-BMD-loss (relative % of baseline year) between 2nd follow-up and baseline; mean follow-up time is 6.5 years

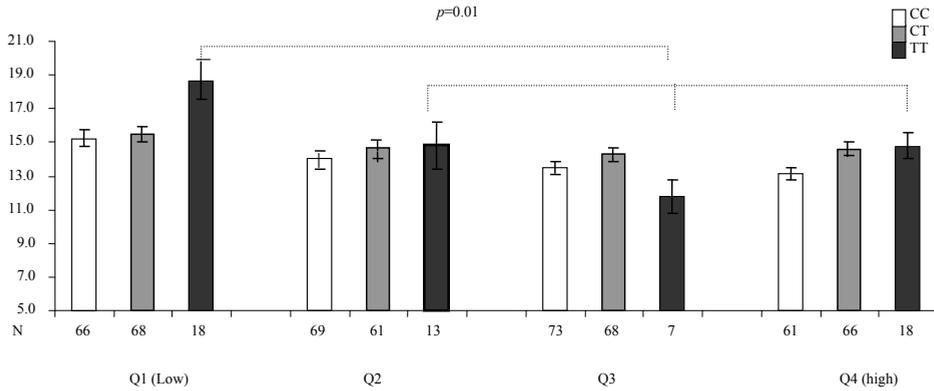


Figure 1. Homocysteine levels by MTHFR genotype (C677T) in quartiles of dietary riboflavin intake in both men and women.

Bars represents back transformed means, with standard errors. Adjustments were done for gender, age and BMI. Hcy levels in TT homozygous subjects in the lowest quartile of dietary riboflavin intake was compared to those with TT genotype in the three remaining quartiles. N denotes numbers of subjects.

We investigated the effect of dietary riboflavin and folate intake on a possible relation between the MTHFR C677T genotype and baseline BMD. For this reason we grouped individuals by quartiles of dietary riboflavin or folate intake, and examined the relationship between MTHFR genotype and baseline BMD (femoral neck and lumbar spine) and bone loss. We observed no effect of the MTHFR genotype on baseline BMD across quartiles of dietary riboflavin or folate intake, neither in FN nor in LS (Table 3). However, in women an effect of riboflavin on the relation between MTHFR genotype and BMD-loss was observed. Women with the TT genotype in the lowest quartile of dietary riboflavin intake had higher rate of BMD-loss as compared to the CC genotype (1.4 vs 0.9% of baseline-year, $p=0.05$). Adjustment for age, height, weight did not affect the results. We were unable to study whether differences in Hcy levels explained the observed association with BMD-loss, because we had a small number of people available that had both baseline Hcy levels and bone loss data.

In men, no effect was observed of dietary riboflavin intake on the relation between the C677T MTHFR genotype and baseline BMD (neither in FN nor in LS) or BMD-loss (Table 3.A).

Dietary folate intake did not influence the relationship between the MTHFR C677T genotype and bone parameters in both men and women (supplementary Table 1).

Fracture incidence

We analysed fracture risk according to quartiles of riboflavin and folate intake, and we did not observe an association (data not shown). We next examined the association between the MTHFR C677T genotype and incident fracture risk according to quartiles of energy-adjusted riboflavin or folate intake. In the lowest quartile of

Table 3. Bone parameters by MTHFR genotype (C677T) within quartiles of dietary riboflavin intake in the Rotterdam Study

<i>Quartiles of dietary riboflavin intake (adjusted for energy intake)</i>																	
	Quartile 1 (low)			Quartile 2			Quartile 3			Quartile 4 (high)			p				
	CC	CT	TT	p	CC	CT	TT	p	CC	CT	TT	p		CC	CT	TT	p
Women																	
N (%)	283 (44.5)	269 (42.3)	84 (13.2)	84 (13.2)	295 (44.8)	302 (45.8)	82 (12.4)	82 (12.4)	290 (44.3)	301 (45.9)	64 (9.8)	64 (9.8)	285 (44.8)	278 (43.7)	73 (11.5)		
FN-BMD (g/cm ²)	0.81 ± 0.12	0.82 ± 0.13	0.81 ± 0.13	0.7	0.83 ± 0.13	0.83 ± 0.13	0.80 ± 0.12	0.4	0.84 ± 0.13	0.83 ± 0.13	0.84 ± 0.14	0.84 ± 0.14	0.2	0.86 ± 0.14	0.86 ± 0.15	0.84 ± 0.13	0.4
LS-BMD (g/cm ²)	1.02 ± 0.18	1.00 ± 0.16	0.99 ± 0.19	0.2	1.04 ± 0.18	1.04 ± 0.19	1.01 ± 0.18	0.6	1.03 ± 0.17	1.03 ± 0.19	1.04 ± 0.18	1.04 ± 0.18	0.9	1.07 ± 0.17	1.06 ± 0.18	1.07 ± 0.16	0.9
N (%)	146 (45.2)	136 (42.1)	41 (13.1)	41 (13.1)	131 (40.4)	165 (50.9)	28 (8.6)	28 (8.6)	153 (45.0)	164 (48.2)	23 (6.8)	23 (6.8)	132 (43.4)	138 (45.4)	34 (11.2)		
FN BMD-loss (%) [†]	-0.85 ± 1.21	-0.86 ± 1.24	-1.36 ± 1.10	0.1 [*]	-0.75 ± 1.01	-0.76 ± 0.95	-0.92 ± 0.99	0.8	-0.86 ± 1.11	-0.86 ± 1.08	-0.69 ± 0.99	-0.69 ± 0.99	0.8	-0.88 ± 1.08	-0.80 ± 1.09	-0.74 ± 1.02	0.5
Men																	
N (%)	205 (0.44)	212 (0.45)	49 (0.11)	49 (0.11)	207 (0.45)	212 (46)	39 (0.09)	39 (0.09)	204 (0.43)	214 (0.48)	52 (0.11)	52 (0.11)	229 (0.49)	189 (0.40)	50 (0.11)		
FN-BMD (g/cm ²)	0.90 ± 0.13	0.89 ± 0.14	0.91 ± 0.12	0.7	0.92 ± 0.13	0.91 ± 0.14	0.9 ± 0.13	0.7	0.92 ± 0.13	0.91 ± 0.13	0.92 ± 0.14	0.92 ± 0.14	0.8	0.94 ± 0.14	0.96 ± 0.13	0.94 ± 0.16	0.5
LS-BMD (g/cm ²)	1.15 ± 0.20	1.13 ± 0.21	1.18 ± 0.21	0.2	1.17 ± 0.19	1.17 ± 0.22	1.16 ± 0.20	1.0	1.15 ± 0.18	1.16 ± 0.21	1.15 ± 0.18	1.15 ± 0.18	0.9	1.16 ± 0.21	1.20 ± 0.19	1.18 ± 0.21	0.1
N (%)	116 (45.7)	107 (41.3)	33 (13.0)	33 (13.0)	109 (47.2)	105 (44.5)	19 (8.3)	19 (8.3)	95 (41.3)	109 (47.0)	27 (11.7)	27 (11.7)	120 (50.6)	98 (40.9)	20 (8.4)		
FN BMD-loss (%) [†]	-0.45 ± 0.97	-0.52 ± 0.79	-0.29 ± 1.17	0.4	-0.35 ± 1.02	-0.55 ± 0.94	-0.42 ± 0.83	0.3	-0.50 ± 0.96	-0.38 ± 0.81	-0.35 ± 0.70	-0.35 ± 0.70	0.4	-0.38 ± 0.92	-0.36 ± 0.73	-0.46 ± 0.86	0.9

Data shown as mean ± SD, *p for ANOVA, FN= femoral neck, LS= lumbar spine, † FN BMD-loss (relative % of baseline year) between 2nd follow-up and baseline; mean follow-up time is 6.5 years, † TT vs CC; p=0.05, Adjusted for age and BMI

Supplementary Table. Bone parameters by MTHFR genotype (C677T) within quartiles of dietary folate intake in the Rotterdam Study

	<i>Quartiles of dietary folate intake (adjusted for energy intake)</i>															
	Quantile 1 (low)				Quantile 2				Quantile 3				Quantile 4 (high)			
	CC	CT	TT	p	CC	CT	TT	p	CC	CT	TT	p	CC	CT	TT	p
Women																
No of genotype	330	314	67	310	318	83	299	325	86	331	314	65				
FN-BMD (g/cm ³)	0.81 ± 0.12	0.81 ± 0.13	0.82 ± 0.13	0.9	0.83 ± 0.14	0.82 ± 0.13	0.82 ± 0.14	0.3	0.83 ± 0.12	0.85 ± 0.13	0.82 ± 0.13	0.3	0.87 ± 0.14	0.86 (0.15)	0.84 ± 0.12	0.3
LS-BMD (g/cm ³)	1.02 ± 0.18	1.00 ± 0.18	1.01 ± 0.18	0.3	1.03 ± 0.18	1.02 ± 0.17	1.01 ± 0.17	0.6	1.04 ± 0.18	1.06 ± 0.18	1.03 ± 0.17	0.5	1.07 ± 0.16	1.07 ± 0.19	1.07 ± 0.20	1.0
FN-BMD-loss	-1.05 ± 1.34	-0.83 ± 1.10	-1.34 ± 1.35	0.1	-0.70 ± 1.05	-0.89 ± 1.02	-0.97 ± 1.05	0.2	-0.83 ± 1.08	-0.74 ± 1.09	-0.93 ± 0.95	0.6	-0.80 ± 0.94	-0.80 ± 1.15	-0.63 ± 0.76	0.1
Fracture																
HR (95% CI) [†]	1.0 (reference)	0.9 (0.6-1.3)	1.4 (0.8-2.5)		0.9 (0.6-1.3)	1.0 (0.7-1.4)	0.6 (0.3-1.1)		1.0 (0.7-1.5)	1.4 (1.0-1.9)	1.4 (0.9-2.3)		1.1 (0.7-1.5)	0.9 (0.6-1.2)	0.9 (0.5-1.6)	
HR (95% CI) [‡]	1.0 (reference)	1.1 (0.6-2.0)	1.2 (0.4-3.0)		1.0 (0.5-1.7)	0.9 (0.5-1.6)	0.7 (0.3-2.0)		1.5 (0.9-2.6)	1.1 (0.6-1.9)	1.8 (0.9-3.6)		0.9 (0.5-1.6)	0.6 (0.3-1.2)	0.9 (0.3-2.5)	
Men																
No of genotype	215	212	47	212	236	62	232	215	39	247	205	52				
FN-BMD (g/cm ³)	0.90 ± 0.13	0.89 ± 0.14	0.91 ± 0.12	0.7	0.92 ± 0.13	0.91 ± 0.14	0.91 ± 0.13	0.7	0.92 ± 0.13	0.91 ± 0.13	0.92 ± 0.14	0.8	0.94 ± 0.14	0.96 ± 0.13	0.94 ± 0.16	0.5
LS-BMD (g/cm ³)	1.15 ± 0.20	1.13 ± 0.21	1.18 ± 0.21	0.2	1.17 ± 0.19	1.17 ± 0.22	1.16 ± 0.20	1.0	1.15 ± 0.18	1.16 ± 0.21	1.15 ± 0.18	0.9	1.16 ± 0.21	1.20 ± 0.19	1.18 ± 0.21	0.1
FN-BMD-loss [§]	-0.51 ± 0.94	-0.43 ± 0.81	-0.39 ± 0.86	0.8	-0.42 ± 1.01	-0.49 ± 0.85	-0.25 ± 1.06	0.4	-0.48 ± 1.00	-0.55 ± 0.82	-0.38 ± 0.93	0.6	-0.28 ± 0.91	-0.33 ± 0.81	-0.50 ± 0.78	0.7
Fracture																
HR (95% CI) [†]	1.0 (reference)	1.1 (0.6-1.9)	1.0 (0.4-2.8)		0.9 (0.5-1.8)	0.5 (0.2-1.1)	0.9 (0.3-2.4)		0.6 (0.3-1.2)	0.8 (0.4-1.5)	0.9 (0.3-2.9)		0.7 (0.3-1.3)	0.8 (0.4-1.6)	1.2 (0.4-3.1)	
HR (95% CI) [‡]	1.0 (reference)	1.0 (0.4-2.5)	0.8 (0.2-3.8)		0.8 (0.3-2.2)	0.3 (0.1-1.2)	0.4 (0.1-3.2)		0.5 (0.2-1.6)	0.7 (0.2-1.8)	0.6 (0.1-4.9)		0.7 (0.3-2.0)	1.2 (0.5-3.0)	1.6 (0.5-6.0)	

Data shown as mean ± SD, *p for ANOVA, Adjusted for age and BMI, FN= femoral neck, LS= lumbar spine, HR=hazard ratio, § FN-BMD-loss (relative % of baseline year) between 2nd follow-up and baseline; mean follow-up time is 6.5 years, † osteoporotic fracture, ‡ fragility fracture, Numbers of subjects with BMD measurements: in men: n=1877, in women: n=2602

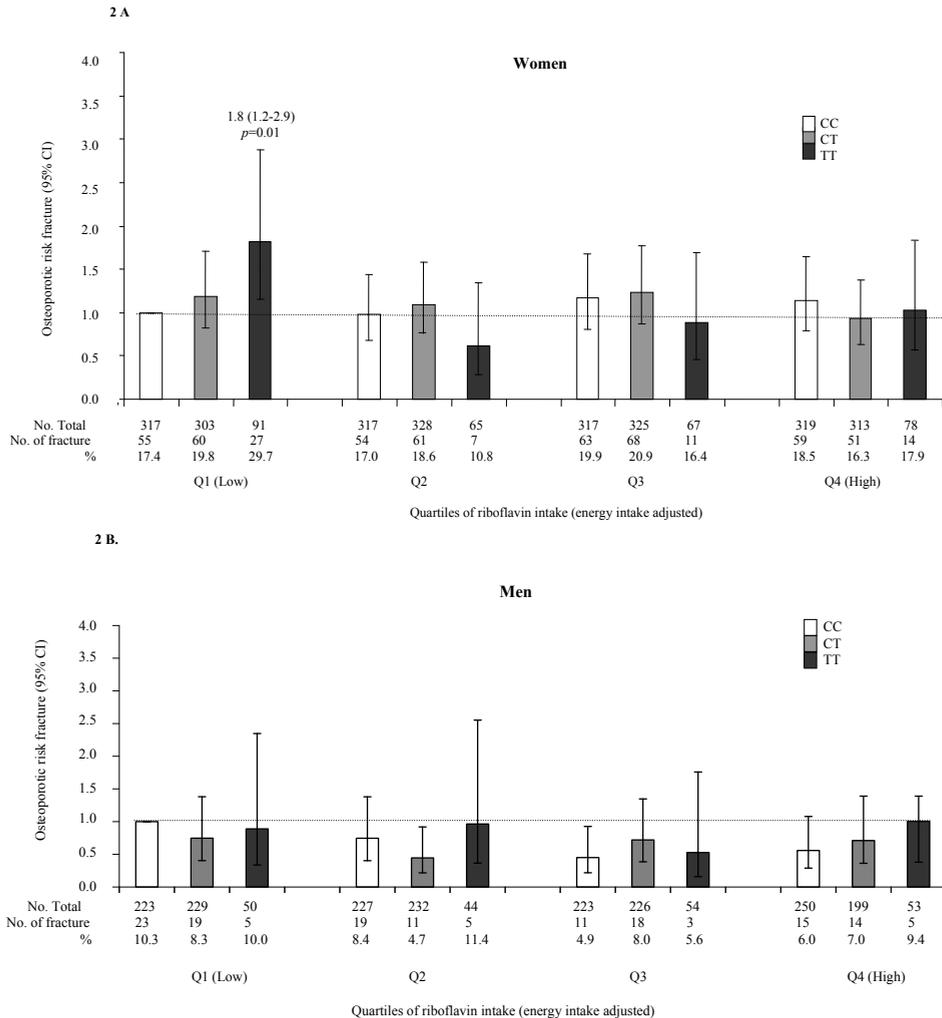


Figure 2. Incident osteoporotic fracture by MTHFR genotype (C677T) in quartiles of dietary riboflavin intake in women (2A) and in men (2B).

Bars represent hazard ratios, with 95% confidence intervals. Adjustments were done for age and BMI

dietary riboflavin intake, T-homozygous women had an almost 2-fold higher risk for incident osteoporotic fracture compared to those with the CC genotype (RR=1.8, 95% CI; 1.2-2.9, $p=0.01$) (p -interaction=0.03) (Figure 2.A). In men we found no interaction between dietary riboflavin intake and the MTHFR C677T genotype with fracture risk (Figure 2.B). The effect of riboflavin intake was even more pronounced for fragility fracture (Figure 3). TT homozygous women in the lowest quartile had a 2.6-fold higher risk of fragility fracture compared to those with the CC genotype (RR=2.6, 95% CI; 1.3-5.1, $p=0.01$). Highly significant interaction was observed between the MTHFR C677T polymorphism, dietary riboflavin intake and fragility fracture risk

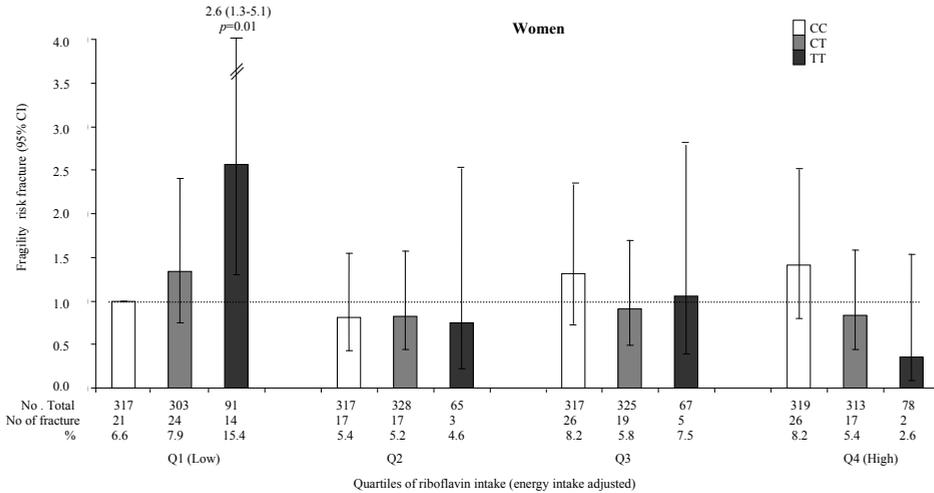


Figure 3. Fragility fracture by MTHFR genotype (C677T) in quartiles of dietary riboflavin intake in women.

Bars represents hazard ratio's, with 95% confidence interval. Adjustments were done for age and BMI

($p=0.0002$) (Figure 3). Adjustment for femoral neck BMD and femoral neck bone loss did not alter these results. Additional correction for co-morbidity factors (such as dementia, diabetes mellitus type II, prevalence of myocardial infarction, smoking status and recent falls) did not affect the results. Similarly, we examined the association between the MTHFR C677T genotype and fracture risk according to quartiles of energy-adjusted dietary folate intake, but we did not observe any influence of dietary folate neither in women nor in men.

In the women who had Hcy values available ($n=416$) we tried to examine whether the riboflavin-intake dependent association between the MTHFR C677T genotype and fracture risk was dependent on Hcys levels. In this small and selected group we observed an increased fracture risk for T-homozygous women in the lowest quartile of riboflavin intake, which was not reaching significance (RR: 4.03, 95% CI; 0.61-26.60). Adjustment for baseline Hcy levels did not essentially change this risk estimate (RR: 3.75, 95% CI; 0.51-27.48).

Population attributable risk

For women in the lowest quartile of riboflavin intake we calculated the population attributable risk (PAR) for the TT-genotype. The risk of fragility fracture that was attributable to MTHFR TT-genotype was estimated 17% in women that had low riboflavin intake. In the total population the PAR for the MTHFR TT-genotype in combination with low riboflavin intake was 5%. For all incident osteoporotic fractures these PARs were 9% and 2.3% respectively. Therefore, based on our results one could argue that, 5% of fragility fractures (or 2.3% of all incident osteoporotic

fractures) in our female population would potentially be prevented if all women are supplemented with riboflavin. This is much higher in women with low riboflavin intake, where 17% of fragility and 9% of all incident osteoporotic fractures might be prevented.

Discussion

This study shows that there is a relationship between the MTHFR C677T genotype and fracture risk depending on dietary riboflavin (vitamin B2) intake. Women homozygous for the T allele had a higher Hcy level and increased risk for fracture than CC homozygotes but only when dietary riboflavin intake was low.

We recently identified elevated Hcy levels as a new risk factor for fracture ⁵. This risk was independent of known risk factors for fracture, such as BMD. In the present study, the increased fracture risk by MTHFR genotype was again independent of BMD or other known risk factors for fracture such as age, dementia, cardiovascular disease and other measures of co-morbidity. This suggests that an elevated Hcy level caused by the MTHFR 677TT genotype in combination with low dietary riboflavin intake is a risk factor predicting fracture independently of other known risk factors and supports the initial results in our observational study ⁸.

Although we observed an effect on fracture, no association between the MTHFR C677T variant and BMD was seen. This is in line with one previous study ³⁵, but is not consistent with other studies ^{16,21} that did show effects of this polymorphism on BMD. We cannot fully explain this difference, however, our study population is older than reported in previous studies. We, therefore, hypothesize that at older age perhaps other mechanisms than homocysteine are important in determining BMD.

We did not find any effect of dietary folate intake on the relationship between the MTHFR and fracture risk in our cohort. It appears that in our population riboflavin intake is a more important determinant compared to folate intake for increased fracture risk in T-homozygous individuals. Our results are consistent with an earlier study that reported a significant effect of riboflavin intake but not folate intake on the MTHFR genotype in determining BMD ²².

Previous research on the dietary regulation of increased Hcy concentrations has implicated both low folate and cobalamin intakes in elevated levels of homocysteine. However, recent studies suggest that among individuals carrying the MTHFR 677 TT genotype, riboflavin status might be a more potent modulator of homocysteine levels than folate status ³⁶. In the Framingham Study, an association between the TT genotype and Hcy was found to be dependent on low folate status ¹⁹. Thus, riboflavin did not seem to be the limiting nutrient. The most likely explanation for the

inconsistencies in the aforementioned studies is that mandatory fortification of flour with riboflavin has been in place since the 1940s in the United States. The impact of this policy in optimizing riboflavin status in the general US population would reduce the extent to which riboflavin is found to be a limiting nutrient in determining homocysteine levels in individuals with the TT genotype.

Several biological mechanisms could explain how elevated Hcy levels are related to fracture risk. It was suggested that Hcy concentrations may interfere with collagen cross-linking, resulting in poor quality of bone and increased susceptibility to fracture³⁷. Recent evidence showed that higher levels of Hcy are correlated with excretion of higher levels of collagen cross-links³⁸. In a pilot study of 100 individuals, we also found that high Hcy levels are not associated with lower levels of collagen cross-links per excreted collagen molecule, but instead found a tendency towards a higher number of collagen cross-links (unpublished data). Yet, these are bone resorption markers, and one expects the Hcy effect on bone (if real) to affect the quality of newly formed bone, something that is not monitored effectively by any of the markers studied so far. In addition, no one knows the exact concentration of Hcy at the environment of the active osteoid, and whether it is sufficient to interfere with collagen cross-links. Interestingly, Paschalis *et al.*³⁹ observed altered cross-links in fragile bone specifically in the micro-environment of the bone forming surfaces, which would support this hypothesis. In addition, Saito *et al.*⁴⁰ recently showed that bone tissue of fracture cases had a lower amount of cross-links and increased Hcy levels as compared to controls. An alternative hypotheses explaining the relationship between Hcy and fracture was recently suggested by showing that Hcy may have a direct effect on bone by stimulating osteoclast formation and osteoclast activity⁴¹. This is in line with our observation that women with the TT genotype had higher BMD loss (possibly reflecting higher osteoclast activity) when dietary riboflavin intake was low. However, this effect was only borderline significant and could not explain the increased risk of fracture seen in these same individuals. We cannot exclude that changes in BMD may occur after the initial fracture, making bone loss a secondary phenomenon rather than a causal factor.

Our results do not prove that elevated homocysteine is the cause of the observed associations. In a subgroup for whom homocysteine levels were available, adjustment of the increased fracture risk for homocysteine levels did not affect the risk estimates. However, since data on homocysteine levels was only available in a small number of individuals, it is difficult to draw any conclusion from this analysis. Nevertheless, it is possible that the higher risk of fracture is not caused by higher Hcy levels, but by other factors related to a higher Hcy level such as impaired DNA methylation^{42,43}.

In men, no association was observed between the MTHFR C677T variant and risk of fracture by dietary riboflavin intake. An explanation for this noticeable difference

in effect by gender is not immediately apparent, however, due to the lower incidence of fracture in men, lack of power to detect an association in men might contribute to this. For studying the effect of low dietary riboflavin intake together with the MTHFR genotype on risk of fracture, the sample size permitted us to detect a relative risk of 2.3 with 80% power for fragility fracture in women. In men we had 80% power to detect a relative risk of 3.2 or more (For all incident fractures the required relative risks are 1.9 in women and 2.5 in men). In addition, men might have a different threshold for dietary riboflavin intake, below which the effect of the MTHFR C677T genotype can become apparent, since in general riboflavin intake was higher in men compared to women. Further studies are needed to elucidate the gender specificity of our findings.

Population stratification might be a confounder in genetic association studies. However, because our study has a population-based cohort design and consists of ethnically homogenous elderly Dutch Caucasian living in a stable area with little emigration/immigration, we believe that stratification is not a major concern in our study.

The strengths of the present study lies within the size of our study population and validated dietary assessment with high response rate. The study has also some limitations. Only baseline dietary intakes were available and not vitamin levels while combination of dietary intake and vitamin levels might provide a better predictive power for vitamin effects on bone health than dietary vitamin intake alone. Although our study is large, it represents a single observation concerning this interaction. Replication of this finding in different populations is necessary to establish its consistency.

In conclusion, while the MTHFR C677T variant had no overall effect on fracture risk, it strongly interacts with dietary riboflavin intake to determine fracture risk in our cohort of elderly Caucasian women. A low dietary riboflavin status results in a higher fracture risk for TT genotype women. This suggests that riboflavin intake modifies the effect of the MTHFR C677T variant on fracture risk. This study highlights the importance of exploring gene-environment interaction in osteoporosis.

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CHAPTER 4.2

Association of Methionine synthase A 2756G polymorphism with low dietary cobalamin intake and osteoporosis in elderly Caucasian

Abstract

An elevated Homocysteine (Hcy) concentration has been identified as a potentially modifiable risk factor for osteoporotic fractures. To gain more insight into the potential underlying mechanism, we studied a polymorphism involved in the remethylation of Hcy in relation to bone mineral density (BMD) and fracture risk. This enzymatic step is fully dependent on cobalamin (vitamin B12).

We examined 4744 men and women who were genotyped for the methionine synthase (MTR) A2756G variant and had data available on nutrient intake and fracture risk. We performed analysis on Hcy levels in 878 individuals, and BMD data were available for 4347 individuals.

In women, the G allele of the MTR A2756G polymorphism was associated with lower femoral neck BMD (GG versus AA/AG; mean difference=0.03 mg/cm², p=0.04), and with lower lumbar spine BMD (GG versus AA/AG; mean difference=0.05 mg/cm², p=0.01). Male individuals with the GG genotype had a higher rate of BMD loss compared with the AA genotype (0.4 vs 0.9% of baseline-year, p=0.01). A similar trend was observed in women, but this did not reach significance. The MTR A2756G GG women had a more than 200% higher risk for fragility fracture compared to women with the AA genotype (RR: 2.16, 95% CI; 1.16-4.00, p=0.02). This increased risk of fracture seemed to be dependent on dietary B12 intake. Only in the presence of low dietary cobalamin intake did female MTR 2756-G homozygotes have a markedly increased risk for fragility fracture (RR: 2.61, 95%CI; 1.25-5.49) and for vertebral fracture (OR: 3.16, 95% CI; 1.05-9.50).

In our cohort of elderly Caucasians the G allele of the MTR A2756G is associated with lower BMD in women and interacts with dietary cobalamin intake to determine fracture risk. These findings need to be replicated in other large cohorts. This study highlights the importance of gene-environment interaction in osteoporosis.

Introduction

Osteoporosis is a multifactorial disease and characterized by low bone mass and microarchitectural deterioration of bone tissue, with as a consequence an increase in bone fragility and susceptibility to fractures¹. Elevated homocysteine (Hcy) concentrations have been identified as a potentially modifiable risk factor for osteoporotic fractures. The Hcy metabolism contains numerous enzymes that require nutritional cofactors, including vitamins B12 (cobalamin), B6 (pyridoxine), B2 (riboflavin) and B11 (folate). Disruptions in the Hcy metabolism can be due to deficiency of these B vitamins or genetic polymorphisms of the enzymes involved in the Hcy pathway, or a combination of the two.

Hcy is an intermediate metabolite of methionine and can be remethylated to methionine by methionine synthase (MTR) MTR uses cobalamin as a cofactor and 5-methyltetrahydrofolate as a methyl donor to catalyze the remethylation of Hcy. Thus, reduced activity of MTR could reduce the remethylation of Hcy resulting in increasing Hcy levels.

One of the polymorphisms in the MTR gene is A2756G (rs1805087). This polymorphism consists of either an aspartic acid (Asp) or a glycine (Gly) at codon 919². Although the direct functional impact of this polymorphism has not yet been established, there is some evidence that this polymorphism changes the activity of the enzyme. In some studies, individuals with the GG genotype have lower Hcy concentrations^{3,5}, but other studies failed to observe an effect of this polymorphism on Hcy levels^{6,7}.

The MTR A2756G variant has been implicated in increased risk of adverse health conditions including connective tissue disease^{8,9}, cardiovascular disease¹⁰, and cancer¹¹; we found no publications on the relation between this polymorphisms and osteoporosis.

To gain more insight into the potential underlying mechanism relating Hcy to osteoporosis, we studied the MTR A2756G polymorphism in relation to BMD, bone loss and fracture risk in a large population-based cohort of elderly Caucasians.

Materials and Methods

Study population

This study was embedded in the Rotterdam Study, a population-based cohort study of men and women in which all residents of the Rotterdam suburb Ommoord aged 55 years and older were invited to take part. The design of the study has been described elsewhere¹². Written informed consent was obtained from all participants

and the Medical Ethics Committee of the Erasmus Medical Center approved the study. Baseline data collection was conducted between January 1990 and June 1993, while the follow-up assessment of BMD was performed between July 1996 and December 1999. A total of 7983 men and women participated in the study (response rate 78%) and for the present analysis we studied 4744 men and women who were genotyped for the MTR A2756G variant and had available data on nutrient intake and fracture risk. Analysis of Hcy levels was performed in a total of 878 individuals and BMD data were available for 4347 individuals.

Dietary intake

Dietary habits were assessed using validated food intake data from a food frequency questionnaire and were available for 5542 men and women. A validation study comparing this questionnaire with a 2-week food diary demonstrated reproducible and valid estimates^{13,14}. Dietary vitamin B intake was adjusted for energy intake, as described elsewhere¹⁵.

Measurement of Hcy levels

Non-fasting blood samples from 878 subjects at baseline were immediately placed on ice and processed within 60 minutes. Serum samples were kept frozen until Hcy levels were measured. Total Hcy levels were determined as a fluorescence derivative with the use of high-pressure liquid chromatography and expressed as micro mol per liter ($\mu\text{mol/L}$)^{16,17}.

Non-vertebral fracture assessment

Fracture events were obtained from the computerized records of the general practitioners (GPs) in the research area. Research physicians regularly followed participant information in the GP's records outside the research area, and made an independent review and encoding of all reported events. Subsequently, a medical expert in the field reviewed all coded events for the final classification of diseases, 10th revision (ICD-10)¹⁸. Additional information on hip fractures was gathered through the Dutch National Hospital Registration system. Fracture follow-up started at baseline and for this study ended on January 1, 2002. Information on incident non-vertebral fractures was available for an average follow-up period of 7.4 ± 3.3 (SD) years ($n=940$ fractures). We studied all incident fractures, while fractures that were considered not to be osteoporotic were excluded (i.e. fractures caused by cancer and all hand, foot, skull and face fractures). Fragility fractures, which are fractures occurring at the hip, pelvis, and proximal humerus ($n=405$)¹⁹, were considered separately.

Vertebral fracture assessment

Both at baseline and at follow-up visits between 1997 and 2001, thoracolumbar radiographs of the spine were obtained. The follow-up radiographs were available for 3469 individuals who survived an average of 6.4 (SD 0.4) years after the baseline visit to the center, and who were still able to come to our research center. All follow-up radiographs were scored for the presence of vertebral fracture by the McCloskey/Kanis method, as described earlier²⁰. If a vertebral fracture was detected, the baseline radiograph was also evaluated. If the vertebral fracture was already present at baseline, it was considered a baseline prevalent fracture. If it was not present at baseline, the fracture was defined as being incident.

Measurement of BMD

BMD (in grams per square centimeter) of the hip and lumbar spine (L2-L4) was measured by dual-energy x-ray absorptiometry (DXA) using a Lunar DPX densitometry apparatus (DPX-L, Lunar Corp. Madison, Wisconsin, USA), under standard protocols. BMD was measured at baseline (between 1990 and 1993) and at a follow-up (between 1996 and 1999). To increase the accuracy of BMD measurements at follow-up, the search and template tools in the comparison mode were used to position the femoral neck region of interest in scans of the same individual using DPX-IQ software. The rate of change in BMD was calculated as the difference between baseline and follow-up, divided by the follow-up time (mean $6.5 \pm$ SD 0.6 years). Within the population that had dietary information, BMD measurements were available for 4323 individuals at baseline and 2211 individuals at follow-up.

Anthropometric measurements and potential confounders

Height (cm) and weight (kg) were measured at the initial examination, in standing position wearing indoor clothes without shoes. Body mass index (BMI) was computed as weight in kilograms divided by height in meters squared (kg/m^2).

The number of falls in the preceding year, and current smoking status were assessed with the use of a questionnaire.

Genotyping

Genomic DNA was isolated from blood samples using standard methods²¹. We utilized TaqMan allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA, USA) for genotyping the MTR A2756G polymorphism. Primer and probes are available upon request.

The assays utilized 2 ng of genomic DNA and took place in 2 microliter reaction volumes. The amplification and extension protocol was as follows: an initial activation step of 10 minutes at 95 degrees preceded 40 cycles of denaturation at 95

degrees for 15 seconds, and annealing and extension at 50 degrees for 60 seconds. Allele-specific fluorescence was then analyzed on an ABI prism 7900HT Sequence Detection System v2.1 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Allele and genotype frequencies of the MTR A2756G polymorphism were tested for Hardy-Weinberg equilibrium proportions with the chi-square test. Analysis of variance (ANOVA) was used to examine the associations between the studied polymorphism and Hcy levels and BMD measurements across genotypes and median of dietary cobalamin intake. Analysis of covariance (ANCOVA) was performed to adjust for possible confounders such as BMI, age and co-morbidity. In case of a consistent trend, shown as an allele dose effect, we performed a linear regression analysis to quantify the association.

Variables were log transformed if they did not meet normality assumptions; this was the case for Hcy levels and dietary cobalamin intake. Dietary cobalamin intake as analysed as a categorical variable with quartile cut-off points. Quartiles and median of dietary cobalamin intake was made in a gender-specific manner and have been energy adjusted.

To estimate the risk for incident fracture risk by genotype we used the Cox proportional hazard models, thereby taking potential differences in follow-up time into account. Adjustments were also made for possible confounders, i.e., age, BMI, current smoking status and recent falls. We investigated possible interactions between dietary cobalamin intake and the MTR A2756G genotypes in relation to fracture by including a product term of the two main effects in a Cox regression model with fracture as the dependent variable.

All p-values less than 0.05 was considered significant. All analyses were done using the SPSS package version 11.

Results

The genotype distributions for all studied polymorphisms were in Hardy-Weinberg proportions.

Table 1 presents data on the baseline characteristics of the study population.

We found a borderline significant association of the MTR genotype with age in women in whom the GG group was two years older than either the GA or AA group. Hcy levels were 1.49 $\mu\text{mol/L}$ higher in male AA carriers compared to the GG genotype with evidence of an allele dose effect (p for trend=0.02, Table 1). A similar trend was seen in women, but was far from significant. When men and women were combined,

Table 1. Baseline characteristics of the study population by the MTR A2756G genotypes in both genders in the Rotterdam Study

	<i>Men</i>				<i>Women</i>			
	AA	AG	GG	P _{ANOVA}	AA	AG	GG	P _{ANOVA}
Number *	1401	563	73		1963	843	80	
Age (y)	67.1 ± 7.3	67.5 ± 7.5	67.6 ± 7.3	0.66	68.0 ± 8.1	67.9 ± 7.9	70.3 ± 7.8	0.04
Height (cm)	174.8 ± 6.6	175.2 ± 6.7	175.2 ± 6.8	0.50	161.9 ± 6.3	162.0 ± 6.5	160.9 ± 6.8	0.39
Weight (kg)	79.1 ± 10.4	79.0 ± 10.3	77.4 ± 9.3	0.40	70.0 ± 11.4	70.4 ± 10.4	69.7 ± 10.6	0.26
Body mass index (kg/m ²)	25.8 ± 2.9	25.7 ± 2.9	25.2 ± 2.8	0.16	26.7 ± 4.1	26.8 ± 3.7	26.8 ± 5.4	0.75
Cobalamin intake (mg/day)	5.7 ± 6.6	5.8 ± 3.8	4.9 ± 2.7	0.38	4.9 ± 4.0	5.0 ± 3.4	4.9 ± 3.5	0.97
Number **	248	91	15		355	153	16	
Hcy level (mmol/L) †	16.2 ± 0.3	15.0 ± 0.4	14.8 ± 1.0	‡0.02	14.5 ± 0.2	14.3 ± 0.3	13.3 ± 1.0	0.50

Data are given as unadjusted mean ± SD for continuous variables

†Geometric mean (SE), adjustment for age and BMI, ‡ p-trend=0.01

* Number of individuals with nutrient intake data, ** Number of individuals with data on Hcy levels.

Hcy levels were 1.12 µmol/l higher in AA carriers compared to the GG genotype although did not reach significance (p=0.17, adjusted for gender and age) (p for trend=0.06).

BMD and genotypes

Table 2 presents data on BMD across the MTR A2756G genotypes. In women, the MTR A2756G polymorphism was significantly associated with both femoral neck BMD (FN-BMD) with evidence for a recessive effect (GG versus AA/AG; mean difference=0.03 mg/cm², p=0.04) and with lumbar spine BMD (LS-BMD): (GG versus AA/AG; mean difference=0.05 mg/cm², p=0.01). A similar pattern was seen in men, although this did not reach significance. In addition, we observed an effect of the MTR genotype on BMD loss in men. Men with the GG genotype had 0.4 vs 0.9% of baseline-year higher rate of BMD loss compared with the AA genotype (p=0.01). Again we found the same trend in women, but this did not reach significance. We then examined a potential effect of dietary cobalamin intake on the association between MTR genotypes and BMD and bone loss, by stratifying the population into two equal groups according to their dietary cobalamin intake. We observed no effect of dietary cobalamin intake on the association between the MTR A2756G genotypes in relation to BMD and bone loss in both genders (data not shown).

Fracture risk and genotypes

Table 3 gives data on fracture risk according to the MTR A2756G genotypes. In men, no evidence for an association was observed. There was a significant association between the MTR A2756G genotype and risk of fracture in women with evidence

Table 2. BMD across the MTR A2756G genotypes in both genders in the Rotterdam Study

	Total population						Men						Women											
	AA		AG		GG		p		AA		AG		GG		p		AA		AG		GG		p	
	Number	Mean	SD	Number	Mean	SD	Number	Mean	SD	Number	Mean	SD	Number	Mean	SD	Number	Mean	SD	Number	Mean	SD	Number	Mean	SD
Number	2959	1251	137	137	137	137	1241	1241	503	503	65	65	65	65	1718	1718	748	748	72	72	72	72	72	72
FN-BMD (g/cm ²)	0.87 ± 0.14	0.88 ± 0.14	0.85 ± 0.14	0.85 ± 0.14	0.85 ± 0.14	0.85 ± 0.14	0.92 ± 0.14	0.92 ± 0.14	0.93 ± 0.13	0.93 ± 0.13	0.91 ± 0.13	0.91 ± 0.13	0.91 ± 0.13	0.91 ± 0.13	0.83 ± 0.13	0.83 ± 0.13	0.85 ± 0.13	0.85 ± 0.13	0.79 ± 0.13	0.79 ± 0.13	0.79 ± 0.13	0.79 ± 0.13	0.79 ± 0.13	0.79 ± 0.13
LS-BMD (g/cm ²)	1.09 ± 0.20	1.10 ± 0.19	1.05 ± 0.21	1.05 ± 0.21	1.05 ± 0.21	1.05 ± 0.21	1.16 ± 0.20	1.16 ± 0.20	1.18 ± 0.20	1.18 ± 0.20	1.13 ± 0.23	1.13 ± 0.23	1.13 ± 0.23	1.13 ± 0.23	1.04 ± 0.18	1.04 ± 0.18	1.06 ± 0.18	1.06 ± 0.18	1.03 ± 0.17	1.03 ± 0.17	1.03 ± 0.17	1.03 ± 0.17	1.03 ± 0.17	1.03 ± 0.17
FN-BMD-loss ‡	-0.66 ± 1.01	-0.67 ± 1.08	-1.00 ± 0.95	-1.00 ± 0.95	-1.00 ± 0.95	-1.00 ± 0.95	-0.40 ± 0.87	-0.40 ± 0.87	-0.46 ± 0.93	-0.46 ± 0.93	-0.87 ± 0.93	-0.87 ± 0.93	-0.87 ± 0.93	-0.87 ± 0.93	-0.84 ± 1.06	-0.84 ± 1.06	-0.83 ± 1.15	-0.83 ± 1.15	-1.14 ± 0.96	-1.14 ± 0.96	-1.14 ± 0.96	-1.14 ± 0.96	-1.14 ± 0.96	-1.14 ± 0.96
GG vs [AA/AG]	AA/AG	AA/AG	GG	GG	GG	GG	AA/AG	AA/AG	AA/AG	AA/AG	GG	GG	GG	GG	AA/AG	AA/AG	AA/AG	AA/AG	GG	GG	GG	GG	GG	GG
Number	4214	4214	137	137	137	137	1744	1744	1744	1744	65	65	65	65	2466	2466	2466	2466	72	72	72	72	72	
Effect size GG vs [AA/AG]																								
FN mean difference (g/cm ²) (%)	0.02 (2.7)	0.02 (2.7)					0.14	0.14	0.01 (0.9)	0.01 (0.9)					0.84	0.84	0.05 (5.8)	0.05 (5.8)						0.04
LS mean difference (g/cm ²) (%)	0.04 (3.7)	0.04 (3.7)					0.01	0.01	0.04 (3.1)	0.04 (3.1)					0.37	0.37	0.06 (5.7)	0.06 (5.7)						0.01
FN-BMD loss mean difference (%)	0.34 (33.6)	0.34 (33.6)					0.004	0.004	0.45 (51.7)	0.45 (51.7)					0.01	0.01	0.32 (27.5)	0.32 (27.5)						0.14

Data shown as mean ± SD, †p for ANOVA, Adjusted for gender, age and BMI, FN= femoral neck, LS= lumbar spine, ‡p for ANOVA, Adjusted for age and BMI, † FN-BMD loss (relative % of baseline year) between 2nd follow-up and baseline; mean follow-up time is 6.5 years.

Table 3. Fracture risk according to MTR A2756G genotypes in both genders in the Rotterdam Study

Types of fracture/ Genotype	Men		Women	
	No. of fractures/ Total no (%)	RR (95% CI)	No. of fractures/ Total no (%)	RR (95% CI)
Osteoporotic				
AA	98/1341 (7.3)	1 (reference)	354/1886 (18.8)	1 (reference)
AG	44/545 (8.1)	1.10 (0.77-1.57)	139/823 (16.9)	0.89 (0.73-1.08)
GG	5/72 (6.9)	0.89 (0.36-2.20)	19/77 (24.7)	1.26 (0.79-1.99)
Fragility				
AA	42/1341 (3.1)	1 (reference)	117/1886 (6.2)	1 (reference)
AG	17/545 (3.1)	0.96 (0.54-1.68)	54/823 (6.6)	1.06 (0.76-1.47)
GG	3/72 (4.2)	1.19 (0.37-3.05)	11/77 (14.3)	2.16 (1.16-4.00)
All Vertebral				
AA	79/802 (9.9)	1 (reference)	128/1052 (12.2)	1 (reference)
AG	34/323 (10.5)	1.08 (0.70-1.65)	53/482 (11.0)	0.87 (0.62-1.24)
GG	3/45 (6.7)	0.66 (0.20-2.18)	7/38 (18.4)	1.53 (0.65-3.59)

Age and BMI adjusted

for a recessive effect. Homozygous carriers of the G allele had a higher risk of fragility fracture compared with A-allele carriers (RR: 2.16, 95% CI; 1.16-4.00, $p=0.02$). Correcting for FN-BMD attenuated the risk slightly (RR: 1.90, 95% CI; 0.99-3.65, $p=0.06$).

To examine the potential effect of dietary cobalamin intake on the relationship between the MTR A2756G polymorphism and fracture risk, participants were grouped according to their dietary cobalamin intake into two equal groups: high versus low cobalamin intake (Table 4). We observed that in the low dietary cobalamin intake group, G homozygous women had a more than 250% increased risk for fragility fracture compared with the AA genotype (RR=2.61, 95% CI; 1.25-5.49, $p=0.01$). The p -value for the interaction was $p=0.6$ and there was a more than 3-fold risk for vertebral fracture compared with the AA genotype (OR=3.16, 95% CI; 1.05-9.50, $p=0.04$) (p -interaction=0.2). Further adjustment for FN-BMD did not change these results. In men we did not observe such an effect of dietary cobalamin intake on the association between fracture and the MTR A2756A polymorphism.

Hcy levels

In a subset of individuals with Hcy level measurements, we investigated whether there was a relationship between the MTR genotype and Hcy levels in two groups: higher or lower than the median dietary cobalamin intake. No effect of cobalamin intake across the genotype groups was found (data not shown).

Table 4. Fracture risk according to MTR A2756G genotype by median of dietary cobalamin

	<i>Osteoporotic</i>		<i>Fragility</i>		<i>Vertebral</i>	
	No. of fractures/ Total no. (%)	RR (95% CI)	No. of fractures/ Total no. (%)	RR (95% CI)	No. of fractures/ Total no. (%)	OR (95% CI)
<i>Men</i>						
Low cobalamin						
AA	44/678 (6.5)	1.0	18/678 (2.7)	1.0	46/415 (11.1)	1.0
AG	26/262 (9.9)	1.48 (0.91-2.41)	9/262 (3.4)	1.23 (0.55-2.79)	21/162 (13.0)	1.22 (0.70-2.12)
GG	3/41 (7.3)	1.05 (0.33-3.39)	2/41 (4.9)	1.71 (0.40-7.51)	1/30 (3.3)	0.29 (0.04-2.16)
High cobalamin						
AA	54/663 (8.1)	1.34 (0.90-2.01)	24/663 (3.6)	1.62 (0.87-3.01)	33/387 (8.5)	0.77 (0.47-1.23)
AG	18/283 (6.4)	1.06 (0.61-1.83)	8/283 (2.8)	1.21 (0.53-2.80)	13/161 (8.1)	0.71 (0.37-1.36)
GG	2/31 (6.5)	1.02 (0.25-4.20)	1/31 (3.2)	1.22 (-.16-9.12)	2/15 (13.3)	1.25 (0.27-5.83)
<i>Women</i>						
Low cobalamin						
AA	184/944 (19.5)	1.0	60/944 (6.4)	1.0	65/535 (12.1)	1.0
AG	73/397 (18.4)	0.91 (0.69-1.19)	26/397 (6.6)	1.00 (0.63-1.59)	28/237 (11.8)	0.96 (0.60-1.55)
GG	11/43 (25.6)	1.22 (0.66-2.25)	8/43 (18.6)	2.61 (1.25-5.49)	5/17 (29.4)	3.16 (1.05-9.50)
High cobalamin						
AA	170/942 (18.0)	0.92 (0.75-1.14)	57/942 (6.1)	0.93 (0.64-1.34)	63/517 (12.2)	1.03 (0.71-1.50)
AG	66/426 (15.5)	0.80 (0.60-1.07)	28/426 (6.6)	1.04 (0.66-1.64)	25/245 (10.2)	0.81 (0.49-1.35)
GG	8/34 (23.5)	1.19 (0.59-2.41)	3/34 (8.8)	1.33 (0.42-4.25)	2/21 (9.5)	0.68 (0.15-3.04)

Age and BMI adjusted

Discussion

In this study we observed that the MTR A2756G polymorphism is associated with BMD, bone loss and fracture risk in women. The genotype-dependent effect on fracture was stronger in the presence of low dietary cobalamin (vitamin B12) intake.

We and others have suggested that elevated Hcy levels are a novel risk factor for osteoporotic fracture independent of BMD^{22,23}. In the present study, the increased fracture risk by G allele of MTR genotype was independent of BMD and was dependent on dietary cobalamin intake. On the other hand, in women, the MTR genotype was not associated with Hcy levels. One explanation for this is that cobalamin may play a more dominant role in bone health compared with Hcy levels this suggests that the MTR 2756GG genotype in combination with low dietary cobalamin intake predicts fracture, independently of elevated Hcy levels.

Our study had limited statistical power because of the low frequency of the MTR GG genotype (3.1%), which is reflected in the wide confidence intervals for effect estimates. Hence, these findings need to be confirmed in larger populations. Our

population is relatively old, which is important given that cobalamin deficiency is more prevalent in the elderly ²⁴. Therefore, the real effect of low dietary cobalamin intake on fracture risk might be even larger than that observed in our study.

Little seems to be known about the relation of Hcy and cobalamin with bone health. Hcy levels are closely linked to cobalamin status, and cobalamin might be directly involved in the bone quality. Evidence for an association between cobalamin and bone emerges from *in vivo* studies. In a study by Carmel *et al.* ²⁵ patients with cobalamin deficiency were found to have low levels of serum osteocalcin, indicating a reduced osteoblast activity, and cobalamin treatment increased serum osteocalcin levels in these patients ²⁵. Osteocalcin is one of the most abundant non-collagenous proteins in bone, and cobalamin treatment increases serum osteocalcin levels in these patients. Osteocalcin is required to stimulate bone mineral maturation ²⁶. Furthermore, it is well documented that osteoblasts produce osteocalcin, which is used as a marker of bone formation. In addition, osteocalcin is released during bone resorption, reflecting a higher bone turnover in these patients. A higher bone turnover indicates impaired bone remodelling resulting in fragile bone which is susceptible to fracture.

The strengths of the present study lie in the size of our study population and the validated dietary assessment with high response rate. The study also has some limitations. Only baseline dietary intakes were available and not vitamin levels. It has been suggested that serum cobalamin, specifically, methylmalonic acid (MMA) or holotranscobalamin (holo-TC) and concentrations are better predictors of cobalamin status than dietary cobalamin intake, particularly in elderly subjects ²⁷. Nevertheless, in Europe the dietary intakes are relatively stable over time, especially among the elderly ²⁸. The validated food frequency test is therefore a good measure for long-term assessment of nutrient intake. Although our study is large, it represents a single observation concerning the effect of this polymorphism in relation to cobalamin intake. Replication of this finding in different populations is necessary to establish its consistency.

In conclusion, the MTR A2756G variant has an effect on fracture risk in women, and this effect is more pronounced in women with low dietary cobalamin intake. Moreover, the MTR A2756G is associated with BMD in women and bone loss in men. These results need to be replicated in other large cohorts, but highlight the importance of gene-environment interaction in osteoporosis.

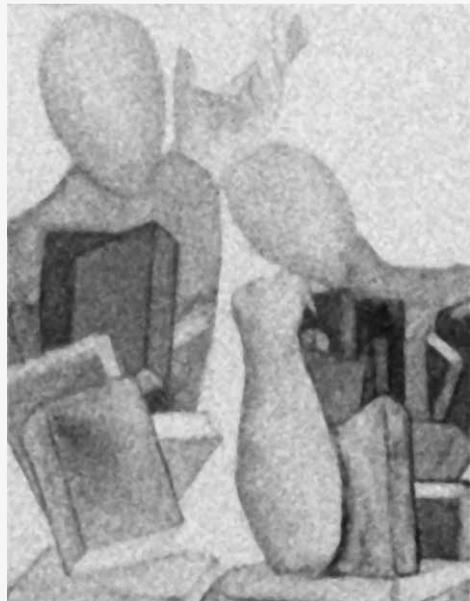
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CHAPTER 5

Genetic and dietary determinants of homocysteine levels in elderly Caucasians



Abstract

Homocysteine (Hcy) is a sulfur amino acid formed by demethylation of methionine, which can be remethylated to methionine, and this requires B vitamins (riboflavin, pyridoxine, folate and cobalamin) as cofactors. We investigated the association between Hcy levels and eight polymorphisms in enzymes involved in the Hcy metabolism. In addition to the influence of dietary vitamin B intake on these associations.

We studied 2208 individuals aged 55 years and over who participated in four independent samples from the Rotterdam Study, and have been genotyped for polymorphisms involved in Hcy metabolism (MTHFR C677T, MTHFR A1298C, RFC G80A, THYMS 28 bp repeat 3R/2R, TCN C776G, MTR A2756G, MTRR A66G, CBS ins68bp). Dietary intake of nutrients was obtained from food frequency questionnaires at baseline.

The MTHFR 677T, MTR 2756A and RFC 80 G variants were associated with Hcy levels. In addition, a stepwise regression approach revealed that, besides the effect of age and gender, the variability in Hcy levels was attributed to the T allele of MTHFR 677 (1.3%, $p=0.001$), the A allele of MTR 2756 (0.2%, $p=0.02$), and the C allele of the MTHFR 1298 (0.2%, $p=0.05$). Furthermore, when nutrient factors were included in the model, the largest part of the variability in Hcy levels was explained by dietary folate intake (3.5%, $p=0.001$) and then the T allele of MTHFR 677 (1.8%, $p=0.001$) and dietary protein intake (0.9%, $p=0.0002$).

Moreover, the MTHFR C677T and RFC G80A polymorphisms separately interact with dietary folate intake to determine Hcy levels among all studied variants and nutrients based on the logic regression approach.

This study shows that MTHFR C677T, MTR A2756G and RFC G80A influence Hcy levels in our population. Furthermore, at baseline the MTHFR C677T polymorphism, dietary folate intake and dietary protein intake are the strongest independent predictors of Hcy levels after age and gender in our cohort. Interestingly, we observed an interaction between the MTHFR C677T and RFC G80A polymorphisms with low dietary folate intake in determining Hcy levels among the other studied variants and dietary intakes in our population.

Introduction

A mild elevation of homocysteine (Hcy) plasma level is an independent risk factor for a wide range of diseases from birth defects ¹ to cardiovascular disease ², cognitive dysfunction ³, osteoporosis and fracture in the elderly ⁴.

Hcy is formed by demethylation of methionine, and can be remethylated to methionine by the enzyme methionine synthase (MTR). This enzyme requires vitamin B12 (cobalamin) as a cofactor, and methionine synthase reductase (MTRR) is necessary to keep MTR active. The methyl group that is necessary for this reaction is derived from the folate (pool in the cell) by an essentially irreversible reaction catalyzed by the enzyme methylenetetrahydrofolate reductase (MTHFR). MTHFR needs vitamin B2 (riboflavin) as a cofactor ⁵. The reduced folate carrier (RFC) plays a role in the transport of 5-methyltetrahydrofolate (5-THF) into cells ⁶. Thymidylate synthase (THYMS) catalyses the reductive methylation and competes with MTHFR for the one-carbon unit of 5,10-methyleneTHF ⁵. Hcy can be broken down in the transsulfuration pathway, in which the first step is performed by the enzyme cystathionine β -synthase (CBS), which needs pyridoxine as a cofactor.

Hcy levels are determined by both genetic and environmental risk factors ⁷⁻¹⁰, heritability estimates for Hcy levels range from 47 to 57% and there is an ongoing debate regarding the relative contribution of each. The main environmental determinants include nutritional deficiencies in vitamin cofactors such as riboflavin ⁴, pyridoxine, folate and cobalamin ¹¹. These vitamins play a crucial role in the remethylation and transsulfuration of Hcy, and the availability of which is determined by dietary intake.

There are several polymorphisms in the genes encoding the enzymes involved in the Hcy metabolism and these have been associated with abnormal Hcy levels. The most extensively studied polymorphism associated with Hcy variability is the MTHFR C677T (Ala 222 Val) ¹². This polymorphism has been reported to explain 4-9% of variations in plasma Hcy levels ^{7,9,13,14}. Other common functional variants of the enzymes involved in Hcy metabolism include MTHFR A1298C (Glu429Ala) ¹⁵, MTR A2756G ¹⁶, CBS 844ins68 (an insertion variant) ¹⁷ and MTRR A66G ¹⁸. However, studies to date have considered only one or a few of these enzymes out of all those known to be involved in the metabolic pathway. Also, associations with Hcy levels on these genetic variants have not been consistent in all studies: some studies showed conflicting findings for MTHFR A1298C ^{19,20} and the MTR A2756G ²¹, and THYMS 28bp repeat 3R/2R ²², and CBS ins68bp ²³, MTRR A66G ¹⁸ and RFC G80A ⁶. This inconsistency in previous studies may in part be due to the small size of most of the studies, or to the unavailability of vitamin B status to examine potential gene-nutrient interactions to determine Hcy levels.

Therefore, in the current study, we investigated a possible association between the studied polymorphisms involved in the Hcy metabolism with Hcy levels in a large population. Moreover, we studied gene-nutrient interactions to address genetic and dietary determinants of Hcy levels in a several independent samples from a large population-based cohort of elderly Caucasian men and women.

Materials and Methods

Study population selection

We analyzed four independent samples from the Rotterdam Study. The Rotterdam Study is a prospective, ongoing population-based cohort study of individuals aged 55 years or older resident in the Ommoord district of the city of Rotterdam, in the Netherlands. The study was designed to investigate chronic, disabling diseases. The rationale and design of the study have been described previously²⁴. The baseline examination included 7983 subjects. The medical ethics committee of the Erasmus Medical Center approved the Rotterdam Study. All the subjects in the Rotterdam Study gave written informed consent.

A random sample of 1028 subjects from the baseline examination (1991-1993) was selected (cohort 1). The sample did not differ in age, gender or education from the population from which it was selected. A second sample of 555 subjects was studied from the first follow-up visit (1995-1996) (cohort 2). Cohort 2 was originally recruited for a study of age-related changes in the brains of elderly persons²⁵ and the exclusion criteria were dementia, blindness, and the presence of standard contraindications to the use of magnetic resonance imaging. The subjects in cohort 2 ranged in age from 60 to 90 years and were randomly selected, with stratification according to age (in five-year age groups) and sex. A third sample of 1087 subjects was studied from the third follow-up visit (1996-1999) (cohort 3). At the same follow-up visit (1996-1999), a fourth sample of 505 women, aged 65 to 75 years was examined. This group had no history of using medication, absence of disorders such as dementia, stroke, hip fracture, and cancer (cohort 4).

To increase the statistical power, the baseline and three independent, non-overlapping follow-up samples of subjects were included in the present study. The non-overlapping samples of these four cohorts included a total of 2015 persons.

Dietary intake

Dietary habits were assessed using validated food intake data from a food frequency questionnaire. A validation study comparing this questionnaire with a 2-week food

diary demonstrated reproducible and valid estimates²⁶. Dietary vitamin B intake was adjusted for energy intake as described elsewhere²⁶.

Measurement of homocysteine levels

Blood samples were immediately placed on ice and processed within 60 minutes, and kept frozen until Hcy levels were measured.

For cohort 1, non-fasting serum samples were obtained, and total Hcy levels were determined as a fluorescence derivative with the use of high-pressure liquid chromatography^{27,28}. For cohort 2, non-fasting sodium citrate plasma samples were obtained. For cohort 3, fasting EDTA-treated plasma samples were obtained. For cohort 4, fasting sodium citrate plasma samples were obtained. For cohorts 2 and 3 total Hcy levels were measured with the use of a fluorescence polarization immunoassay on an IMx analyzer (Abbott Laboratories), which for cohort 4 Hcy levels were measured with LC/MS-MS.

Measurements

Height (cm) and weight (kg) were measured at the initial examination, in standing position wearing indoor clothes without shoes. Body mass index (BMI) was computed as weight in kilograms divided by height in meters squared (kg/m²).

Genotyping

Genomic DNA was isolated from blood samples using standard methods²⁹. The 33R, 23R, and 33R genotypes of the THYMS gene and 28 bp repeat and del-del, ins-del and ins-ins of 68bp in the CBS gene were detected using PCR products and were analysed by Gene Scan with some modifications. We utilized TaqMan allelic discrimination Assays-By-Design (Applied Biosystems ABI 3100, Foster City, CA) for genotyping the MTHFR C677T, and A1298C, the RFC G80A, the TCN C776G, the MTR A2756G and the MTRR A66G polymorphisms. Primer and probes are available upon request.

The assays utilized 2 ng of genomic DNA and 2 microliter reaction volumes. The amplification and extension protocol was as follows: an initial activation step of 10 minutes at 95 degrees preceded 40 cycles of denaturation at 95 degrees for 15 seconds and annealing and extension at 50 degrees for 60 seconds. Allele-specific fluorescence was then analyzed on an ABI prism 7900HT Sequence Detection System v2.1 (Applied Biosystems, Foster City, CA).

Statistical analysis

Allele and genotype frequencies of the studied polymorphisms were tested for Hardy-Weinberg equilibrium proportions with the χ^2 test.

Variables were log transformed if they did not meet normality assumptions, this was the case for Hcy levels and dietary folate intake.

Analysis of variance (ANOVA) was used to examine the associations between each variant and Hcy levels. Analysis of covariance (ANCOVA) was performed to adjust for possible confounders such as BMI, and age. In case of a consistent trend, shown as an allele dose effect, we performed a linear regression analysis to quantify the association.

Multivariable linear regression methodology was used to determine the significant independent predictors that influence Hcy levels.

Intakes of B vitamins were adjusted for total energy intake using the linear residual regression method of Willett and Stampfer³⁰. Gender-specific tertiles of dietary B vitamin intakes were calculated. Reference groups were those with the lowest vitamin B intake.

To explore the relation of Hcy levels with genotypes within a complete dataset, standard deviation scores were calculated separately for each subject in each cohort. Then the four cohorts were combined to increase statistical power. The standard deviation score was calculated with the formula $(\text{Hcy}_i - \text{Hcy}_m) \div \text{SD}$, where Hcy_i is the natural-log-transformed Hcy level in the individual subject, Hcy_m is the mean natural-log-transformed Hcy level in the cohort, and the SD is the standard deviation of the natural-log-transformed Hcy level in the cohort. P-values lower than 0.05 were considered significant. All analyses were done using the SPSS package version 11.

To explore possible interactions between polymorphisms and/or nutrient intake, we performed logic regression^{31,32} that is available as an R package (version 1.4.0; <http://bear.fhrc.org/~ingor/logic/>). The regression model was used to investigate the effect of multiple polymorphisms involved in Hcy metabolism. We set 100,000 iterations in the simulated annealing algorithm, therefore the number of acceptances/rejections during the iteration process was optimal. Each polymorphism is coded into two binary covariates: X1d (dominant) = 1 if a person has at least one variant allele and X1r (recessive) = 1 if the person has two variant alleles; otherwise both are 0. Corresponding to the dominant and recessive effects of polymorphisms. In the logic regression we divided the intake of each dietary B vitamin (energy adjusted) to low and high with regard to the median intake.

Logic regression was successfully applied to select the best model as described else where^{31,32}.

Results

The baseline characteristics of our study populations are presented in Table 1.

Table 1. General characteristics of the study population

Characteristic	Cohort 1 (n=1026)	Cohort 2 (n=555)	Cohort 3 (n=1087)	Cohort 4 (n=505)
Total number				
Number of women (%)	602 (58.7)	279 (50.3)	692 (63.7)	500 (100)
Age (years)	70.51 ± 8.62	73.56 ± 7.90	73.86 ± 7.63	68.43 ± 3.84
Body mass index (kg/m ²)	26.37 ± 3.74	26.33 ± 3.55	26.45 ± 12.07	27.32 ± 4.53
Dietary intakes				
Riboflavin (vitamin B2) (mg/day)	1.55 ± 0.54	-	-	-
Pyridoxine (vitamin B6) (mg/day)	1.60 ± 0.41	-	-	-
Folate (vitamin B11) (µg/day) (min-max)	215.5 (79.5-1352.5)	-	-	-
Cobalamin (vitamin B12) (µg/day) (min-max)	5.1 (0.99-105.6)	-	-	-
Protein intake	79.72 ± 18.52	-	-	-
Energy intake (kj/day)	8189.88 ± 2038.41	-	-	-
Hcy levels (µMol/L) (max-min)	15.20 (0.26-60.9)	11.36 (5.2-40.0)	10.49 (3.4-54.6)	9.90 (3.50-23.50)

Values are unadjusted means ± standard deviations or numbers with percentages

Genotype distributions for all polymorphisms were consistent with those predicted by Hardy-Weinberg equilibrium.

Table 2 shows the deviations from the population mean Hcy levels for each single polymorphism, when all subjects in the four independent samples were pooled. Subjects homozygous for the T allele of the C677T MTHFR polymorphism had 0.43 µmol higher Hcy levels as compared with the CC genotype group with evidence for an allele dose effect (p -trend<0.0001). In addition, homozygotes for the G allele of the G80A RFC had 0.12 µmol higher Hcy levels compared with A homozygotes, with evidence for an allele dose effect (p -trend=0.03). Similarly, homozygotes for the A allele of the A2756G MTR gene, had 0.103 µmol higher Hcy levels than the 2756 MTR GG variant (p -trend=0.005).

Table 3 shows the Hcy levels according to the MTHFR C677T, RFC G80A, and the MTR A2756G polymorphisms in the four samples separately.

In a multi locus approach by logic regression, still the MTHFR C677T was significantly associated with Hcy levels and the other polymorphisms add no more effect (data not shown).

Genetic factors modulating the variations in determining Hcy levels

Stepwise multiple linear regressions were performed to identify independent determinants of Hcy levels (Table 4). First, we examined the effects of genotypes on Hcy levels. In the total study population 10.9% of the variability in Hcy levels was explained by this model. Of these age and gender explained 9.2%, (P <0.0001),

Table 2. Deviation of mean Hcy levels according to polymorphisms in genes in Hcy metabolism

<i>Gene</i>	<i>SNP</i>	<i>Genotype</i>	<i>N</i>	<i>Deviation from mean (Hcy)</i>	<i>p-ANOVA</i>	<i>p-trend</i>
MTHFR	C677T	CC	887	-0.05 ± 0.03	1.6*10 ⁻⁹	1.0 *10 ⁻⁹
		CT	854	0.09 ± 0.03		
		TT	216	0.38 ± 0.06		
	A1298G	AA	1064	0.07 ± 0.03	0.66	
		AG	906	0.06 ± 0.03		
		GG	252	0.003 ± 0.06		
THYMS	2R/3R	2R2R	228	-0.02 ± 0.07	0.31	0.13
		2R3R	494	0.01 ± 0.05		
		3R3R	293	0.11 ± 0.06		
RFC	G80A	GG	794	0.10 ± 0.03	0.11	0.03
		GA	1077	0.04 ± 0.03		
		AA	339	-0.02 ± 0.05		
TCN	C776G	CC	670	0.01 ± 0.04	0.13	
		CG	1088	0.10 ± 0.03		
		GG	428	0.02 ± 0.05		
MTR	A2756G	AA	1513	0.10 ± 0.02	0.008	0.005
		AG	618	-0.04 ± 0.04		
		GG	74	-0.003 ± 0.11		
MTRR	A66G	AA	704	0.08 ± 0.04	0.41	
		AG	1081	0.05 ± 0.03		
		GG	417	0.001 ± 0.05		
CBS	Ins68bp	0	1431	0.05 ± 0.03	0.48	
		1	281	-0.02 ± 0.06		
		2	20	0.14 ± 0.22		

Values are means ± standard error
Adjusted for gender, age, and BMI.

and the MTHFR C677T 1.3%, ($p < 0.0001$), MTR A2756G 0.2%, ($p = 0.02$), and MTHFR A1298C 0.2%, ($p = 0.05$).

Then, we assessed the independent effects of genetic and nutrient factors in determining Hcy levels in cohort 1. In this population, the variability in Hcy levels was attributed to age and gender 9.5%, ($p < 0.0001$), to dietary folate intake 3.5%, ($p = 0.001$), MTHFR C677T 1.8%, ($p = 0.0002$), and to protein intake 0.9%, ($p = 0.01$) and energy intake 0.9%, ($p = 0.0002$, Table 4).

The same analysis could not be performed for cohorts 2, 3 and 4 of the Rotterdam Study, because data on dietary intake were not collected at the same time that Hcy levels were determined.

Table 3. Hcy levels according to polymorphisms in genes in Hcy metabolism

Gene	SNP	Genotype	Cohort 1		Cohort 2		Cohort 3		Cohort 4	
			N	Hcy levels	N	Hcy levels	N	Hcy levels	N	Hcy levels
MTHFR	C677T	CC	415	14.50 ± 0.21	226	11.00 ± 0.21	434	10.08 ± 0.15	204	9.31 ± 0.17
		CT	395	15.23 ± 0.22	225	11.27 ± 0.22	420	10.57 ± 0.16	236	9.62 ± 0.17
		TT	86	16.79 ± 0.52	59	12.27 ± 0.47	115	11.76 ± 0.35	46	10.34 ± 0.41
	p-ANOVA		2.0*10 ⁻⁶		0.04		1.5*10 ⁻⁷		0.05	
	p-trend		1.9*10 ⁻⁵		0.02		6.1*10 ⁻⁶		0.01	
RFC	G80A	GG	322	15.24 ± 0.25	190	11.83 ± 0.25	339	10.64 ± 0.18	178	9.51 ± 0.19
		GA	422	14.85 ± 0.21	230	11.01 ± 0.21	471	10.41 ± 0.15	230	9.68 ± 0.17
		AA	142	14.87 ± 0.37	76	10.82 ± 0.37	137	10.11 ± 0.28	81	9.02 ± 0.27
	p-ANOVA		0.45		0.02		0.26		0.1	
	p-trend				0.01		0.1			
MTR	A2756G	AA	604	15.23 ± 0.18	335	11.46 ± 0.19	656	10.63 ± 0.13	320	9.72 ± 0.15
		AG	244	14.57 ± 0.27	149	11.13 ± 0.27	256	9.93 ± 0.20	154	9.24 ± 0.20
		GG	31	14.08 ± 0.74	19	9.84 ± 0.67	31	10.59 ± 0.60	15	9.93 ± 0.70
	p-ANOVA		0.06		0.07		0.01		0.1	
	p-trend		0.02		0.05					

Values are means ± standard error, Adjusted for gender, age, and BMI, * Values have been back log transformed.

Effect of genetic factors and dietary B vitamin intake on Hcy levels

Since MTHFR C677T and dietary folate intake explained most variance in Hcy levels in the latest stepwise model, and they also mentioned in the biological pathway of Hcy metabolism, therefore, we tested possible interaction between these factors by performing a stratified analysis. The association between the MTHFR C677T variant and Hcy levels were modified by dietary folate intake at baseline. The genotype effect was larger in the low folate intake group with differences between the CC and TT group (mean difference=5.22 µmol/l, (35%), (p<0.0001). This difference was 1.47 µmol/l, (11.0%) in the high folate intake group (p=0.1). In both groups an allele dose effect was observed and this only reached significant for the low intake group (Figure 1A). In addition, an interaction between dietary folate intake and the RFC genotype was observed (p-interaction=0.04, Figure 2B). While the RFC G80A AA homozygotes had a 0.27µmol/l (2%) higher Hcy levels than GG genotype in the low folate group, the AA group had a 2.09 µmol/l, (16.6%) lower Hcy levels in higher folate group. Thus, the difference in Hcy level for the AA group between the low and high folate intake group was 2.64 µmol/l, (19.8%), (p=0.003).

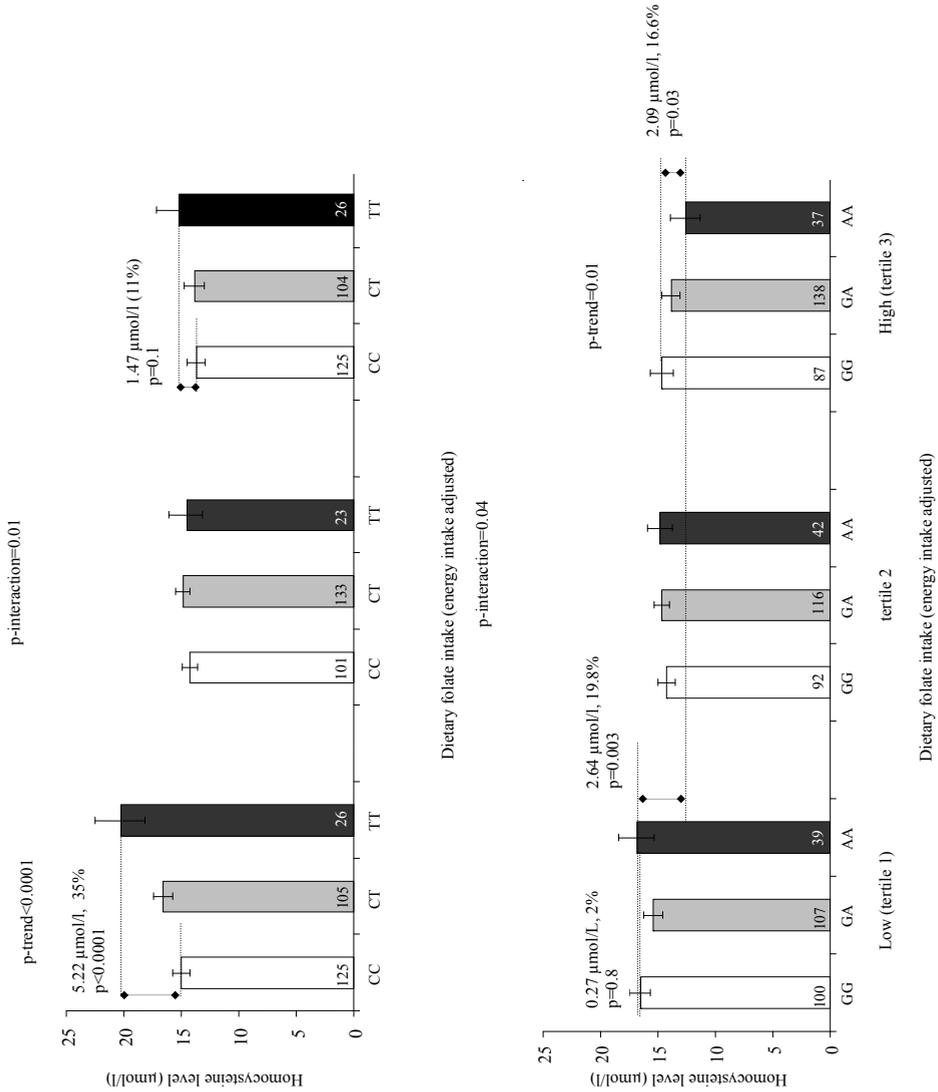


Figure 1A, 1B. Association between dietary folate intake and the MTHFR C677T variant in relation to Hcy level. Adjusted for gender, age and BMI. The dietary folate intake adjusted for energy intake. Bars represent means with standard errors.

Table 4. Independent contributors to variations in determining Hcy levels

Factors	<i>Total population</i>	
	Percentage	P-value
<i>Polymorphisms</i>		
Age and gender	9.2	<0.0001
MTHFR C677T	1.3	<0.0001
TCN C776G	-	-
MTHFR A1298C	0.2	0.05
MTR A2756G	0.2	0.02
MTRR A66G	-	-
RFC G80A	-	-
Total for this model	10.9	
<i>Baseline (Cohort 1)</i>		
<i>Polymorphisms and nutrients</i>		
Age and gender	9.5	<0.0001
MTHFR C677T	1.8	0.0002
MTHFR A1298C	-	-
MTR A2756G	-	-
MTRR A66G	-	-
RFC G80A	-	-
Protein intake	0.9	0.01
Energy intake	0.9	0.0002
Folate intake	3.5	0.001
Cobalamin intake	-	-
Riboflavin	-	-
Pyridoxine intake	-	-
Total for this model	16.6	

Effect of genetic factors and dietary B vitamin intake on Hcy levels by logic regression

To confirm the observed interaction we performed a logic regression analysis within R statistical package on those individuals who had data available for all studied polymorphisms and nutrient intake (n=704). In this analysis the best model was a model in which both MTHFR and RFC interact with dietary intake of folate in determining Hcy levels (Figures 2).

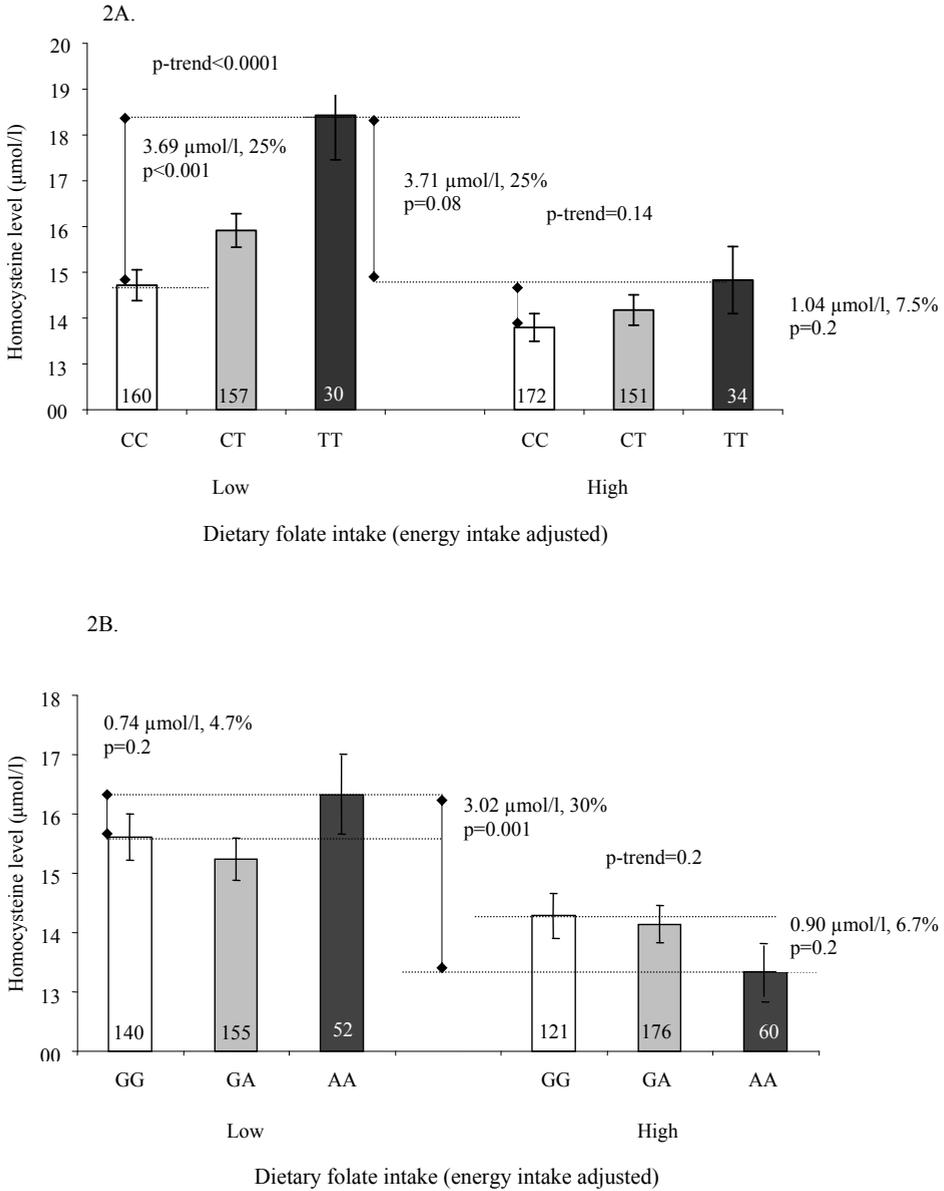


Figure 2A, 2B. Association between dietary folate intake and the MTHFR C677T variant in relation to Hcy level
 Adjusted for gender, age and BMI.
 Low indicates below median of dietary folate intake and high indicates above median. The dietary folate intake adjusted for energy intake.
 Bars represent means with standard errors.

Discussion

In the present study populations, we described that Hcy levels were significantly different among subjects on the basis of genotypes for the MTHFR C677T, the MTR A2756G, and the RFC G80A polymorphisms. Furthermore, we observed a significant interaction between dietary folate intake and the C677T MTHFR and G80A RFC variant.

In agreement with previous studies^{14,33}, we confirmed that the 667T allele of the MTHFR gene is associated with higher levels of Hcy. While this is not so surprising, since MTHFR is a key regulatory enzyme in Hcy metabolism^{34,35}, we observed no difference in Hcy levels by the MTHFR 1298 genotypes. This variant alone may not significantly affect the Hcy levels¹⁵, but perhaps interacts with the 677 to determine Hcy levels. However, in our multi-locus approach within our population it not appears to interact with the MTHFR C677T polymorphism.

We observed that the MTR A2756G A allele was associated with elevated Hcy levels in an allele dose-dependent manner. This suggests that the MTR 2756A variant may diminish the MTR function, resulting in reduction of the conversion of Hcy to methionine and consequently a higher level of Hcy. The polymorphism results in the substitution of an aspartic acid (A2756), a moderate helix maker, by a glycine, a strong helix maker (2756G). The polymorphism lies close to the cobalamin binding domain of MTR¹⁶, therefore functional consequences as a result of different protein structure is conceivable.

All of the studies with fewer than 500 subjects failed to see an association between MTR genotype and Hcy levels^{21,36-39}. However, two reports with larger numbers of subjects (>500) have noted a modest association between the presence of this variant and fasting Hcy concentrations^{23,40,41}. We confirm this association in our large population-based sample of > 2000 individuals.

We observed an association between RFC G80A genotype and Hcy levels consistent with the study of Chango *et al.*⁶. RFC is the transporter for folate in the form of 5-methyl-tetrahydrofolate⁴². The RFC-1 (G 80 A) polymorphism, at position 80, in exon 2, changes an arginine (G allele) into a histidine (A allele)⁶. The RFC-1 A allele (histidine) is reported to have a higher affinity for reduced folate than the G allele⁶. Thus, in the presence of the G80 allele, the intestinal absorption of dietary folates might be impaired thereby increasing Hcy levels. In this light, our finding of the interaction of dietary folate intake with the RFC G80A in determining Hcy levels could be biologically plausible. The presence of the G80 allele impaired the intestinal absorption of dietary folate and resulted in increasing Hcy levels in subjects who have low dietary folate intake (below median). This indicates that under biological stress condition (low folate intake) possible differences caused by these polymorphisms might became evident as slight increase in Hcy levels.

The multivariate analysis showed that besides the effect of MTHFR 677, also MTR 2756 plays a role in determining Hcy levels. Furthermore, a minor effect of the MTHFR 1298 was observed, for which the explanation is unknown.

Application of multi-locus analysis allowed us to identify interacting polymorphisms that contribute to the variation of increasing Hcy levels. The presence of interacting polymorphisms and the effect of identified genetic variants were small, yet they confirmed the complex character of elevated Hcy. This illustrates the necessity for considering not only one, but also all the genetic polymorphisms of the enzymes involved in the Hcy metabolism. Nevertheless, we note that we have limited power to pick up such associations, because this approach was feasible in 70% of the individuals.

Several intervention studies have provided evidence for the importance of B vitamins in Hcy metabolism ^{43,44}. In our study population, cohort 1, dietary folate intake was the most important dietary determinant of Hcy levels at baseline, consistent with previous studies ⁴⁴. Folate is used as a substrate, where it donates a methyl group for the remethylation of Hcy to methionine. Consistent with our results, several lines of evidence indicate that there is an relation between protein and Hcy levels ⁴⁵. Unlikely, to some studies ⁴⁶, we did not find an effect of cobalamin on Hcy levels independent of the other dietary B vitamin intake. It is possible that the levels of cobalamin are not related to its intake. In the elderly, the uptake of cobalamin is impaired which could influence the relation between dietary intake of cobalamin and Hcy levels ⁴⁷.

The strength of our study lies in the large study population. However, there are some limitations. Only baseline dietary intakes were available and no circulating vitamin B levels, while a combination of dietary intake and vitamin levels might provide a better predictive power for vitamin effects on Hcy levels than dietary vitamin intake alone. Moreover, there were considerable differences in the techniques used to assess Hcy levels in the four cohorts. These differences in method are known to influence the measurement of homocysteine levels ⁴⁸.

In summary, we showed that MTHFR C677T, MTR A2756G and RFC G80A polymorphisms influence Hcy levels separately. Furthermore, our results indicate that the MTHFR C677T polymorphism, dietary folate intake and dietary protein intake are the strongest predictors of Hcy levels besides age and gender. Moreover, by applying a multi-locus approach, we identified interactions between MTHFR C677T and RFC G80A with low folate intake in increasing Hcy levels. While our results require confirmation in other cohorts, this suggests that genotype driven intervention could target those individuals most likely to benefit by reducing Hcy levels.

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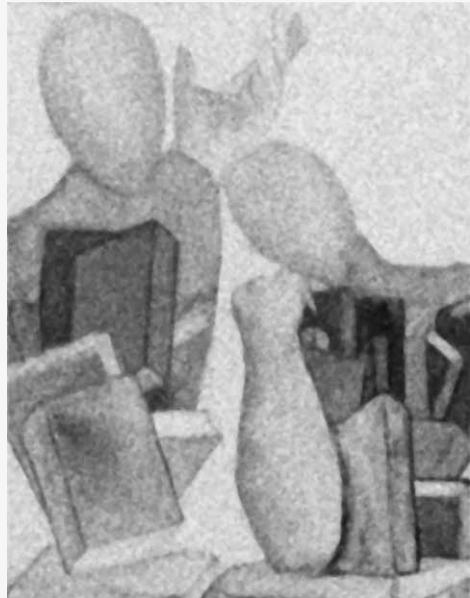
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CHAPTER 6

Gene polymorphisms determining in bone phenotypes

The -1997 G/T and the Sp1 polymorphisms in the Collagen Type I alpha 1 (COL1A1) gene in relation to changes in femoral neck BMD and the risk of fracture in the elderly: The Rotterdam Study



Abstract

The COLIA1 Sp1 polymorphism has been found to be associated with bone mineral density (BMD) and fracture. A promoter polymorphism, the -1997 G/T has been associated with BMD. In this study, we examined whether these polymorphisms alone and in the form of haplotypes influence bone parameters and fracture risk in a large population-based cohort of elderly Caucasian.

We determined the COLIA1 -1997 G/T (promoter) and the Sp1 G/T (intron) polymorphisms in 6280 individuals and inferred haplotypes. Femoral neck BMD and BMD change were compared across the COLIA1 genotypes at baseline and at follow-up (mean follow-up 6.5 years). We also investigated the relationship between the COLIA1 polymorphisms and incident non-vertebral fractures which were recorded during a mean follow-up period of 7.4 years. Vertebral fractures were assessed by radiographs on 3456 genotyped individuals.

Femoral neck BMD measured at baseline was 3.8% lower in women carrying two copies of the T-Sp1 allele (p for trend = 0.03). No genotype dependent differences in BMD-loss were observed. In women homozygous for the T allele of the Sp1 polymorphism the risk of fragility fracture increased 2.3 times (95% CI: 1.4-3.9, p = 0.001). No such association was observed with the promoter polymorphism. In men no association with either the Sp1 or the -1997 G/T promoter polymorphism was seen with BMD, or fracture. High linkage disequilibrium (LD) (D' =0.99, r^2 =0.03) exists between the two studied polymorphisms. We observed three haplotypes in our population: haplotype 1 (G promoter-G intron) frequency (f) = 69%, haplotype 2 (G promoter-T intron) f = 17.6% and haplotype 3 (T promoter-G intron) f = 13.4%. Haplotype 2 was associated with increased risk of fragility fracture in women 2.1 (95% CI: 1.2-3.7, p = 0.001).

We confirm that the COLIA1 Sp1 polymorphism influences BMD and the risk of fracture in postmenopausal Caucasian women. In contrast, we found no independent effect of the -1997 G/T promoter polymorphism on BMD, or fracture.

Introduction

Osteoporosis is a multifactorial disease with both genetic and environmental determinants. It is characterized by a reduction in bone mineral density (BMD) and microarchitectural deterioration of bone tissue which leads to an increased risk of fracture in later life ^{1,2}. Being a predictor of bone fragility and susceptibility to fracture, BMD is used for the diagnosis of osteoporosis ^{3,4}. The risk of fracture is not only dependent on BMD but also on geometry, architecture, material properties and mass distribution ⁵.

The skeletal determinants of osteoporotic fracture risk such as BMD, bone loss and bone geometry are all subject to strong genetic influences ^{2,6,7}. It has been estimated from twin studies that between 60-80% of the variance in BMD is attributable to genetic factors ^{8,9}. Several genes are thought to be involved in the pathogenesis of osteoporosis. Collagen type I alpha 1 “(COLIA1)” is one of the most prominent candidate genes, which has been consistently associated with osteoporosis in different populations ¹⁰⁻¹². COLIA1 encodes the alpha 1 chain of collagen type I, which is the most abundant structural protein in the bone matrix, rare mutations in this gene cause osteogenesis imperfecta a mendelian disorder presenting with moderate to severe bone fragility ^{13,14}.

Previously, Grant *et al.* ¹⁵ identified a relatively common guanine to thymidine (G→T) polymorphism in the first intron of COLIA1. This polymorphism affects one of the binding sites of the transcription factor Sp1 and results in increased expression of collagen type I alpha 1 in bone matrix in T carriers ¹¹. We and others have shown that the T allele is associated with osteoporosis, lower BMD ^{10,15} and increased fracture risk ^{10,12,16,17}. Moreover, in a very large prospective meta-analysis of individual data, we observed that the Sp1 polymorphism in the COLIA1 gene is associated with reduced BMD and incident vertebral fractures independent of BMD ¹⁸. In addition to the Sp1 polymorphism, Garcia-Giralt *et al.* ¹⁹ described two polymorphisms within the COLIA1 promoter region: the -1997 G/T and -1663 indelT. The study showed in a small cohort of postmenopausal Spanish women that the T-allele of the -1997 G/T polymorphism was significantly associated with 7.5% decreased lumbar spine BMD and 12% decreased femoral neck BMD. Furthermore, they analyzed compound genotypes including three polymorphic sites. However, this small study of the promoter polymorphisms in women investigated the relation in form of compound genotypes and did not extend their study to examine haplotypes of promoter and Sp1 polymorphisms, and most importantly, did not analyze fractures, the clinically most relevant endpoint of osteoporosis.

Therefore, we investigated the influence of both COLIA1 polymorphisms independently and in the form of haplotypes in relation to baseline femoral neck BMD,

change in BMD with follow-up and risk of vertebral and non-vertebral fractures in a large population-based cohort of elderly Caucasian men and women.

Materials and Methods

Study Population

This study was embedded in the Rotterdam Study, a population-based cohort study in which all residents of the Rotterdam suburb Ommoord aged 55 years and older were invited to take part in. The design of the study has been described elsewhere²⁰. Written informed consent was obtained from all participants and the Medical Ethics Committee of the Erasmus Medical Center approved the study. Baseline data collection was conducted in January 1990 and June 1993, while two follow-up assessments were performed between July 1993 and January 1996 and from July 1996 until December 1999. A total of 7983 subjects participated in the study (response rate 78%) and for the present study we examined 6280 individuals who were genotyped.

Study Design

This study was performed in three steps. In the first step, we performed a cross-sectional analysis where we examined the relation between the genotype and baseline BMD (n = 5737). In the second step, we performed a longitudinal analysis to study change in BMD between baseline and the second follow-up (mean 6.5 ± SD 0.6 years, n = 2670). In the third step we looked at the relation between *COL1A1* polymorphisms and the risk of incident fracture. We studied incidence of non-vertebral fracture (mean follow-up 7.4 ± 3.3 SD years, n=6280) and vertebral fractures assessed by radiographs both at baseline (1990-1993) and at follow-up visit, between 1997 and 1999, thoracolumbar radiographs of the spine were available for 3469 individuals in a mean follow-up of 6.4 years.

Measurements

Bone mineral density (g/cm²) of the hip and L2-L4 of the lumbar spine were measured by dual-energy x-ray absorptiometry (DXA) using a Lunar DPX- densitometry (DPX-L) (Lunar Radiation Corporation, Madison, WI, USA) and re-analysed with DPX-IQ software, under standard protocols. Methods, quality, assurance accuracy, and precision issues of the DXA measurements have been described previously²¹. The relative change of BMD from baseline was estimated as the difference in BMD between assessment periods divided by the BMD at baseline.

Height (cm) and weight (kg) were measured with a stadiometer at the initial examination, in standing position wearing indoor clothes without shoes. Body mass

index (BMI) was computed as weight in kilograms divided by height in centimeters squared (kg/cm^2).

Fracture Follow-up

Information on incident non-vertebral fractures was collected from baseline (1990-1993) until January 1, 2002 (mean follow-up 7.4 ± 3.3 SD years, $n=6280$).

Non-vertebral fracture events were retrieved from computerized records of the general practitioners (GPs) in the research area. Research physicians regularly followed participant information in GPs records outside the research area and made an independent review, encoding all reported events. Subsequently, a medical expert in the field reviewed all coded events for final classification. We excluded fractures that were considered non-osteoporotic fractures; caused by cancer and high trauma fractures, including fractures of the hand, foot, skull and face. Subsequently, we analysed separately “fragility” fractures, which were defined as any fracture of the hip, pelvic and proximal humerus that had occurred with minimal trauma at older age (mean age >75 years).

Vertebral fracture assessment

Both at baseline and at first follow-up, between 1997 and 1999, thoracolumbar radiographs of the spine were obtained. The follow-up radiographs were available for 3456 individuals, who survived an average of 6.4 (SD 0.4) years after the baseline visit and who were still able to come to our research center. All follow-up radiographs were scored for the presence of vertebral fracture by the McCloskey/Kanis method, as described previously²².

Genotyping

Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. 1-2 ng genomic DNA was dispensed into 384-well plates using a Caliper Sciclone ALH3000 pipetting robot (Caliper LS, Mountain View, CA, USA). Genotypes were determined using the Taqman allelic discrimination assay. The Assay-by-Design service (www.appliedbio-systems.com) was used to set up a Taqman allelic discrimination assay for the COL1P1R-1997 polymorphism (Primers Fw: GCCTCCGGAGGGTGTCA Rv: AAGGAGAGCAATTCTTACAGGTGTCT, Probes FAM-CCTGAGGGATGGAA-MGB, VIC-CCTGAAGGATGGAA-MGB). The PCR reaction mixture included 1-2 ng of genomic DNA in a 2 μl volume and the following reagents: FAM and VIC probes (200 nM), primers (0.9 μM), 2x Taqman PCR master mix (ABgene, Epsom, UK). Reagents were dispensed in a 384-well plate using the Deerac Equator NS808 (Deerac Fluidics, Dublin, Ireland). PCR cycling reaction was performed in 384 well PCR plates in an ABI 9700 PCR system (Applied Biosystems Inc., Foster City, CA,

USA) and consisted of initial denaturation for 15 minutes at 95° C, and 40 cycles with denaturation of 15 seconds at 95° C, and annealing and extension for 60 seconds at 60° C. Results were analysed by the ABI Taqman 7900HT using the sequence detection system 2.22 software (Applied Biosystems Inc., Foster City, CA, USA). To confirm the accuracy of genotyping results, 332 (5%) randomly selected samples were re-genotyped with the same method. No inconsistencies were observed. For Sp1 (intron1) an assay was set up using primer express software version 2.0. Forward and reverse primer sequences were 5'-GTTGTCTAGGTGCTGGAGGTT-3' and 5'-GGCGAGGGAG-GAGAGAAGG-3'. The PCR reaction mixture included 5 ng of genomic DNA in a 4 µl volume and the following reagents: FAM-CCCGCCCACATTCCTGG-MGB probes (250 nM), TET-CCCGCCCCATTCCTGG-MGB probes (500 nM), primers (300 nM), 2x Taqman PCR master mix (Applied biosystems Inc.). PCR cycling reaction were performed in 384 wells PCR plates in an ABI 9700 PCR system (Applied biosystems Inc.) and consisted of initial denaturation for 15 minutes at 95°C, and 40 cycles with denaturation of 15 seconds at 95°C and annealing and extension for 60 seconds at 60°C. Results were analysed by the ABI Taqman 7900HT using the sequence detection system 2.1 software (Applied biosystems). To confirm the accuracy of the genotyping results 332 randomly selected samples were genotyped for a second time with the same method. All polymorphisms had an error rate lower than 1%.

Statistical Analysis

Hardy-Weinberg equilibrium of the COLIA1 polymorphisms genotypes was tested using the GENEPOP-package²³. Linkage disequilibrium between each pair of alleles at both polymorphic loci was calculated as D' and r^2 ²⁴.

We stratified all analyses by gender considering peak bone mass, changes in BMD and fractures, follow age and sex -specific patterns. Baseline BMD and BMD rate of change were compared across COLIA1 polymorphisms using univariate analysis of variance (ANOVA). Corrections were made for age and BMI. Trend analysis assuming an underlying additive genetic model was done for the presence of zero, one, or two copies of the associated allele, incorporating the genotype variable as a continuous term in a general linear regression model. For the analysis of non-vertebral fracture follow-up data, we computed the incidence rates of fracture among genotypes and used Cox proportional hazards model adjusting for age and BMI to estimate risk of fracture. For vertebral fractures, odd ratios with 95% confidence intervals (95% CI) were calculated using logistic regression models, since no data on the exact time of occurrence could be determined.

We used the HaploStats (available at <http://cran.r-project.org/>) to estimate the frequency of inferred haplotypes and investigate the association of haplotypes with BMD and the risk of fractures. We restricted the analysis to haplotypes with an

inferred frequency of more than 0.02. The first haplotype, which was most frequent, was used as reference.

Significant *p*-values were considered 0.05 or lower. Finally, model assumptions were verified and model residuals were checked for goodness-of-fit. SPSS 11.0 (SPSS, Chicago, IL, USA) was used for the analyses.

Results

Allele and genotype frequencies of the -1997 G/T and Sp1 polymorphisms were in Hardy-Weinberg equilibrium proportion ($p=0.61$ and $p=0.10$ respectively). General characteristics of the study population at baseline and follow-up are shown in Table 1.

Table 1. General characteristics of the study population at baseline and second follow-up

	Women		Men	
	Baseline	2 nd follow up	Baseline	2 nd follow up
Number	n=3374	n=1724	n=2452	n=1287
Age (years)	68.3 ± 8.2	72.7 ± 6.8	67.6 ± 7.7	72.2 ± 6.5
Height (cm)	161.1 ± 6.8	160.6 ± 6.4	174.6 ± 6.8	174.0 ± 6.7
Weight (kg)	69.3 ± 11.4	70.3 ± 12.2	78.2 ± 10.8	79.5 ± 11.3
Body mass index (kg/m ²)	26.7 ± 4.1	27.2 ± 4.4	25.6 ± 3.0	26.3 ± 3.2
Femoral neck BMD (g/cm ²)*	0.83 ± 0.14	0.80 ± 0.13	0.92 ± 0.14	0.90 ± 0.14
Lumbar spine BMD (g/cm ²)	1.03 ± 0.18	-	1.16 ± 0.20	-
FN- BMD change	n=1527		n=1143	
FN-BMD change (relative % of baseline year) [†]	-0.84 ± 1.09		-0.45 ± 0.92	

Values are means ± standard deviation

Anthropometric measurement based on 5826 individuals at baseline and 3011 individuals at follow-up

*BMD measurements based on 5737 individual at baseline and 2670 individual at second follow-up

[†] Femoral neck (FN) BMD change has been measured between second follow-up and baseline

Second follow-up measurements were performed on average 6.5 (SD 0.6) years after baseline

Baseline BMD by COL1A1 genotypes

In both genders, age, height, weight, and BMI did not differ significantly between genotypes for the -1997 G/T and Sp1 polymorphisms (data not shown). Table 2 shows the BMD values according to COL1A1 genotypes in men and women. Femoral neck BMD was 3.8% lower (mean difference=0.03 g/cm², $p=0.09$) in women homozygous carriers of the Sp1 T allele as compared to non-carriers, with evidence for an allele dose effect (p for trend=0.03) (Table 2). No association was found between the -1997 promoter polymorphism and lumbar spine BMD or femoral neck BMD in men or women (Table 2). We did not observe any significant association between

Table 2. BMD measurements by COLIA1 genotypes at baseline and follow-up

	<i>Promoter -1997 G/T</i>				<i>Intron 1 Sp1 G/T</i>			
	GG	GT	TT	[†] <i>p</i>	GG	GT	TT	[†] <i>p</i>
<i>Men</i>								
<i>BMD</i>	n=1663	n=494	n=37		n=1484	n=655	n=55	
Femoral neck (g/cm ²)	0.92 ± 0.13	0.92 ± 0.14	0.90 ± 0.14	1.00	0.92 ± 0.14	0.92 ± 0.14	0.90 ± 0.12	0.45
Lumbar spine (g/cm ²)	1.16 ± 0.19	1.17 ± 0.20	1.15 ± 0.22	0.96	1.17 ± 0.19	1.16 ± 0.20	1.16 ± 0.20	0.91
Number	n=668	n=233	n=16		n=606	n=293	18	
FN-BMD change (relative % of baseline year) [‡]	-0.46 ± 0.91	-0.36 ± 0.93	-0.38 ± 0.60	0.62	-0.39 ± 0.93	-0.56 ± 0.87	-0.30 ± 0.82	0.01
<i>Women</i>								
<i>BMD</i>	n=2157	n=710	n=53		n=1971	n=858	n=91	
Femoral neck (g/cm ²)	0.83 ± 0.14	0.84 ± 0.13	0.85 ± 0.13	0.22	0.83 ± 0.13	0.82 ± 0.14	0.80 ± 0.14	[‡] 0.09
Lumbar spine (g/cm ²)	1.03 ± 0.18	1.04 ± 0.18	1.01 ± 0.18	0.69	1.04 ± 0.18	1.04 ± 0.19	1.01 ± 0.20	0.52
Number	n=820	n=298	n=24		n=773	n=340	n=29	
FN-BMD change (relative % of baseline year) [‡]	-0.84 ± 1.11	-0.83 ± 1.01	-0.78 ± 1.03	0.85	-0.85 ± 1.08	-0.80 ± 1.04	-0.74 ± 1.49	0.55

Values are expressed as mean plus/minus standard deviation. Adjustments for age and BMI. [†]*p* for ANOVA. [‡]

For trend linear regression: *p* = 0.03

Femoral neck (FN) BMD change has been measured between second follow-up and baseline

the Sp1 and -1997 promoter polymorphisms with changes in BMD during follow-up in men or women (Table 2).

Risk of fracture by COLIA1 genotypes

The relation between risk of fracture and COLIA1 Sp1 polymorphism is shown in Table 3. Women with two copies of the T allele of the Sp1 polymorphism had 2.3 times higher risk of fragility fracture (95% CI; 1.4-3.9, *p* = 0.001). Adjustment for femoral neck BMD did not essentially modify the association. A similar association was observed in men homozygous carriers of the Sp1 T allele, which was borderline significant (RR = 2.3, 95% CI; 0.9-5.8, *p* = 0.07). For the -1997 promoter polymorphism no association was found with any type of fracture in either men or women (Table 3).

Haplotype analysis

High LD exists between the -1997 G/T and Sp1 polymorphisms as assessed by *D'* measure (*D'* = 0.99, *r*² = 0.03). We observed in our population three -1997 G/T, Sp1 G/T haplotype alleles (Figure 1): haplotype 1 (G promoter-G Intron), 69.0%; haplotype 2 (G promoter-T Intron), 17.6%; and haplotype 3 (T promoter-G Intron), 13.4%. Haplotype 4 (T promoter-T Intron) was not present (Figure 1). The strong linkage disequilibrium (LD) between the -1997 G/T and Sp1 G/T polymorphism

Table 3. Risk of fractures by COLIA1 genotypes

COLIA1 geno- types	Types of fracture	Event (%)			Risk Ratio (95% CI)		
		GG	GT	TT	GG (Reference)	GT vs Reference	TT vs Reference
<i>Men</i>							
Promoter -1997 G/T							
	Non-vertebral	147/1952 (7.5)	49/592 (8.3)	1/46 (2.2)	1.0 (Reference)	3.18 (0.45-22.74)	3.44 (0.47-29.94)
	<i>Fragility</i>	72/1952 (3.7)	14/592 (2.4)	1/46 (1.8)	1.0 (Reference)	1.46 (0.20-10.55)	0.94 (0.12-7.14)
	Vertebral	100/1056 (9.5)	35/340 (10.3)	3/28 (10.7)	1.0 (Reference)	1.07 (0.71-1.61)	1.23 (0.36-4.18)
Intron 1 Sp1 G/T							
	Non-vertebral	91/1254 (7.3)	39/574 (6.8)	4/44 (9.1)	1.0 (Reference)	0.97 (0.71-1.33)	1.40 (0.65-3.00)
	<i>Fragility</i>	36/1254 (2.9)	14/574 (2.4)	2/44 (4.5)	1.0 (Reference)	0.94 (0.58-1.51)	2.34 (0.94-5.85)
	Vertebral	68/719 (9.5)	31/350 (8.9)	2/22 (9.1)	1.0 (Reference)	0.83 (0.55-1.24)	1.59 (0.60-4.22)
<i>Women</i>							
Promoter -1997 G/T							
	Non-vertebral	528/2721 (19.4)	182/879 (20.7)	8/63 (12.7)	1.0 (Reference)	1.53 (0.76-3.09)	1.66 (0.82-3.36)
	<i>Fragility</i>	216/2721 (7.9)	73/879 (8.3)	4/63 (6.3)	1.0 (Reference)	1.13 (0.42-3.05)	1.19 (0.44-3.26)
	Vertebral	159/1320 (12.0)	52/445 (11.7)	5/35 (14.3)	1.0 (Reference)	0.98 (0.70-1.37)	1.31 (0.49-3.45)
Intron 1 Sp1 G/T							
	Non-vertebral	279/1626 (17.2)	121/710 (17.0)	17/76 (22.4)	1.0 (Reference)	1.05 (0.90-1.23)	1.34 (0.92-1.23)
	<i>Fragility</i>	98/1626 (6.0)	42/710 (5.9)	10/76 (13.2)	1.0 (Reference)	1.01 (0.78-1.31)	2.33 (1.39-3.87)
	Vertebral	98/891 (11.0)	51/401 (12.7)	3/34 (8.8)	1.0 (Reference)	1.21 (0.88-1.65)	1.37 (0.56-3.35)

Adjustments for age and BMI

and the virtual nonexistence of haplotype 4, suggests there is absence of ancestral recombination in the region. We observed a borderline association ($p=0.06$) between haplotype 2 (G promoter–T Intron) and femoral neck BMD in women. Women who carry haplotype 2 (G promoter–T Intron) had lower (-0.01 mg/cm²) femoral neck BMD compared with haplotype 1 (G promoter–G Intron).

The relation between risk of fracture and COLIA1 haplotypes is shown in Table 4.

Women with haplotype 2 (G_{promoter}–T_{Intron}) had 2.1 times higher relative risk of fragility fracture ($p=0.03$), in men the increase in risk is 2.0 ($p=0.31$). These results were essentially unchanged after adjustment for femoral neck BMD. For haplotype 3 (T_{promoter}–G_{Intron}) we found no association with any type of fracture in either men or women.

Discussion

In this large population-based study we found that the Sp1 polymorphism influences the risk of fragility fracture in elderly women with a similar yet, not significant effect

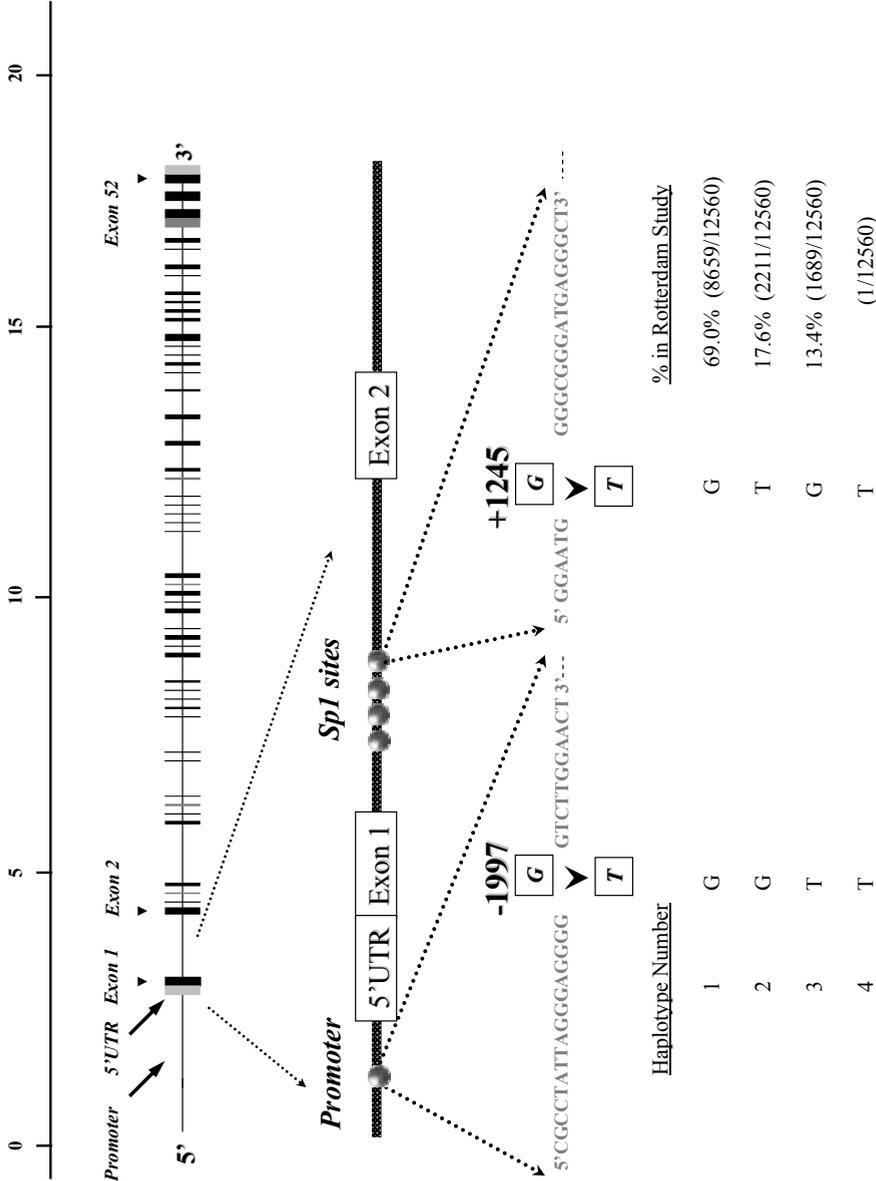


Figure 1. Schematic representation of the COLIA1 gene with the structural-1997 G/T polymorphism in the promoter region and G/T Sp1 polymorphism at binding site. With observed haplotype frequencies in the Rotterdam Study²⁷.

Table 4. Risk of fracture by COLIA1 haplotypes

Types of Fracture	Men			Women		
	OR (95 % CI)			OR (95 % CI)		
	Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 1	Haplotype 2	Haplotype 3
Non-vertebral	1 (Reference)	1.29 (0.55-3.02)	0.00 (0.00-∞)	1 (Reference)	1.31 (0.83-2.07)	0.61 (0.29-1.29)
Fragility	1 (Reference)	2.01 (0.71-5.67)	0.00 (0.00-∞)	1 (Reference)	2.12 (1.23-3.66)	0.80 (0.29-2.23)
Vertebral	1 (Reference)	1.64 (0.62-4.31)	1.19 (0.35-4.00)	1 (Reference)	1.23 (0.51-2.95)	1.31 (0.50-3.42)

Adjustments for age and BMI

Haplotype1: (G_{promoter}-G_{intron})

Haplotype2: (G_{promoter}-T_{intron})

Haplotype3: (T_{promoter}-G_{intron})

in men. Similarly, women homozygous for the T-allele had 3.8% lower BMD at baseline. The -1997 G/T polymorphism showed no independent effect on fracture risk or BMD levels in both genders. The haplotype analysis showed an association with BMD and fracture in women, which appeared to be driven by the effect of the Sp1 polymorphism.

A study in Spain¹⁹ showed that the -1997 G/T polymorphism located in the promoter region of COLIA1 gene, was associated with BMD in postmenopausal women of Spanish origin. In addition, analysis of compound genotypes of the three studied polymorphisms (-1997 G/T, Sp1, and -1663 indelT) suggested that the lowest value for BMD corresponded to GG homozygous at -1997 and heterozygous at the other two loci. Furthermore, in another report by the same group they observed a possible functional mechanism for the -1997 G/T polymorphism²⁵. Our population-based study suggests there is no independent effect of the -1997 polymorphism on BMD and the risk of fractures.

Recently, in a study by Stewart *et al.*²⁶ examined the three polymorphisms of the COLIA1 gene in forms of haplotypes in postmenopausal women. In contrast with our study, they observed that there is an association between reduced BMD values and the promoter -1997G/T polymorphism. Since the promoter, -1997G/T polymorphism is in strong linkage disequilibrium with the Sp1 polymorphism, the observed association of haplotype 2 derived by the SP1 polymorphism.

We showed that the association between fragility fractures and Sp1 polymorphism is significant only in women. We also found that the association between fragility fracture and the Sp1 polymorphism to be independent of femoral neck BMD. A possible explanation for an increased risk of fracture is due to the different number of fracture between men and women. There are a higher number of fractures in women as compared to men (13.2% in women and 4.5% in men). This suggests that other underlying biological mechanisms beyond BMD levels such as the role of micro-architecture, and composition of mineral crystals in bone tissue which, might

explain the increased fracture risk^{11,27,28}. Biomechanical testing of bone samples from heterozygous individuals with the GT genotype showed reduced bone strength compared to homozygous GG genotype and a slight reduction in mineralization of bone^{11,29}. Presence of the T allele in the COLIA1 Sp1 binding site leads to an abnormal relative level of COLIA1/COLIA2, which may reduce bone quality and quantity¹¹. Accordingly, we assume that a weaker network of abnormal collagen cross-linking may generate a three-dimensional unstable condition that may be responsible for its relatively greater risk of fragility fracture in the elderly women homozygous for the Sp1-T allele. Similarly, it is likely that the Sp1 polymorphism is driving these associations since evidence of functionality of this polymorphism has been reported previously.

In Genetic Markers for Osteoporosis (GENOMOS) Study, which is the largest study examining the Sp1 polymorphism (n=20,786) in relation to osteoporosis, an association between the Sp1 polymorphism and 1.3 times incident risk of vertebral fractures was also observed. An effect of the -1997 promoter polymorphism due to power limitations cannot be fully excluded, and should be subject to study in the larger population like GENOMOS.

Our present study has some limitations. Survival bias may play a role in this study if individuals who were lost to follow-up were dependent to genotype. Considering this selection bias, a possible relationship of the COLIA1 polymorphisms with changes in BMD cannot be fully excluded.

In conclusion, we observed an increased risk of fragility fractures in women carriers of the COLIA1 Sp1 T-allele. In contrast, the -1997 G/T polymorphism by itself appears to have no influence on fracture, BMD in postmenopausal women. Though the role of power limitations cannot be excluded.

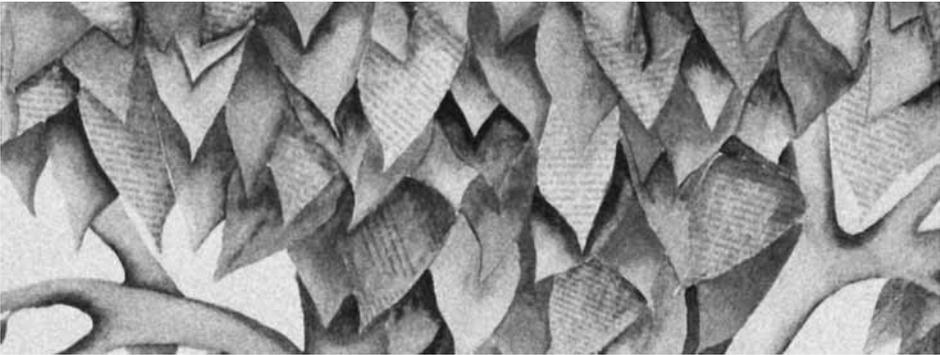
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CHAPTER 7

General discussion



The aim of this thesis was to determine the importance of genetic variability and dietary determinants of homocysteine (Hcy) metabolism in relation to osteoporosis in the elderly population. The current chapter will provide a more general discussion and includes the main findings and background of this thesis. Finally, clinical implications of our studies are discussed and suggestions are made for future research.

Main findings

First, we performed a systematic review on all published studies that investigated the relation between Hcy levels, B vitamin determinants of Hcy levels, and bone health. We found strong evidence that an elevated Hcy level predicts a higher risk for fracture, and moderate evidence that cobalamin (vitamin B12) and folate (vitamin B11) status is associated with bone mineral density (BMD) (chapter 2).

By investigating an effect of dietary B vitamins intake on bone phenotypes in the Rotterdam Study, we found that low dietary intake of both riboflavin and pyridoxine was associated with low BMD. In addition, high intake of pyridoxine was associated with a decreased risk of fracture (chapter 3).

In chapter 4, we describe genetic association studies in the Rotterdam Study on the relation to BMD and fracture risk with two polymorphisms that affect the amino acid sequence of two key enzymes (MTHFR and MTR) in the Hcy metabolism. The MTHFR Ala222Val (C677T) was investigated together with dietary intake of two established co-factors of the MTHFR enzyme: i.e., folate and riboflavin. We observed that MTHFR Ala222Val interacts with dietary riboflavin intake to determine fracture risk in women. When dietary riboflavin was low (first quartile), TT-homozygous women had higher Hcy levels and also increased 2.6 times fracture risks. Compared to AA homozygotes GG genotype of the MTR A2756G polymorphism was associated with low 5.1% lower BMD and 2.2 times higher fracture risk in women. The effect of the GG genotype on fracture risk was stronger in the presence of low (below median) dietary cobalamin intake.

In chapter 5 we described analyses in the Rotterdam Study that showed that Hcy levels were associated with the MTHFR C677T, MTR A2756G, and the RFC G80A polymorphisms. Furthermore, by applying a multi-locus approach including the six studied polymorphisms (MTHFR C677T and A1298C, RFC G80A, TCN C776G, MTR A2756G, and MTRR A66G) and dietary intake of B vitamin co-factors (vitamin B2, B6, B11 and B12), an interaction was identified between MTHFR C677T and RFC G80A with dietary folate intake in determining Hcy levels. In the low dietary folate intake (first tertile) 222Val MTHFR homozygotes and 80G RFC carriers had higher Hcy levels. In the low folate intake group, homozygotes for the A allele of the RFC G80A

polymorphism had 2% higher Hcy levels than GG genotype and in the high folate intake group (third tertile) the RFC 80A homozygotes had 16.6% lower Hcy levels. In the low folate group (first tertile) the T allele homozygotes (222ValVal) of the MTHFR C677T polymorphism had 35% higher Hcy levels compared with CC genotype.

Given the potential importance of the COLIA1 gene as a genetic marker of osteoporosis outcomes, we examined two polymorphisms in this gene (chapter 6). We observed an increased risk of fragility fractures in women that were homozygous carriers of the COLIA1 Sp1 T-allele. In contrast, the -1997 G/T polymorphism by itself appears to have no influence on fracture or BMD in postmenopausal women.

Methodological considerations

Study population: The Rotterdam Study

One of the major strengths of our approach was that we used a large population-based cohort study with a long follow-up period. Yet, population stratification could be a possible confounder when allele frequency of the studied polymorphism and disease frequency differ between two sub-populations which are treated as one homogenous population. However, 98% of our population is Caucasian, as was defined by questionnaires in which the ethnicity of the parents and grandparents of the subjects was documented. We therefore think this will not be a major problem in the analyses.

Study design

In this thesis several study designs were used. First, the studies on the relation between candidate genes and fracture risk had a longitudinal design. An important aspect of the longitudinal evaluation of fracture risk in time is the underlying cause for those associations. Also, a prospective design was used to examine the effect of dietary B vitamin intake on the association between candidate gene polymorphisms and incident fracture risk. Because of the prospective design, by assessing of the dietary B vitamin intake at baseline (which is before the fracture event) a causal effect relationship between B vitamin intake and fracture risk can be determined. We used a cross-sectional design only in studies that assessed the effect of polymorphisms on Hcy levels and BMD. Although technically this is a cross-sectional design, genetic studies in general can be considered prospective, since polymorphisms are stable and present in the individuals throughout life. Unlike circulating levels of Hcy, genetic markers do not essentially change during the lifetime of a person.

Some methodological issues should be considered in the interpretation of our findings. In chapters 3, 4 and 5 of this thesis, food frequency questionnaires were

used to assess B vitamin status. One may argue that plasma B vitamins levels would reflect B vitamin status better than food frequency questionnaires. However, in Europe the dietary intakes are relatively stable over time, especially among the elderly¹. The validated food frequency questionnaires are therefore a good measure for long-term assessment of nutrient intake. Furthermore, in this thesis all dietary intake has been corrected for energy intake by using the residual method as described elsewhere². In addition, plasma B vitamins may not be the best functional marker for B vitamin status³. For example studies have shown that up to 50% of subjects with low intracellular vitamin B12 status have normal plasma vitamin B12 levels⁴. Also, measuring vitamin levels only once (for example, in our study at baseline) may not reflect the accurate and long-term reservoir of B vitamins. Another method for accurate assessment of B vitamins status is to measure B vitamin metabolites, such as methylmalonic acid for B12 and RBC-folate for folate. This method might be a better indicator for assessing B vitamin status than studying solely dietary B vitamin intake, or plasma (vitamin B12 and folate) levels of the B vitamins.

In this thesis, we measured Hcy levels once at baseline, which can lead to a measurement error due to transient fluctuations in Hcy levels caused by treatment, disease, age, or changes in the diet. Therefore, to determine the long term Hcy status, it might be useful to measure Hcy levels at different time points during follow-up⁵.

Given the fact that BMD is a good predictor of bone fragility and susceptibility to fracture, and that the peak bone mass is strongly under genetic influences, BMD was used to study osteoporosis (chapters 3, 4 and 6). We determined BMD by applying dual-energy x-ray absorptiometry (DXA) using a Lunar DPX- densitometry (DPX-L). We applied an accurate technique and used standard protocols to minimize measurement bias, especially in repeated measurement of BMD to examine BMD changes. We reanalysed with one uniform version (DPX-IQ) all scans the image acquisition of which had been done. Using a longitudinal measurement of BMD in a large prospective population-based cohort is an important tool to study bone health. Longitudinal measurements are helpful in establishing causality for the observed change and can be considered extensively for future study. However, the causes of bone loss are multifactorial and thus are also influenced by environmental factors (e.g., calcium intake, sun exposure for vitamin D status and physical activity), in addition to a variety of conditions and medications that may limit or accelerate bone loss. Thus, even longitudinal measurements still may not be an accurate marker for studying bone quality without taking these factors into consideration.

The assessment of incident events (i.e., fractures) is an important advantage of our study design. In the fracture studies in this thesis (chapters 3, 4, and 6) incident fracture data of the Rotterdam Study are well documented by the accurate register of general practitioners in the Dutch healthcare system. Furthermore, analysis of the in-

cident non-vertebral fractures allows to investigate time-to-event and age-dependent relationships.

Association of Hcy and fracture risk with bone health

So far, it is not clear whether the association between Hcy and fracture risk is mediated through BMD or not. To address this question, we explored the findings from previously published studies in a systematic review (chapter 2) and found strong evidence that an elevated Hcy level predicts a higher risk for fracture in the elderly population. However, only a part of the observational studies (4 out of 8) have addressed the role of BMD.

The studies that did try to answer this question, observed that the Hcy levels were associated with fracture risk independent of BMD. However, the fracture risks observed were generally about two times higher for the highest quartile of Hcy levels versus the lowest. So, if these risks were caused solely by low BMD, the difference between mean BMDs in high versus low Hcy must be fairly large (i.e., close to 1 SD difference), given the fact that one SD difference in BMD will generally result in a two times higher fracture risk ⁶. The reports that studied only the relation between Hcy levels and BMD (and not fracture) yield conflicting results. The studies that did observe an association between Hcy and BMD reported a modest (0.2 SD) between extreme groups. Taken together these studies suggest that the association between Hcy and fracture risk is most likely not mediated through BMD. It seems more plausible that differences in BMD, if any, might be a consequence of the relation between Hcy levels and fracture risk rather than a cause.

B vitamins involved in the Hcy pathway and bone health

In chapter 3, we studied the Hcy related B vitamin intake in relation to bone health. We studied the four major B vitamins involved in the Hcy metabolism (vitamin B2, B6, B12 and folate), in contrast to all previous studies which only examined one or two of these vitamins, (generally, vitamin B12 and folate). We observed a positive and independent relation between dietary intake of both riboflavin (vitamin B2) as well as pyridoxine (vitamin B6) with BMD. Furthermore, we observed in the Rotterdam Study that high dietary intake of pyridoxine was associated with a significantly decreased risk of fracture. This effect was not modified by factors related to co-morbidity or by dietary intake of other B vitamins and this effect was independent of BMD.

In addition, we observed that the decreased fracture risk by dietary intake of pyridoxine was independent of Hcy levels. However, since Hcy levels were available only for a small subgroup of our population and were measured only once, we cannot fully address the question as to whether or not the protective effect of dietary pyridoxine on fracture risk is mediated through lowering Hcy levels or not.

Although the mechanism underlying this association is still unclear, pyridoxine could have an effect on bone through the enzyme lysyl oxidase. Pyridoxine serves as an essential co-factor for lysyl oxidase, a key enzyme for the formation of enzymatic cross-links in collagen in bone^{7,8}. Mice studies showed that pyridoxine deficiency results in a low amount of cross-link intermediates and impaired cross-link formation in bone⁷. Yet, pyridoxine is also known to be an important co-factor in many enzymatic steps⁹⁻¹², which open the possibility that pyridoxine exerts its effects not directly on bone but through some enzymatic reactions in the body, including many involved in the synthesis or catabolism of neurotransmitters including γ -aminobutyrate synthesis (GABA)¹⁰. Therefore, pyridoxine deficiency could affect neuromuscular control in the locomotor system and thus, increase the risk of falling, and thereby increase fracture rates. However, in our study we did not observe an effect of dietary pyridoxine intake on the rate of falls, which makes the latter less likely explanation for our observations.

Genetic studies on the association on Hcy and bone health

The observational studies reviewed in chapter 2, did not prove a causal relationship between Hcy and fracture risk. Observational epidemiological studies suffer from many potential biases, from confounding and reverse causation, and this limits their ability to robustly identify causal associations. Therefore, we have utilized the Mendelian randomization approach to determine the magnitude of causal relationships between elevated Hcy levels and fracture risk. Mendelian randomisation provides a method to assess the causal nature of some environmental exposures, by studying an association between a disease and a DNA polymorphism that mimics the biological link between a proposed exposure and disease. To examine whether Hcy levels have a causal relation to fracture risk, we studied polymorphisms known to alter the biological availability of a dietary B vitamin or the activity of enzymes that affect the Hcy levels. We examined the effects of these polymorphisms on different aspects of bone health.

The most extensively studied polymorphism associated with variability in Hcy level is the MTHFR C677T polymorphism. This variant results in an alanine (Ala) to valine (Val) substitution at position 222 of the MTHFR protein, giving rise to a thermolabile enzyme with reduced activity¹³. The MTHFR C677T genotype has been associated

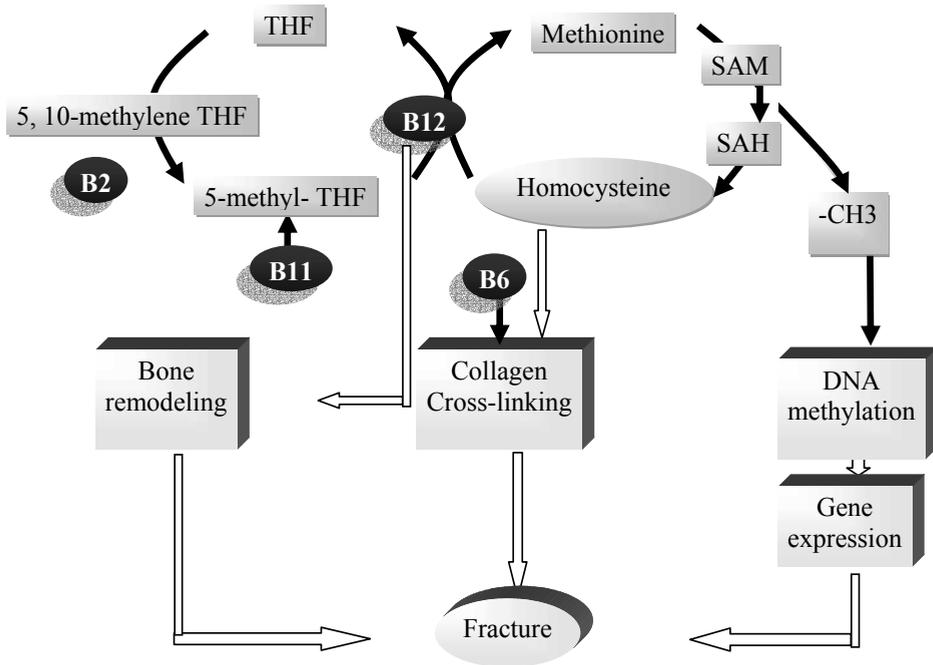


Figure 1. Possible biological mechanisms explain how elevated Hcy levels are related to fracture risk.

B2 (riboflavin), B6 (pyridoxine), B11 (folate), B12 (cobalamin): B vitamins

THF=Tetrahydrofolate, SAM:S-adenosylmethionine, SAH: S-adenosyl-homocysteine, CH3: methyl

with increased fracture risk¹⁴, and weakly with reduced BMD, in some studies¹⁵⁻¹⁷, while, other studies have identified interactions with diet^{14,16,18}.

In order to be active, MTHFR needs to bind to a co-factor, flavin adenine dinucleotide (FAD), a derivative of riboflavin (vitamin B2)¹⁹. The MTHFR enzyme converts 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (5MTHF), which is required for remethylation of Hcy to methionine (see Figure 1). The Val allele of the MTHFR Ala222Val polymorphism has less binding capacity for the FAD, thereby reducing the enzyme activity especially in case of shortage of riboflavin. Previous studies have shown that the MTHFR 677 T allele is only associated with higher Hcy levels if folate and/or riboflavin is low^{20,21}. Given this background it was reasonable to examine the association of MTHFR C677T in greater detail in relation to dietary intakes of B vitamins. Chapter 4 presents an examination in the Rotterdam Study in which we observed the MTHFR Ala222Val to interact with low dietary riboflavin intake to increase fracture risk in women. This increased fracture risk was independent of BMD or other known risk factors for fracture or potential confounders such as age, dementia, cardiovascular disease and other measures of co-morbidity. This suggests that an elevated Hcy level caused by the MTHFR 222ValVal homozygous

genotype in combination with low dietary riboflavin intake is a risk factor predicting fracture independently of other known risk factors.

Our results did not prove that elevated Hcy is the cause of the observed associations with increased fracture risk. In a small subgroup for whom Hcy levels were available, we found that adjustment of the increased fracture risk for Hcy levels did not affect the risk estimates.

In order to get more insight into the potential underlying mechanism relating elevated Hcy levels to fracture risk, we studied another gene, the MTR gene, involved in the remethylation of Hcy (chapter 4). The MTR gene is located on chromosome 1q43 and uses both vitamin B12 as a co-factor and 5-methyltetrahydrofolate as a methyl donor to catalyze the remethylation of homocysteine. This enzymatic step is fully dependent on vitamin B12.

The MTR A2756G polymorphism results in an (Asp) to (Gly) substitution at position 919 of the protein and has been reported to result in a small decrease in Hcy levels²²⁻²⁴. In our study the MTR 919GlyGly homozygous genotype was associated with 5.1% lower BMD and 2.2 times higher fracture risk in women. The effect of the MTR 919 Gly allele on fracture risk was stronger in the presence of low dietary cobalamin (below median) (vitamin B12) intake. This might suggest that the MTR 919GlyGly genotype in combination with low dietary cobalamin intake is a risk factor predicting fracture independently of an elevated Hcy level. One explanation is that cobalamin may play a more dominant role in bone health compared with Hcy levels. However, our study had limited statistical power because of the low frequency of the MTR 919GlyGly genotype (3.1%). Hence, these findings need to be confirmed in other large populations, before a final conclusion can be drawn.

Genetic and dietary determinants of Hcy level

From twin studies the heritability of Hcy levels has been estimated at 47-57%^{25,26}. Studies often have indicated that the MTHFR C677T polymorphism accounts for 4-9% of variations in plasma Hcy levels²⁶⁻²⁹. Therefore, this leaves a major part of the genetic contribution in the variation of Hcy levels unexplained, which is the rationale to continue the search for determinants of Hcy, as described in chapter 5 of this thesis. Based on the literature and biological background we selected the most promising potentially functional genetic variants involved in Hcy metabolism at the time the work on this thesis started. Our selection include MTHFR C677T (Ala222Val), MTHFR A1298C (Glu429Ala)³⁰, MTR A2756G (Asp919Gly)²⁴, CBS 844ins68 (68-bp insertion)³¹, MTRR A66G (Met22Ile)³², TCN C776G (Pro259Arg)^{33,34}, RFC G80A (His27Arg)³⁵, and the THYMS 28-bp repeat in the 5' UTR³⁶. We show that variance in Hcy levels

among subjects were significantly associated with the MTHFR 677T, the MTR 2756G, and the RFC 80A variants. The Hcy metabolism is complex, and multiple genes are involved that encode several key enzymes involved in its pathway. In this light we applied a multi-locus analysis to identify interacting polymorphisms that contribute to explain variation in Hcy levels together with related B vitamin co-factors (chapter 5). We identified interactions between the MTHFR 677T and RFC 80 A variants with low folate intake determining Hcy levels.

Our study is limited because we analysed only eight DNA variants in seven genes. There are for example, other genetic variants located in the genes involved in Hcy transsulfuration or the remethylation pathway which also deserve more attention. Recently, in a large study population of approximately 10,000 subjects 13 polymorphisms involved in Hcy metabolism and related B vitamin co-factors or metabolites were examined ²³. Among those, only four variants were associated with Hcy levels including the MTHFR 677T and MTR 2756G, which were also observed to be associated to Hcy levels in our study population (chapter 5).

In addition to the variants that are described in this thesis, which have been chosen based on the most promising genetic variants in the Hcy metabolism at the time the work on this thesis was started, other variants may also affect Hcy levels. For example, betaine-homocysteine methyltransferase (BHMT) G716A (Arg 239 Gln) ³⁷, and the nicotinamide N-methyltransferase gene (NNMT) ³⁸ could be candidates for further studies.

Candidate genes involved in regulating the bone matrix

A large number of studies have explored the potential associations between candidate gene polymorphisms and various osteoporosis-related phenotypes. The MTHFR C677T is involved in bone metabolism (via influencing Hcy levels) and collagen type I alpha 1 (COL1A1) is the most abundant protein of bone matrix. Both have been considered as candidate genes for osteoporosis by influencing bone quality rather than quantity. Therefore, in this thesis, besides the polymorphisms involved in Hcy metabolism we examined COL1A1 polymorphisms to examine the relationship between each of the two polymorphisms in the COL1A1 with bone end points. The COL1A1 gene, which encodes the alpha I chain of type 1 collagen, is one of the most extensively studied candidate genes for susceptibility to osteoporosis ^{39,40}. Rare mutations in this gene cause osteogenesis imperfecta, a Mendelian disorder presenting with moderate to severe bone fragility ^{41,42}. A polymorphism affecting an Sp1 binding site within a key regulatory region of COL1A1 ⁴³ has been reported to be associated with BMD and susceptibility to osteoporotic fracture ⁴⁴⁻⁴⁷.

Studies have indicated that the COLIA1 Sp1 polymorphism, located in the first intron of the gene, is a functional variant that affects bone quality rather than mineralization³⁹. In keeping with this, the association with fracture in many studies has been found to be independent of BMD⁴⁰. Yet, there are several other polymorphisms in the COLIA1 gene, including several in the promoter region that are potentially functional⁴⁸. This study showed that the -1997 G/T polymorphism located in the 5'promoter region of COLIA1 gene was associated with BMD in postmenopausal women. However, fractures were not analyzed in this study⁴⁸. Given the potential importance of the COLIA1 gene as a genetic marker of osteoporosis outcomes, we investigated the influence of both COLIA1 polymorphisms independently and in the form of haplotypes in relation to femoral neck BMD, changes in BMD, and risk of fracture (chapter 6). We observed an increased risk of fragility fractures in female carriers of the COLIA1 Sp1 T-allele. In contrast, the -1997 G/T polymorphism by itself appears to have no influence on fracture or BMD in postmenopausal women. This suggests that the associations observed so far are explained by the Sp1 polymorphism and not by (LD with) the -1997 C/T polymorphism.

There is yet another polymorphism in the COLIA1 5'promoter region, the -1663 indelT variant, which was found to be correlated to fracture risk^{48,49}. In a small case-control study of Scottish women this variant was found to be increased in frequency among hip fracture cases and was associated with a 27.5% reduction in bone strength compared with the common haplotype⁵⁰. However, this observation needs replication in larger studies in order to confirm the results.

Possible biological mechanisms

Several biological mechanisms are available that could explain how elevated Hcy levels are related to fracture risk. Figure 1 shows these possible mechanisms, which include collagen cross-linking, DNA methylation, and bone remodeling.

Collagen cross-linking

A mechanistic role of high Hcy concentrations in a deteriorating bone metabolism was suggested early in patients with homocystinuria, a genetic disorder characterized by severely elevated Hcy levels and several clinical manifestations, including early onset of osteoporosis⁵¹. It was suggested that high Hcy concentrations may interfere with collagen cross-linking, resulting in poor quality of bone and increased susceptibility to fracture⁵².

However, several studies showed that higher levels of Hcy are correlated with excretion of higher levels of collagen cross-links^{7,53-55}. In a pilot study of 100 elderly

individuals from the Rotterdam Study (50 high and 50 low Hcy levels) we also found that high Hcy levels are not associated with lower levels of collagen cross-links per excreted collagen molecule. Instead, we found a tendency towards a higher number of collagen cross-links (presented in chapter 1). However, these are excreted bone resorption markers, and one expects the Hcy effect on bone (if real) to affect the quality of newly-formed bone, something that is not monitored effectively by any of the markers studied so far. In addition, we do not know the exact local concentration of Hcy at the environment of the active osteoid, and whether it is sufficient to interfere with collagen cross-links. Interestingly, Paschalis *et al.*⁵⁶ observed lower cross-links in fragile bone of osteoporotic patients specifically in the micro-environment of the bone forming surfaces, which would support this hypothesis. In addition, Saito *et al.*⁵⁵ recently showed that bone tissue of fracture cases had a lower amount of cross-links and increased Hcy levels as compared to controls⁵⁵.

Furthermore, there is a possible effect of pyridoxine on bone, as referred to earlier in this discussion. Some reports suggest that pyridoxine deficiency may lead to impaired cross-link formation, because pyridoxine serves as an essential co-factor for lysyl oxidase, therefore resulting in increased bone fragility.

Bone remodelling

It has been suggested that Hcy may have a direct effect on bone by stimulating osteoclast formation and osteoclast activity *in vitro*. Thus, high Hcy levels might be associated with higher bone resorption⁵⁷ resulting in a disturbance of bone remodeling which, in turn leads to more fragile bone. Accordingly, Koh *et al.*⁵⁸ showed a direct activation of osteoclast formation and activity by elevated Hcy levels. This is in line with our observation that women with the MTHFR 222 Val/Val homozygous genotype had a higher BMD change (possibly reflecting higher osteoclast activity) especially when dietary riboflavin (vitamin B2) intake was low (chapter 4.2).

However, another *in vitro* study⁵⁹ suggested that elevated Hcy levels decreases osteoblast activity. They studied Hcy at high doses (100 and 500 μMol) and demonstrated that elevated Hcy levels inhibits production of osteocalcin, however it is questionable whether Hcy levels within normal or mildly elevated range (16-30 μMol) has an effect on osteoblast function.

Hcy levels are also closely linked to cobalamin status, and cobalamin might be directly involved in bone remodeling⁶⁰. Evidence for such an association between cobalamin and bone remodelling emerges from *in vivo* studies. In a study by Carmel *et al.* patients with cobalamin deficiency were found to have low levels of serum osteocalcin. Osteocalcin is one of the most abundant non-collagenous protein in bone, and cobalamin treatment increases serum osteocalcin levels⁶⁰, reflecting a

higher bone turn over in these patients. Higher bone turn over indicates impaired bone remodelling resulting in fragile bone and susceptible to fracture.

Recently, experimental studies by Herrmann *et al.* ^{57,61} have been carried out to clarify the role of high Hcy level in bone. They suggested that Hcy may have a direct effect on bone by stimulating osteoclast formation and osteoclast activity *in vitro*. They support the hypothesis of a Hcy-induced stimulation of bone resorption and decreased bone formation by cell culture experiments. They reported that Hcy induced a dose-dependent increase in osteoclast activity and therefore, with higher bone resorption ⁵⁷ resulting in disturbance of bone remodeling which in turn leads to more fragile bone. Moreover, in a recent study conducted by the same group the authors hypothesized that elevated Hcy levels might be associated with higher bone turnover marker. They reported that in the presence of high Hcy levels osteocalcin levels decrease (up to 34%), suggesting decreased osteoblast activity in the presence of an unchanged osteoclast activity. Their results suggest a mechanistic role of Hcy in bone metabolism. Since these studies are *in vitro* and we do not know whether the elevated Hcy levels created in these experimental studies are lethal for human bone cells or not, this issue needs to be investigated *in vivo* (human cells).

Additional biochemical data within larger sample sizes are needed to establish if Hcy truly interacts with extra-cellular matrix proteins leading to structural changes and a reduced bone stability. Therefore, studies on animal and cell culture experiment are needed to clarify the mechanistic role of Hcy in bone metabolism.

DNA methylation

As is known from the Hcy metabolic pathway (Figure 1) Hcy is produced as a result of methylation reactions. Thus, higher Hcy levels in serum could reflect the methylation status. In that respect elevated Hcy levels may be an indirect indicator of elevated S-adenosylhomocysteine (SAH) and compromised cellular methylation capacity. It has been shown that higher circulating SAH levels associated with decreased methylation of lymphocyte DNA ^{62,63}. In addition, folate treatment has been shown to restore DNA methylation to normal levels and correct the patterns of abnormal gene expression both in animal models ⁶⁴ as well as in humans ⁶⁵. Thus, the decreased methylation associated with high Hcy levels may alter gene regulation, which could eventually lead to disease. This hypothesis was supported by studying a gene, Proliferation Associated SNF2-like Gene (PASG), showed that disruption of a protein that facilitates DNA methylation, causes global hypomethylation, developmental growth retardation and a premature aging phenotype, including osteoporosis ^{66,67}.

Clinical implications of the findings in this thesis

Based on our results (chapter 4.1) one could argue that 5% of fragility fractures (or 2.3% of all incident osteoporotic fractures) in our female MTHFR 222 ValVal homozygous genotype population could be prevented by receiving supplementary riboflavin. The proportion of subjects with a fracture that could be prevented is much higher in females with the MTHFR 222 ValVal homozygous genotype with low riboflavin intake, where 17% of fragility and 9% of all incident osteoporotic fractures might be prevented by riboflavin supplementation. Thus, our findings provide novel options for the possible prevention of osteoporosis, and also suggest an intervention strategy.

The MTHFR 222ValVal homozygous genotype is quite frequent in the Caucasian population (10%) and our results on the gene-environment interaction suggest the possible importance of providing vitamin B2 (e. g., by fortification of food or by supplementation) in the Netherlands. Thus, our findings provide a clinical rationale to consider the role of riboflavin supplementation in the prevention of osteoporosis in the elderly. Randomized double-blind placebo-controlled trials are needed to confirm the suggested preventive effect of riboflavin on fractures in elderly, in particular those with the MTHFR 222 ValVal genotype.

Moreover, the observed association between high dietary pyridoxine intake and low fracture risk suggests that pyridoxine may play a role in bone health. However, our data do not provide compelling evidence to support advice concerning the optimal overall pyridoxine intake in the elderly population. Therefore, it might be useful to perform a randomized double-blind placebo-controlled trials to confirm the protective effect of pyridoxine in the elderly in relation to fracture risk.

Thus, the findings of our studies (chapters 3 and 4) have some potential clinical implications. The identification of the polymorphisms that explain population variance in Hcy metabolism and their possible interaction with related B vitamin co-factors might lead to insights regarding the etiology of osteoporotic fracture, and may also provide preventive targets.

Future Studies

In this thesis we focused on variants in genes that are expected to be involved in Hcy metabolism. However, we studied only seven genes, and only a few variants per gene. Yet, for each gene many more (functional) variants exist and many more genes are likely to be involved in the Hcy metabolism. In future studies, the role of other polymorphisms regulating the enzymes and co-factors in this pathway should also

be investigated. In particular, studying a possible biologically plausible interaction of these polymorphisms with related co-factors for the relevant enzymes is certainly of interest.

Moreover, because most of our analyses were based on a single-center study and statistical power is limited to identify the contribution of all relevant candidate genes. Therefore, international multi-center studies collecting data on DNA and Hcy status are necessary. In addition, such large studies are necessary to reveal other polymorphic enzymes involved in Hcy metabolism but with (even) smaller effects. Finally, larger studies are required to obtain sufficient interactions between these gene and dietary factors.

1- Genome wide association

Due to the rapid developments in the genotyping technology, we are now capable of assessing more than 500,000 single nucleotide polymorphisms (SNPs) in a single DNA sample. This SNP-chip technology will allow us to evaluate most of all existing variations in the human genome in relation to disease, or particular outcomes such as Hcy level. Interestingly, the Rotterdam Study will soon be screened with these high-density chips. This will allow to identify new pathways in relation to osteoporosis, and identification of novel genes in the Hcy pathway that are involved in osteoporosis. Yet, a candidate gene approach still would be the next step to confirm any novel region, including replication of association across several similar cohorts.

The candidate gene approach offers an important tool to assess genotype-phenotype relationships, and benefits from well-founded assumptions related to biological plausibility of the genes, comparatively low cost, and ease of analysis. Given the wealth of knowledge on the Hcy metabolic pathway this system offers excellent opportunities to study gene-environment interactions. The Hcy pathway is a well recognized and studied pathway and epidemiological and clinical studies have documented that nutrient intakes and their deficiency are an important cause of increased Hcy levels. Therefore, another interesting line of research in the future would be to study gene-environment interaction especially for the Hcy pathway.

2- Randomized double-blind placebo-controlled trial

A randomized double-blind placebo-controlled trial is needed to further elucidate the role of the four B vitamins (B2, B6, B12, and folate) in bone health. Since a common cause of elevated Hcy levels is poor B vitamins status, most notably of vitamin B12 and folate, which together with Hcy are involved in the biological mechanism linking B vitamin status to fractures, we would suggest to examine first vitamin B12 and folate.

Furthermore, the individuals who are at high risk of fracture (for example elderly ≥ 70 years living at nursery home) with initial high Hcy levels ($\geq 15 \mu\text{mol/l}$), would be randomly selected in this trial to receive vitamin B12 and folate or placebo, after which Hcy levels, BMD and fracture will be systematically assessed.

3-System biology and Multi-locus approach

In complex phenotypes such as osteoporosis, fracture risk and Hcy level, multiple genes generally act through complex networks involving gene-gene and gene-environment interaction. Accordingly, variants in each gene explain only a limited proportion of the phenotype thereby, making it difficult to define to study which gene and how to assess such interaction. Therefore, multi-locus analysis is a logical approach to study the relation between Hcy pathway and bone health. Application of multi-locus analysis allows identification and ranking of interacting polymorphisms and environmental factors that most prominently contribute to the variation of increasing Hcy levels.

System biology seeks to integrate different levels of information to understand how biological systems function by the study of the relationships and interactions between its various parts. It offers the potential to understand the complexity all factors determining Hcy levels in relation to osteoporosis. There is much knowledge about the Hcy metabolism which makes this approach possible. Especially, after the implementation of genome wide association to identify the most important genetic factors system biology in research on Hcy metabolism and bone health will be important. For example to help not only in the selection of suitable candidate genes that can be studied simultaneously, but also to evaluate and rank candidate genes in regions linked or associated to Hcy levels in relation to osteoporosis.

The complexity of the Hcy pathway illustrates the necessity for considering not only one gene, but multiple genetic polymorphisms of the enzymes and cofactors currently known to be involved in the Hcy metabolism. Finally, such combined genetic effects of the most prominent genes involved in the Hcy pathway should be studied *in vitro* and *in vivo* test systems to understand the underlying biological mechanism.

5- Recommendations

Several recommendations can be made based on the present thesis. First, more attention should be paid to the identification of the most prominent genes involved in the complex Hcy pathway. Second, progress in this area can be expected from the genome wide association (GWA) studies (and replication effects) focussed on Hcy level and related endpoints. Third, there is a need to understand the underlying

biological mechanisms in order to implement targeted intervention by combining genetic information with requirements for specific co-factors.

Fourth, placebo controlled randomized trials are advised to evaluate possible cause-effect relationship between B vitamins and fracture risk. Such studies are likely to profit from genotype-based patient selection based on the gene-environment interaction we have documented. Yet, given the relatively modest effect for each variant large confirmation studies are mandatory. In the end personalized nutritional advice and the use of dietary supplements which are based on genetic information that is related to the requirements for specific co-factors, might help to reduce the incidence of osteoporotic fracture in the individual.

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Osteoporosis is a skeletal disorder which predisposes a person to increased risk of fracture. Recently, a mildly elevated homocysteine (Hcy) levels has been found to predict fracture risk independent of bone mineral density (BMD) and other known risk factors. Since this finding, different nutritional determinants of Hcy, i.e. the vitamins B2 (riboflavin), B6 (pyridoxine), B12 (cobalamin) and B11 (folate), have been investigated in relation to bone health. Despite these investigations, the pathogenesis underlying the association between Hcy and fracture risk is not fully understood.

The general aim of this thesis was to study the association between Hcy and osteoporosis by examining genetic and dietary determinants of Hcy status in relation to bone health. All the studies were performed within the Rotterdam Study, a population-based cohort study among 7,983 individuals aged 55 years and older.

Chapter 1 offers a general introduction to the studies and topics dealt within this thesis.

In chapter 2 we present a systematic review of the previously published studies on the relationship between Hcy levels, related B vitamins and bone phenotypes. Based on this systematic review it is still difficult to draw any definite conclusion due to heterogeneity in the methods of assessment and lack of a standardized threshold for categorizing low B-vitamin status. However, there are convincing data to support an association between Hcy levels and fracture, and also data were found that suggest an association between BMD and vitamin B12 and folate status.

In chapter 3 the relationship between dietary B vitamin intake and bone phenotypes was investigated. It was shown that increased dietary riboflavin and pyridoxine intake are associated with higher BMD. Furthermore, we reported that risk of fracture is reduced when dietary pyridoxine intake is high; this relationship was independent of BMD or co-morbidity.

Chapter 4 consists of two studies investigating the association between two polymorphisms involved in Hcy remethylation and bone phenotypes.

In chapter 4.1 we show that the MTHFR C677T variant interacts with dietary riboflavin intake to influence fracture risk in women. It was observed that neither the MTHFR C677T polymorphism nor low riboflavin intake alone was associated with fracture risk or BMD. However, in the lowest quartile of riboflavin intake, female 677-T homozygotes had a higher risk for incident fractures. Furthermore, in the lowest quartile of dietary riboflavin intake, T-homozygous individuals (men and women combined) had higher Hcy levels compared with C-homozygotes. These results do

not, however, prove that elevated Hcy is the cause of the observed associations: In a subgroup for whom Hcy levels were available, adjustment of the increased fracture risk for homocysteine levels did not affect the risk estimates.

Chapter 4.2 describes the association between the MTR A2756G polymorphism, BMD, bone loss and fracture risk in women. We found that the G allele of the MTR A2756G polymorphism was associated with lower femoral neck BMD, and with lower lumbar spine BMD. Male individuals with the GG genotype had a higher rate of BMD loss compared with the AA genotype. A similar trend was observed in women, but did not reach significance. The MTR A2756G GG women had a more than 200% higher risk for fragility fracture compared to women with the AA genotype. This increased risk of fracture seemed to be dependent on dietary B12 intake. Only in the presence of low dietary cobalamin intake did female MTR 2756-G homozygotes have a markedly increased risk for fragility fracture. Since this is a single center study and because the risk genotype has a low frequency in the population (3%), these findings need to be replicated in other large cohorts.

The studies described in chapter 4 underline the influence of gene-environment interactions in the Hcy metabolism.

In chapter 5, we address the association between Hcy levels, eight polymorphisms in enzymes and proteins and dietary vitamin B intake involved in Hcy metabolism in a multivariate analysis model. It is shown that MTHFR C677T, MTR A2756G and RFC G80A influence Hcy levels in our study population. Furthermore, at baseline the MTHFR C677T polymorphism, dietary folate intake and dietary protein intake are the strongest independent predictors of Hcy levels after age and gender in our cohort. Interestingly, we observed an interaction between the MTHFR C677T and RFC G80A polymorphisms with low dietary folate intake in determining Hcy levels among the other studied variants and dietary intakes in our population. The study described in this chapter illustrates that Hcy metabolism is complex, resulting not only from underlying genetic variability and variability in environmental factors, but also from interaction between these factors.

Chapter 6 describes the effect of one of the most prominent candidate genes in osteoporosis, the COL1A1, which has been consistently associated with osteoporosis in different populations. We show that the COL1A1 Sp1 polymorphism is associated with BMD and fracture. However, in contrast to earlier reports from relatively small study populations, the -1997 G/T polymorphism showed no independent effect on fracture risk or BMD measures.

In the general discussion, chapter 7, the main findings of the thesis are addressed. The results indicate that Hcy is associated with fracture. However, due to the generally modest effects of genetic variations on Hcy levels, in addition to limited knowledge on the pathogenetic mechanisms by which Hcy influences bone health,

additional research in the molecular field is needed before one can draw any definite conclusion regarding causality. Nevertheless, our findings provide a clinical rationale for a riboflavin, pyridoxine, folate and B12 replete, if not supplemented, diet, in the aim to prevent osteoporosis in the elderly. Finally, our studies show that there is a need for better biological explanations in order to implement more targeted interventions. In the end personalized nutritional advice and the use of dietary supplements which are based on genetic information that is related to the requirements for specific co-factors, might help to reduce the incidence of osteoporotic fracture in the individual.

SAMENVATTING

Osteoporose (botontkalking) is een stoornis van het skelet dat iemand gevoeliger maakt voor het krijgen van fracturen. Recent is gebleken dat een mild verhoogde homocysteïne (Hcy) waarde een voorspellende waarde heeft voor de kans op fracturen, ongeacht de waarde van de botmineraal dichtheid (BMD) en andere bekende risicofactoren. Sinds deze bevinding zijn verschillende voedingsdeterminanten van Hcy onderzocht in relatie tot de gezondheid van het bot. De onderzochte determinanten zijn de volgende B-vitamines: B2 (riboflavine), B6 (pyridoxine), B12 (cobalamine) en B11 (foliumzuur). Ondanks deze studies wordt het onderliggende mechanisme van de associatie tussen Hcy en fractuurrisico nog onvolledig begrepen.

Het doel van dit proefschrift was de associatie tussen Hcy en osteoporose te bestuderen middels het bestuderen van de relatie tussen determinanten van Hcy (zowel genetische als voedings determinanten) en de gezondheid van het bot. Alle studies zijn uitgevoerd in de Rotterdam Studie, een groot bevolkingsonderzoek onder ongeveer 8000 mensen van 55 jaar en ouder die allen in de wijk Ommoord in Rotterdam woonden op het moment dat de studie begon.

Hoofdstuk 1 biedt een algemene introductie in de studies en onderwerpen die in dit proefschrift worden besproken.

In hoofdstuk 2 presenteren wij een systematische overzicht van de gepubliceerde studies over de relatie tussen Hcy waarden, gerelateerde B vitamines en botfenotypes. Gebaseerd op deze systematische review is het nog steeds moeilijk om een definitieve conclusie te trekken over de relatie tussen B-vitamines en gezondheid van het bot. Dit komt met name door de verschillende methodes die gebruikt zijn voor de statistische analyse en het bepalen van de B-vitamine status. Echter, er is overtuigende data beschikbaar die een associatie ondersteunt tussen de waarde van Hcy en fracturen en tevens is er data beschikbaar dat wijst op een verband tussen BMD en vitamine B12 en folaat status legt.

In hoofdstuk 3 werd de relatie tussen inname van vitamine B via het dieet en botfenotypes onderzocht. Hierin werd getoond dat een verhoogde inname van riboflavine en een hogere inname van pyridoxine geassocieerd zijn met een hogere BMD. Daarnaast rapporteerden wij dat het fractuurrisico gereduceerd is bij een hoge inname van pyridoxine; deze relatie was onafhankelijk van de BMD of co-morbiditeit.

Hoofdstuk 4 bestaat uit twee studies waarin de associatie tussen twee polymorfismen, welke betrokken zijn bij remethylering van het Hcy, en botfenotypes werd onderzocht.

In hoofdstuk 4.1 tonen wij dat de MTHFR C677T variant interactie vertoont met riboflavine inname en zo het fractuurrisico in vrouwen beïnvloedt. Er werd gezien dat zowel het MTHFR C677T polymorfisme, als een lage riboflavine inname niet geassocieerd was met het fractuurrisico of de BMD wanneer ze los van elkaar werden bestudeerd. Echter, in het laagste kwartiel van riboflavine inname, hadden vrouwelijke 677-T homozygoten een hoger risico op incidente fracturen. Ook hadden dezelfde vrouwen hogere Hcy waarden vergeleken met C-homozygoten. Deze resultaten bewijzen echter niet dat een verhoogd Hcy de oorzaak is van de geobserveerde associaties: in een subgroep van mensen voor wie Hcy waarden beschikbaar waren, beïnvloedde correctie van het verhoogde fractuurrisico voor verschillen in homocysteïne waarden de risicoschatting niet.

Hoofdstuk 4.2 beschrijft de associatie tussen een DNA variant in het gen wat codeert voor het enzyme methionine synthase (MTR A2756G) en gezondheidskenmerken van het bot, zoals BMD, botverlies en fractuurrisico. Wij vonden dat het G allel van het MTR A2756G polymorfisme geassocieerd was met een lagere BMD in de femurhals en in de lumbale wervelkolom. Mannen met het GG genotype hadden een hogere snelheid van BMD verlies vergeleken met het AA genotype. Een gelijksoortige trend werd geobserveerd in vrouwen, maar bereikte het significantieniveau niet. De MTR A2756G GG vrouwen hadden een meer dan 200% hoger risico op een fractuur vergeleken met vrouwen met het AA genotype. Dit verhoogde fractuurrisico leek afhankelijk te zijn van diëtaire B12 inname. Alleen in de aanwezigheid van een lage diëtaire cobalamine inname hadden deze vrouwen een duidelijk verhoogd risico op fracturen. Aangezien het risico genotype een lage frequentie in de populatie heeft (3%), dienen deze bevindingen in andere grote cohorten gerepliceerd te worden. De studies die beschreven worden in hoofdstuk 4 onderstrepen het belang van gen-omgeving interacties in het Hcy metabolisme.

In hoofdstuk 5 bespreken wij de associatie tussen Hcy waarden, 8 polymorfismen in enzymen en diëtaire inname van Hcy-gerelateerde B vitamines. Hierin wordt getoond dat 3 polymorfismen Hcy waarden in onze studiepopulatie beïnvloeden: MTHFR C677T, MTR A2756G en RFC G80A. Verder zijn, na leeftijd en geslacht, het MTHFR C677T polymorfisme, folaat inname en eiwit inname de sterkste onafhankelijke voorspellers van Hcy waarden. Wij zagen bovendien een interactie tussen zowel MTHFR C677T als het RFC G80A polymorfisme met een lage diëtaire folaat inname in het bepalen van Hcy waarden. De studie die in dit hoofdstuk wordt beschreven is illustratief voor de complexiteit van het Hcy metabolisme, wat niet enkel veroorzaakt wordt door de onderliggende genetische variabiliteit en verschillen in omgevingsfactoren, maar ook door interactie tussen deze factoren.

Hoofdstuk 6 beschrijft het effect van één van de meest prominente kandidaatgenen in osteoporose, het COL1A1, welke consistent geassocieerd is met osteoporose

in verschillende populaties. Wij laten zien dat het COL1A1 Sp1 polymorfisme geassocieerd is met BMD en fracturen. Maar, in tegenstelling tot eerdere bevindingen uit relatief kleine studiepopulaties, liet het -1997 G/T polymorfisme geen onafhankelijk effect op fractuurrisico of BMD metingen zien.

In de algemene discussie, hoofdstuk 7, worden de hoofdbevindingen van dit proefschrift besproken. De resultaten wijzen erop dat Hcy geassocieerd is met fracturen. Echter, vanwege het over het algemeen bescheiden effect van genetische variaties op Hcy waarden, maar ook vanwege de beperkte kennis over het pathogenetisch mechanisme over de manier hoe Hcy de gezondheid van het bot beïnvloedt, is aanvullend onderzoek in het moleculaire vakgebied nodig voordat men conclusies kan trekken over causaliteit. Desalniettemin geven onze bevindingen een klinische rationale voor een aanvulling van riboflavine en pyridoxine in het dieet, met als doel het voorkomen van osteoporose in ouderen. Tot slot laten onze studies zien dat betere biologische verklaringen nodig zijn om meer gerichte interventies te kunnen implementeren. Concluderend zouden persoonlijk voedingsadvies en het gebruik van voedingssupplementen, gebaseerd op genetische informatie die gerelateerd is aan de benodigdheden van specifieke co-factoren, de incidentie van osteoporotische fracturen mogelijk

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LIST OF PUBLICATIONS

Nahid Yazdanpanah, André G. Uitterlinden, Jan Lindemans, Huibert A.P. Pols, John P. A. Ioannidis, and Joyce B.J. van Meurs. The association between homocysteine, related B vitamins and bone health: a systematic review and a meta-analysis.

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