The Interplay of Genes and Diet in Metabolic Diseases and Aging

Studies on Obesity, Osteoporosis and Survival

Maria Carola Zillikens

Acknowledgements

The studies presented in this thesis were conducted at the Department of Internal Medicine in collaboration with the Departments of Epidemiology and Clinical Genetics of the Erasmus Medical Center (MC) Rotterdam, The Netherlands. The Rotterdam Study was funded by the Erasmus MC and Erasmus University, the Netherlands Genomics Initiative (NGI)/the Netherlands Organization for Scientific Research (NWO), the Netherlands Organization for the Health Research and Development (ZonMw), the Research Institute for Diseases in the Elderly (RIDE), the Ministry of Education, Culture and Science, the Ministry for Health, Welfare and Sports, the European Commission (DG XII), and the Municipality of Rotterdam. The Erasmus Rucphen Family (ERF) study was supported by grants from NWO, Erasmus MC and the Center for Medical Systems Biology (CMSB). The contribution of the participants, general practitioners, and pharmacists of the Rotterdam Study and the ERF study are gratefully acknowledged.

The studies described in Chapter 8 were performed within the Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) Consortium. CHARGE members include the Rotterdam Study (RS), the Framingham Heart Study (FHS), the Cardiovascular Health Study (CHS), the Atherosclerosis Risk in Communities (ARIC) Study, the Age, Gene/Environment Susceptibility (AGES) Reykjavik Study and the and the European Special Population Network (EUROSPAN).

The studies described in Chapter 9 were performed within the setting of the GEnetic Factors of OSteoporosis (GEFOS) consortium, including the Rotterdam Study, the ERF Study, the Twins UK (TUK) Study, the deCODE Genetics (dCG) Study and the Framingham Osteoporosis Study (FOS). The GEFOS consortium has been funded by the European Commission.

Financial support for publication of this thesis was kindly provided by Procter & Gamble, Andromedical Research, GE Healthcare Lunar, Nutricia Advanced Medical Nutrition, Ipsen, Servier, Novartis, Ely Lilly, AMGEN, Novo Nordisk, Schering-Plough, Sanofi Aventis, Boehringer Ingelheim, Pfizer, Genzyme and the Dutch Society for Calcium and Bone Metabolism (NVCB).

Layout and print: Optima Grafische Communicatie, Rotterdam
Cover: M.C. Zillikens
ISBN 978-90-8559-001-9

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De Wisselwerking tussen Genen en Voeding bij Metabole ziekten en Veroudering
Studies naar Obesitas, Osteoporose en Overleving

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.G. Schmidt
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 7 oktober om 15.30 uur

door

Maria Carola Zillikens

geboren te Arnhem

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. A.G. Uitterlinden
	Prof.dr. B.A. Oostra
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	Dr. E.J.G. Sijbrands

To my parents

To Lex, Rogier,
Celeste and Eline

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Chapter 1

Introduction and outline of the thesis

INTRODUCTION

Obesity and osteoporosis are common and complex disorders with important consequences for human health and for society. The two conditions are intimately linked, as evidenced by epidemiological studies showing that obesity protects from osteoporosis while low body weight poses a strong risk factor [1, 2]. Obesity, defined as a body mass index (BMI) of 30 kg/m^2 and over, has become a global epidemic and represents an important risk factor for type 2 diabetes mellitus, hypertension, cardiovascular disease, stroke, some types of cancer and disability. Osteoporosis, a skeletal disorder characterized by loss of bone strength and proneness to fractures, is a major health threat to hundreds of millions of elderly individuals worldwide and prevalence will continue to rise as populations age. Osteoporosis and obesity result from an interaction between genetic factors and environment. Although the growing prevalence of obesity is most likely driven by changing lifestyles encompassing increased caloric intake and decreased energy expenditure through physical activity, individual susceptibility varies widely and is strongly influenced by genetic factors. Heritability estimates for BMI, the most widely used parameter of obesity, range from 30 to 70% in family- and twin studies [3]. There are also genetic influences on obesity-related traits like total body fat mass, lean mass and measures of fat distribution [4-8], but the heritability of these parameters is less clear than that of BMI. A large contribution of genes has also been documented for the susceptibly to develop osteoporosis. Heritability estimates for bone mineral density (BMD), the most widely used parameter for osteoporosis, range between 50 and 85% [9]. The age-adjusted heritability of osteoporotic fractures is smaller (between 25 and 50%) and may be independent to that of BMD [10], possibly due to other important factors associated with fractures such as falls.

Sex Differences

Although the importance of sex as a key determinant in health and illness has been recognized for a long time [11], systematic studies of sex-differences in medicine are still rare. There is a well known sexual dimorphism in human body composition with adult males having greater body height, total lean mass and bone mineral density, and a lower fat mass than females [12]. Moreover, there are differences in fat distribution with males showing more upper body or so-called "android" or "central abdominal" distribution of adipose tissue and females a more peripheral or "gynoid" distribution with bigger hips and thighs.

Specific fat depots may confer differential metabolic risk. In particular, central abdominal fat may be more strongly associated with the development of metabolic risk factors and cardiovascular disease as compared with lower body subcutaneous fat [13-15]. Evidence

for sex-specific genetic effects on body composition, however, is scarce since few studies have systematically investigated potential sex-differences in the genetic architecture of body composition. This knowledge is important for the design of genetic studies and also for studies on the relationship of body composition with common diseases like cardiovascular disease and type 2 diabetes. Although the vast majority of studies on osteoporosis have been performed in women, sex differences are well known for example in disease prevalence and patterns of risk factors [16], while sex-specific genetic effects have been reported for BMD [17].

Relationship between obesity and osteoporosis

The incidence of both obesity and osteoporosis increases with age but, paradoxically we, obesity appears to protect from osteoporosis. There is a clear positive association between body weight and body mass index (BMI) with bone mineral density (BMD) as well as increased fracture risk with low BMI. [1, 2, 18]. Possible explanations for higher BMD in heavier people include the well known mechanical loading influence on bone health, i.e., the weight-bearing effect of both fat and lean mass, while lean mass is also thought to influence bone through muscle mediated effects of physical exercise. Body composition may also influence bone health through other physiological processes. For example, adipose tissue may influence BMD through the production of hormones and adipokines by adipocytes. There is ongoing controversy about the relative importance of the fat and lean components of the body to BMD [18, 19]. In postmenopausal women fat mass has been shown most consistently to be positively related to BMD, possibly mediated by higher estrogen levels, which are normally very low after menopause. However, several discrepancies can be found in the literature, e.g., fat mass being important in young but not in old women and in old but not in young men [20]. Some researchers even claim the existence of a detrimental effect of body fat on bone [21, 22].

The effect of fat distribution on BMD is far from clear. Studies on the relationship between body fat distribution and BMD have yielded conflicting results, probably because most of them included a small number of subjects and/or were not population based and used different techniques for measurement of BMD and fat distribution. Also, the effect of adiposity on the relationship between fat distribution and BMD was not always considered as a confounder, potentially leading to an overestimation of the effect of abdominal fat distribution on BMD.

Environmental influences

Important environmental (lifestyle) factors contributing to the development of obesity are excessive caloric intake and low physical activity. Interestingly, diet and physical

activity are also related to osteoporosis. Other lifestyle factors, such as a decreased exposure to sunlight (to ensure adequate production of vitamin D) and excessive use of cigarettes, alcohol and caffeine, can negatively influence BMD. Dietary factors that have been favourably associated with osteoporosis are the intake of calcium and vitamin D, but other nutrients may also play a role. These include the amount and type of proteins and the acid/base balance of the diet [23] and potentially the intake of boron, silicium and vitamin K, although hard evidence for the role of these nutrients is lacking [24]. Recently, mildly elevated homocysteine concentrations were identified as a novel and potentially modifiable risk factor for age-related osteoporotic fractures, independent of BMD [25, 26]. Homocysteine levels are influenced by nutritional factors, in particular by dietary intake of the B vitamins folate, riboflavin, pyridoxine and cobalamin, which serve as co-factors or substrates for the enzymes involved in the homocysteine metabolism. It is not known whether these B vitamins can lower the risk of osteoporotic fractures by decreasing homocysteine levels.

Genetic studies

Despite the high heritability of body mass index and bone mineral density, knowledge on the genetic background of obesity and osteoporosis is still very limited. Several approaches are used to find genes that are associated with common disease or traits including genome-wide linkage analysis, candidate genes analysis and genome-wide association studies (GWAS). The purpose of these genetic studies is providing new routes to understanding the aetiology of disease, as well as identifying tools for better disease prediction, prevention and treatment.

Genome-wide linkage analysis examines the whole genome in a hypothesis-free approach with a limited number (usually a few hundred) of genetic markers in populations of related individuals to identify an approximate chromosomal location of genetic variation related to a disease or trait. This technique has been very successful at the identification of the genetic variation underlying single-gene (monogenic/Mendelian) disorders but much less successful for common human diseases or traits. Monogenic disorders leading to early, severe, obesity affect the central regulation of appetite. These conditions are rare, except for mutations in the melanocortin 4 receptor (*MC4R*) that account for about 5% of morbidly obese patients (BMI > 40 kg/m^2)[27]. Monogenetic conditions with abnormal low BMD (e.g., osteogenesis imperfecta, osteoporosis pseudoglioma symdrome) or high BMD (e.g., pycnodysostosis, sclerosteosis and Van Buchem's disease) have led to the identifications of genes related to collagen production (e.g. collagen type 1A1 (*COL1A1*)), low-density lipoprotein receptor-related protein 5 (*LRP5*), *cathepsin K* and the sclerosteosis (*SOST*) gene[28-30]. Such genetic discoveries have

attracted commercial interest and resulted in the development of new medication for osteoporosis, currently being tested in clinical trials.

In the candidate gene approach a particular known gene of interest (e.g., based on predicted function) is assessed in a case control or population-based setting for association of one or more genetic variants in the gene with the trait or disease of interest. Many genes with plausible candidacy have been associated with measures of obesity and osteoporosis. The 2005 Human Obesity Gene Map update reports over 1.100 studies, including 550 genes, markers and chromosomal regions apparently linked to or associated with obesity phenotypes [27]. However, only very few of these associations have been replicated in independent large-scale studies. The same holds for osteoporosis, where many candidate gene association studies have been underpowered [31]. Since the launch of the Genetic Markers for Osteoporosis (GENOMOS) consortium in 2003, systematic replication has been performed in 18,000 - 45,000 subjects of several candidate genes including estrogen receptor alpha (*ESR1*) [32], collagen type 1A1 (*COL1A1*) [33], vitamin D receptor (*VDR*) [34], transforming growth factor beta 1 (*TGFB1*) [35] and low-density lipoprotein receptor-related protein 5 and 6 (*LRP5* and *LRP6*) [36]. These studies have convincingly established the association of *ESR1* with fractures and of *LRP5* with BMD and fractures.

Genome-wide association studies have only recently been introduced to the field of genetic epidemiology research, owing to developments like the completion of the reference sequence of the human genome [37] and the International HapMap [38]. Combined with recent advances in micro-array genotyping technology these developments have facilitated the large-scale assessment of common genetic variation in thousands of individuals [39]. During the past 2 years, more than 200 GWA studies have reproducibly described over 300 common genetic variants associated with common diseases and traits, albeit each explaining only a small fraction of the genetic variation[40, 41].

SIRT1 as an example of the candidate gene approach

An intriguing new candidate gene for metabolic diseases and survival is *SIRT1*, a conserved protein that belongs to the group of NAD^+-dependent histone deacetylases named Sirtuins, which are related to longevity in lower organisms like yeast, flies, and worms [42, 43]. SIRT1 has also been implicated in life span extension during caloric restriction in model organisms [44-46]. Dietary restriction is one of the most robust methods for extending lifespan and delaying age-related disease among various species, including yeast, flies and rodents and potentially non-human primates [47, 48]. SIRT1 protects cells against oxidative stress and ageing and it also has an important function in endocrine signalling, specifically in glucose and fat metabolism [49-53]. It interacts with peroxisome proliferator-activated receptor-gamma (PPARγ) to repress its transcriptional activity, leading to inhibition of adipogenesis during fasting and activation of lipolysis [49]. Treat-

ment of mice with resveratrol, a naturally occurring SIRT1 agonist present in red wine and grapes, was recently shown to prevent diet-induced obesity and insulin resistance and increase their survival [54, 55]. *SIRT1* is thus a strong candidate gene for obesity as well as for survival. Its activity may be modulated by dietary factors such as precursors for NAD^+, i.e., dietary niacin (vitamin B3) intake and nicotinamide riboside which was recently discovered as a SIRT stimulator present in milk [56]. But also antioxidant vitamins and caloric intake might in theory modify *SIRT1* expression or activity and thus interact with genetic variations in *SIRT1*. These potential interactions have not been studied in humans so far.

OUTLINE OF THE THESIS

In this thesis, epidemiological, genetic and nutritional studies are presented on the metabolic diseases obesity and osteoporosis, and on survival. In a family-based study, the heritability of body composition is estimated as well as the existence of sex-specific genetic effects. Then, studies are presented estimating the relationship body fat distribution and bone mineral density and between the intake of dietary B vitamins and bone mineral density and fractures. As an example of the candidate gene approach, studies are reported investigating the *SIRT1* gene in relation to obesity and survival and in interaction with diet. Last, genetic studies on obesity and osteoporosis are presented using the newest technique in gene finding, i.e., genome-wide association. In the general discussion the findings are placed into perspective and suggestions for future research are given. The thesis ends with a summary and conclusions.

Chapter 2 addresses the sex-specific heritability of various body composition parameters estimated by anthropometry and total body DXA in the Erasmus Rucphen Family (ERF) study. Based on the well-known sexual dimorphism in body composition it is hypothesized that there may be sex-specific genetic effects influencing these traits. A statistical tool is used, called variance decomposition procedures, to partition variation of body composition into genetic and environmental components common to both sexes and to men and women separately and the correlation is calculated between genetic components in men and women.

Chapter 3 presents the results of our study on the association between bone mineral density (BMD) and various measures of body fat distribution obtained by anthropometry and total body DXA in the ERF study. We also report whether body mass index (BMI) and serum insulin and adiponectin levels influence the relation between fat distribution and BMD.

Chapter 4 shows the relation between the intake of dietary homocysteine-related B vitamins (riboflavin, pyridoxine, folate and cobalamin) and BMD and the risk of fracture

in 5,304 elderly individuals from the large population-based cohort of the Rotterdam study. It is of clinical relevance to know if the intake of B vitamins associated with homocysteine levels can explain the relationship of homocysteine levels with fractures, because supplementing B vitamins in the elderly could be a low cost and probably safe measure to prevent osteoporotic fractures.

Chapter 5 describes the results of the analysis on the association between *SIRT1* genetic variation and BMI, the risk of obesity and longitudinal weight change in elderly subjects from the Rotterdam study and in subjects from the ERF study.

Chapter 6 presents the results of the study on the association of *SIRT1* genetic variation with mortality in elderly subjects from the Rotterdam study and additionally in subjects with increased oxidative stress (type 2 diabetes and smokers), where SIRT1 might be very important to protect cells against oxidative stress. In addition, we studied the interaction between *SIRT1* genetic variants and dietary intake of niacin (vitamin B3), a precursor for NAD^+ that SIRT1 needs.

Chapter 7 addresses a potential gene-diet interaction on BMI at the *SIRT1* locus in subjects from the Rotterdam study. Specifically, an interaction is investigated with dietary intake of energy, fat, calcium, milk, antioxidant vitamins (betacarotene, vitamin C and E) and niacin. Data in the subjects from the ERF study is used for replication of findings with milk intake.

Chapter 8 describes the results of a study using genome-wide association (GWA) to identify novel common variants influencing BMI and central abdominal fat as reflected in waist circumference (WC). GWA data on BMI and WC are meta-analysed in 31,373 individuals of Caucasian descent from eight population-based studies within the Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) consortium.

Chapter 9 reports a meta-analysis of GWA data on the lumbar spine and femoral neck BMD in 19,195 Caucasian subjects from five studies, within the setting of the GEnetic Factors of OSteoporosis (GEFOS) consortium with the purpose to identify novel loci influencing BMD.

Chapter 10 contains a general discussion and presents future perspectives

Chapter 11 concludes with a summary and conclusions of the thesis.

Chapter 12 presents the summary and conclusions in Dutch

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Chapter 2

Sex-specific genetic effects influence variation in body composition

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Diabetologia 2008;51:2233-41

ABSTRACT

Background Despite well-known sex differences in body composition it is not known whether sex-specific genetic or environmental effects contribute to these differences.
Methods We assessed body composition in 2,506 subjects from a young Dutch genetic isolate participating in the Erasmus Rucphen Family study by dual-energy X-ray absorptiometry and anthropometry. We used variance decomposition procedures to partition variation of body composition into genetic and environmental components common to both sexes and to men and women separately and calculated the correlation between genetic components in men and women.
Results After accounting for age, sex and inbreeding, heritability ranged from 0.39 for fat mass index to 0.84 for height. We found sex-specific genetic effects for fat percentage (fat%), lean mass, lean mass index (LMI), and fat distribution, but not for BMI and height. Genetic correlations between sexes were significantly different from 1 for fat%, lean mass, LMI, android fat, android:gynoid fat ratio and waist: hip ratio (WHR), indicating that there are sex-specific genes contributing to variation of these traits. Genetic variance was significantly higher in women for the waist-, hip- and thigh circumference and WHR, implying that genes account for more variance of fat distribution in women than in men. Environmental variance was significantly higher in men for android:gynoid fat ratio.
Conclusions Sex-specific genetic effects underlie sexual dimorphism in several body composition traits. The findings are relevant for studies on the relationship of body composition with common diseases like cardiovascular disease and type 2 diabetes and for genetic association studies.

ABBREVIATIONS

h^2	heritability
ρG	genetic correlation
σ	standard deviation
σE	environmental standard deviation
σE_F	environmental standard deviation female
σE_M	environmental standard deviation male
σG	genetic standard deviation
σG_F	genetic standard deviation female
σG_M	genetic standard deviation male
$\sigma^2 E$	environmental variance
$\sigma^2 E_F$	environmental variance female
$\sigma^2 E_M$	environmental variance male
$\sigma^2 G$	genetic variance
$\sigma^2 G_F$	genetic variance female
$\sigma^2 G_M$	genetic variance male
DXA	dual-energy x-ray absorptiometry
ERF	Erasmus Rucphen Family study
E × S	environment by sex interaction
fat%	fat percentage
FMI	fat mass index
G × S	genotype by sex interaction
LMI	lean mass index
WHR	waist:hip ratio
WTR	waist:thigh ratio

INTRODUCTION

Obesity has become a global epidemic and represents an important risk factor for type 2 diabetes mellitus, hypertension, cardiovascular disease, stroke, some types of cancer and disability. Although the growing prevalence of obesity is most probably caused by increasing energy intake and decreasing energy expenditure by physical activity, individual susceptibility varies widely and is strongly influenced by genetic factors. Twin, adoption and family studies have shown that between 40% – 80% of inter-individual variation of BMI is heritable [1-3]. There are also significant genetic influences on obesity-related traits like total body fat mass, lean mass and measures of fat distribution [4-8], but the heritability of these variables is less clear than that of BMI.

Although the importance of sex as a key determinant in health and illness has been recognized for a long time [9], systematic studies of sex differences in medicine are still lacking. There is a well-known sexual dimorphism in human body composition with adult men having greater body height, total lean mass and bone mineral mass, and a lower fat mass than women [10]. Moreover, there are differences in fat distribution with men showing more upper body or so-called "android" distribution of adipose tissue and women a more peripheral or "gynoid" distribution with bigger hips and thighs.

Given the large genetic effects on body composition, it is possible that different (although partly overlapping) genes contribute to variation in body composition in men and women and/or that the genetic determinants of body composition may be modulated by sex-specific hormonal, environmental, and nutritional factors. Despite its genetic determination, sex can also be considered an environmental factor that can modify both the penetrance and expressivity of a wide variety of traits [11, 12]. Evidence for sex-specific genetic effects (or a genotype by sex interaction) on body composition, however, is scarce since few studies have systematically investigated potential sex-differences in the genetic architecture of body composition. Studies on sex differences in heritability of BMI have shown inconsistent results. Some studies found evidence for a higher heritability of BMI in women than in men [2, 3, 12-14] or the other way around [15-17] or no difference [18, 19]. In the Diabetes Heart Study [7], heritability estimates were larger in women for BMI and lean mass but not for fat percentage (fat %) assessed by dual-energy x-ray absorptiometry (DXA). However, these differences were considered not significant considering the large standard errors. Sex-specific differences in heritability can be caused by differences in additive genetic variance, non-genetic (environmental) variance or total phenotypic variance. Using variance decomposition procedures, also known as variance components analysis, variation in traits can be partitioned into genetic and environmental components and genetic correlations can be calculated between men and women. Using this procedure, Comuzzie et al [20] found evidence for genotype by sex interaction for several anthropometric variables in Mexican Americans, but no data were available in this study on fat and lean mass.

The aim of the present study was to determine heritability of a large set of body composition variables determined by DXA and anthropometry in a large extended pedigree from a genetically isolated population in the Netherlands and to explore potential sex-specific differences in the relative influence of genetic and environmental factors on body composition. Specifically, we considered the following questions: (1) are there different genes that contribute to the variation of body composition in men and women (i.e. are there qualitative sex differences) and (2) is the magnitude of the genetic and/or environmental variation larger in one sex than in the other (i.e. are there quantitative sex differences in genetic and/or environmental variances)?

METHODS

Study population

This study was carried out within the Erasmus Rucphen Family (ERF) study, a family-based cohort study that is embedded in the Genetic Research in Isolated Populations (GRIP) program in the South West of the Netherlands. The aim of this program is to identify genetic risk factors in the development of complex disorders [21-23]. Genealogical records demonstrated that almost all of the inhabitants of this isolated population could be traced back to about 150 individuals who founded this community around 1750. The population is characterized by minimal immigration up until the last few decades. About 20,000 inhabitants are now scattered over eight adjacent villages. Genealogical information on this population was reconstructed using church and municipality records and is currently available in the form of a large database including over 63,000 individuals records.

For the ERF study, 22 families that had at least five children baptised in the community church between 1850 and 1900 were identified with the help of genealogical records. All living descendants of these couples and their spouses were invited to take part in the study. Data collection started in June 2002 and was finished in February 2005. In this study, we focused on the 2,506 participants for whom complete phenotypic and genealogical information was available. PEDSTATS [24] was used to produce summary statistics of the pedigree and correlations of the variables by pair type.

The Medical Ethics Committee of Erasmus Medical Center Rotterdam approved the study. Written informed consent was obtained from all participants.

Data collection

At the research center, located within the community, extensive clinical examinations were performed, including the collection of fasting blood samples, anthropometric measurements, and personal interviews. A research physician obtained information on medical history, medication use, smoking and alcohol use in a personal interview.

Anthropometric measurements

Height and weight were measured with the participant dressed in light underclothing. BMI was calculated from these data as weight (kg)/ height^2 (m^2). Circumferences of the waist, hip and thigh were measured on uncovered skin using a tape measure with the participant in the upright position. Waist circumference was measured halfway between the rib cage and the pelvic bone. Hip circumference was measured at the maximal

circumference of the hips. Thigh circumference was measured mid-way between the upper border of the patella and the inguinal fold on the right leg. The waist:hip ratio (WHR) and waist:thigh ratio (WTR) were calculated from these measurements. Figure 1a shows the sites of circumference measurements.

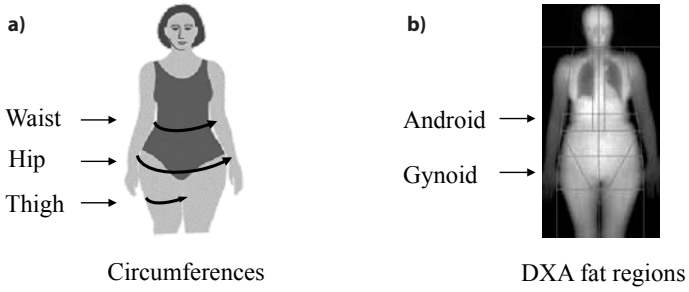

Figure 1
(a) The sites of the circumference measurements.
(b) The regions of "android" and "gynoid" fat assessed by DXA.

Dual-energy x-ray absorptiometry (DXA) measurements

DXA scans were performed using a Prodigy total-body fan-beam densitometer and analyzed with the encore 2005 software v.9.3 (DPX; Luna, Madison, WI, USA). Total body scans were auto-analyzed with this software, in which an algorithm that divides body measurements into areas corresponding to head, trunk, arms and legs is implemented. Total body fat mass (g), lean mass (g), and regional fat mass were obtained from total body scans. The trunk region was limited by an upper horizontal border below the chin (neck cut), vertical borders lateral to the ribs, and a lower border by the iliac crest. The arm region was limited by cuts that cross the arm sockets, as close to the body as possible and separate the arms and hands from the body. The leg region is limited above by the oblique lines passing through the hip joint, and cuts that separate the hands and forearms from the legs and a center leg cut which separates the right and left leg. Two additional regions were defined using the software provided by the manufacturer; the so-called "android" and "gynoid" region. The "android" region has a lower boundary at the pelvis cut and the upper boundary above the pelvis cut by 20% of the distance between the pelvis and neck cuts. The lateral boundaries are the arm cuts. The "gynoid region" has an upper boundary between the upper part of the greater trochanters and a lower boundary defined at a distance equal to twice the height of the "android region". The lateral boundaries are the outer leg cuts. The android and gynoid fat mass and android:gynoid fat ratio were calculated from these measurements. A schematic representation of the android and gynoid region is shown in Figure 1b. All analyzes were

verified by a trained technician who performed adjustments when necessary. Daily quality-assurance tests were performed with a calibration block supplied by the manufacturer. Repeated measurements on the calibration block had coefficients of variation less than 1%.

Definitions

Body weight = total fat mass + total lean mass + bone mineral content. Body mass index (BMI) = body weight (kg)/ height^2 (m^2). Fat mass index (FMI) = total fat mass (kg)/ height^2 (m^2). Lean mass index= total lean mass (kg)/ height^2 (m^2). Total body fat percentage (fat%): (total fat mass / body weight) x 100.

Statistical analysis

Baseline characteristics for men and women were compared using Mann-Whitney U test in SPSS for Windows, version 11.0. Inbreeding coefficients were computed with PEDIG software [25], using all the available genealogical information. Spearman correlations between inbreeding values and the body composition phenotypes were calculated using SPSS. General linear models were used to test phenotypic association between various body composition variables. In order to satisfy distributional assumptions, the phenotype distributions were normalized by natural log or square root transformation to ensure normally distributed residuals. A full pedigree-variance components approach based on maximum likelihood methods as implemented in the SOLAR 2.1.2 (Sequential Oligogenic Linkage Analysis Routines) software package was used to estimate the heritability of body composition variables [26]. Univariate quantitative genetic analysis was performed to partition the phenotypic variance of body composition variables into additive genetic and environmental variance components using maximum likelihood variance decomposition methods [27, 28].

The phenotypic variance of the body composition variables, which reflects the inter-individual variation, was partitioned into its additive genetic (σ^2G) and residual environmental (σ^2E) variance components [29]. Heritability was estimated as the ratio of the additive genetic variance to the sum of the additive genetic and environmental variance, that is including sources of residual variance as measurement error: h^2 = Additive σ^2G / (σ^2G + σ^2E). Dominance variance, which, in conjunction with additive and environmental variance, comprises broad sense heritability, was not estimated. Dominance effects are more easily modeled in twin than in family studies but they are difficult to model in extended pedigrees. Similar to the assumptions made in current genome-wide association studies, we assumed additive effects.

To evaluate sex-specific effects on the variation of body composition variables we used a standard sex-limitation modeling approach that allows testing for specific patterns of interaction, such as genotype by sex interaction (G x S) [20, 30-32]. The test for G x S interactions is based on hypotheses concerning the nature of the variance–covariance relationship of a trait between male–female relative pairs The expected genetic covariance (or shared heritability) between a male and female relative pair is defined as covariance (G_M, G_F) = $2f\,\rho G_{(M,F)}\,\sigma G_M\,\sigma G_F$ where f is the coefficient of kinship between the two individuals, $\rho G_{(M,F)}$ the genetic correlation between the expressions of the trait in the two sexes, and σG_M and σG_F the genetic SDs for men and women.

In the absence of a G x S interaction (i.e., the null hypothesis), the genetic correlation between male and female relative pairs should be 1 ($\rho G_{(M,F)} = 1.0$) and male and female genetic SDs will be equivalent ($\sigma G_M = \sigma G_F$). Conversely, if there is G x S interaction, the genetic correlation between the sexes, $\rho G_{(M,F)}$ will be significantly <1.0 (implying qualitative sex differences) and/or the genetic SDs will be significantly different between the sexes ($\sigma G_M \neq \sigma G_F$), implying quantitative sex differences in the genetic variance. Additionally, environment by sex interactions (E x S), or quantitative differences in the environmental variance, would be indicated by the environmental SDs not being equal between the sexes ($\sigma E_M \neq \sigma E_F$).

To evaluate G x S interactions influencing body composition traits, a full model was fitted in which ρG, σG_M, σG_F, σE_M and σE_F variables were freely estimated using maximum-likelihood methods. In addition, we fitted three nested models, in which one of these variables was constrained, as follows: model 1, in which the genetic correlation between men and women was constrained to one (ρG= 1); model 2 in which genetic SDs were constrained to be equal ($\sigma G_M = \sigma G_F$); and model 3, in which environmental SDs were constrained to be equal ($\sigma E_M = \sigma E_F$). Likelihood ratio tests (LRT) were used to test whether the nested models fit the data significantly worse than the full model. The LRT statistic follows an asymptotic X^2 distribution with 1 df when comparing the full model with the nested models, in which the SDs were constrained to be equal (full model against model 2; and full model against model 3). However, in the case of the model that restricted the genetic correlation to 1, this assumption does not hold since the genetic correlation was constrained to the upper boundary of the parameter space (ρG=1). As a result of this constraint, the test statistic is not distributed as an asymptotic χ_1^2 distribution, but rather as a 1/2:1/2 mixture of a χ_1^2 distribution and a point mass at zero [31-33].

Three basic inferences concerning the nature of sex-based interactions can be made. (1) rejection of the model constraining the genetic correlation between the groups to 1 ($\rho G_{(M,F)}$= 1.0) implies that different genes or a different subset of genes contribute to the variance in body composition variables in men and women; (2) rejection of the model constraining the genetic SDs of the groups to be equal ($\sigma G_M = \sigma G_F$) implies that the magnitude of the genetic effect is different between the two sexes; and (3) rejection of

the model constraining the environmental SDs of the groups to be equal ($\sigma E_M = \sigma E_F$) implies an interaction between the residual environment and the sex.

All analyses were adjusted for age and inbreeding quartiles (given that the distribution of inbreeding coefficients was not normally distributed) and for sex when heritability was estimated for both men and women.

RESULTS

Table 1 shows the number of pairs of relatives present in the large pedigree. The average number of generations in the pedigree used for analysis was 5.6 (range 4 - 7) and the average family size was 113.9 (range 34 - 268). The average number of women per family was 63.9 (range 18 - 145) and of men 50.1 (range 13 - 123).

Table 1. Number of relative pairs within the pedigree of the ERF study population

	Pairs				
	Total	Phenotyped subset	Female-Female	Male-Male	Opposite sex
Pedigree members	4,283	2,506			
Number of families	22	22			
Founders	1,389	479			
Non-founders	2.894	2,027			
Relative pairs					
Sib-pairs	3,436	1,454	459	285	710
Half-Sibs	101	53	19	8	26
Cousins	13,614	5,906	1,782	1,201	2,923
Parent-Child	5,788	1,560	508	303	749
Grandparent-Grandchild	5,556	156	61	23	72
Avuncular	10,741	2,851	883	611	1,357

Number of relative pairs in column 3, 4 and 5 denotes the number for which phenotypic information is available for both members of the pairs

Table 2 shows the general characteristics of the study population. As expected men had significantly increased body height, weight, lean mass and LMI, while body fat (total fat, FMI and fat%) was lower than in women. Android fat was significantly higher in men while gynoid fat was higher in women. Thus, the android:gynoid fat ratio was higher in men as well as the WHR.

The median inbreeding coefficient (interquartile range) was 0.002 (0.008). Inbreeding was significantly correlated with some traits, namely: height (Spearman $r = -0.163$, $p < 0.01$), weight ($r = -0.082$, $p < 0.01$), fat mass ($r = -0.043$, $p < 0.05$), lean mass ($r = -0.076$, $p < 0.01$),

gynoid fat mass (r = - 0.042, p< 0.01) and thigh circumference (r = - 0.037, p< 0.05), but not with BMI, FMI, fat %, LMI , android fat mass, android:gynoid ratio, waist and hip circumference, WHR and WTR.

Table 2. General characteristics of the ERF study population

	Women	Men	p
Number	1,405	1,101	
Age (years)	47.5 ± 14.5	48.2 ± 14.2	< 0.01
Height (m)	1.62 ± 0.06	1.75 ± 0.07	< 0.01
Weight (kg)	69.3 ± 13.2	83.2 ± 13.9	< 0.01
Body mass index (kg/m2)	26.4 ± 4.9	27.3 ± 4.2	< 0.01
Total fat mass (kg)	26.5 ± 9.8	22.3 ± 8.9	< 0.01
Fat mass index (kg/m2)	10.1 ± 3.7	7.3 ± 2.9	< 0.01
Fat percentage (%)	37.4 ± 7.5	26.0 ± 7.0	< 0.01
Total lean mass (kg)	39.9 ± 4.9	57.9 ± 7.0	< 0.01
Lean mass index (kg/m2)	15.2 ± 1.7	19.0 ± 1.9	< 0.01
Android fat mass (kg)	2.34 ± 1.2	2.57 ± 1.2	< 0.01
Gynoid fat mass (kg)	5.09 ± 1.54	3.61 ± 1.3	< 0.01
Android:Gynoid fat ratio	0.45 ± 0.15	0.71 ± 0.20	< 0.01
Waist circumference (cm)	81.5 ± 12.2	93.7 ± 11.7	< 0.01
Hip circumference (cm)	101.3 ± 9.3	99.3 ± 7.3	< 0.01
Thigh circumference (cm)	39.1 ± 1.04	39.3 ± 0.96	< 0.01
Waist:hip ratio	0.80 ± 0.08	0.94 ± 0.08	< 0.01
Waist:thigh ratio	1.63 ± 0.20	1.85 ± 0.20	< 0.01

Values are presented as percentage or mean ± SD

Table 3 shows the genetic, residual environmental variance and heritability estimates for men and women combined after accounting for age, sex and inbreeding. Heritability estimates ranged from 0.39 for FMI to 0.84 for body height. The heritability estimate for body weight (0.52) was higher than that for BMI (0.44) possibly because weight is related strongly to the highly heritable trait body height while BMI is corrected for height. A similar trend was seen was for the heritability estimate of fat mass (0.46) versus FMI (0.39) and lean mass (0.57) versus LMI (0.45). Heritability estimates of circumference measurements were between 0.40 (for waist) and 0.48 (for thigh). The heritability estimates of the circumference ratio's WHR and WTR were 0.40 and similar to the fat distribution variable estimated by DXA in the form of the android:gynoid fat ratio (0.43).

Correlations for each trait by pair type can be found in the Electronic supplementary material (ESM) Table 1 (www.springerlink.com/content/xn6293535815362t).

Table 3. Heritability estimates and variance components of the body composition parameters in 2,506 male and female ERF participants

	σ^2G	σ^2E	h^2
Height (m)	28.44 (1.78)	5.56 (0.98)	0.84
Weight (kg)	1.56 (0.15)	1.44 (0.12)	0.52
Body mass index (kg/m2)	8.59 (1.01)	11.11 (0.95)	0.44
Total fat mass (kg)	0.40 (0.04)	0.47 (0.04)	0.46
Fat mass index (kg/m2)	5.96 (0.73)	9.43 (0.70)	0.39
Total fat mass (%)	21.33 (2.41)	29.13 (2.12)	0.42
Lean mass (kg)	9.35 (0.86)	7.01 (0.63)	0.57
Lean mass index (kg/m2)	1.82 (0.21)	2.25 (0.19)	0.45
Android fat mass (kg)	5.65 (0.63)	7.44 (0.55)	0.43
Gynoid fat mass (kg)	5.29 (0.54)	5.58 (0.44)	0.49
Android:gynoid fat ratio	0.95 (0.11)	1.24 (0.09)	0.43
Waist circumference (cm)	0.64 (0.08)	0.95 (0.07)	0.40
Hip circumference (cm)	0.30 (0.03)	0.36 (0.03)	0.46
Thigh circumference (cm)	0.46 (0.05)	0.51 (0.04)	0.48
Waist:hip ratio	1.20 (0.14)	1.77 (0.12)	0.40
Waist:thigh ratio	0.32 (0.04)	0.46 (0.03)	0.40

Estimates were adjusted for sex, age and inbreeding. Values are presented as variance (SE)
σ^2G: genetic variance; σ^2E: environmental variance; h^2: additive heritability

Tables 4 and **5** show the results of the sex-specific genetic analyses on the variation of the body composition traits, including the heritability estimates for women and men separately with the sex-specific estimates of genetic and environmental variances (σ^2) and the correlation between genetic components of body composition variables in men and women (ρG). We found evidence for sex-specific genetic effects for all traits except for height, BMI, gynoid fat mass and the WTR, while for body weight and fat mass and FMI this evidence was borderline significant (p=0.06) (Table 5). The genetic correlations between men and women were significantly different from 1 for fat%, lean mass, LMI, android fat, android:gynoid fat ratio and WHR (Table 5). This indicates that different genes or a different subset of genes contribute to the variance of these body composition variables in men and women. The genetic variance was significantly higher in women than in men for the waist, hip and thigh circumference and for WHR (Tables 4 and 5). For the WHR, the genetic correlation was, in addition, significantly different from 1. This indicates that for the circumference measurements we found no evidence that different genes contribute to variation in these body composition variables but these same genes account for a larger magnitude of the genetic effect in women than in men. Evidence for an E x S interaction was found only for the android:gynoid fat ratio, with men showing significantly greater environmental variance for this trait than women (p< 0.01). For lean mass, total fat mass and gynoid fat mass differences were of borderline significance (p=0.05–0.07).

Table 4. Sex-specific variance components for the body composition parameters in 2506 ERF participants

	Women			Men			
	$\sigma^2 G$	$\sigma^2 E$	h^2	$\sigma^2 G$	$\sigma^2 E$	h^2	ρG
Height (m)	1.08 (0.09)	0.18 (0.06)	0.86	1.29 (0.12)	0.21 (0.08)	0.86	0.96 (0.05)
Weight (kg)	1.92 (0.24)	1.35 (0.19)	0.59	1.31 (0.23)	1.31 (0.20)	0.50	0.89 (0.10)
BMI (kg/m^2)	10.44 (1.58)	11.38 (1.42)	0.48	7.43 (1.46)	9.38 (1.35)	0.44	0.86 (0.12)
Total fat mass (kg)	0.48 (0.06)	0.36 (0.05)	0.57	0.38 (0.08)	0.51 (0.07)	0.43	0.82 (0.11)
FMI (kg/m^2)	15.81 (2.26)	14.86 (1.93)	0.52	11.40 (2.32)	16.5 (2.15)	0.41	0.81 (0.12)
Total fat mass (%)	28.33 (4.02)	24.42 (3.36)	0.54	19.79 (3.90)	27.7 (3.61)	0.42	0.74 (0.12)
Lean mass (kg)	0.09 (0.01)	0.05 (0.01)	0.64	0.12 (0.02)	0.08 (0.01)	0.59	0.84 (0.09)
LMI (kg/m^2)	1.79 (0.27)	1.82 (0.23)	0.50	2.47 (0.43)	2.17 (0.37)	0.53	0.74 (0.12)
Android fat mass (kg)	0.07 (0.01)	0.06 (0.01)	0.54	0.06 (0.01)	0.07 (0.01)	0.43	0.75 (0.12)
Gynoid fat mass (kg)	0.06 (0.01)	0.04 (0.01)	0.59	0.05 (0.01)	0.06 (0.01)	0.44	0.87 (0.11)
Android:gynoid fat ratio	1.07 (0.14)	0.72 (0.11)	0.60	1.34 (0.23)	1.36 (0.20)	0.50	0.57 (0.11)
Waist circ. (cm)	0.90 (0.13)	0.89 (0.11)	0.50	0.50 (0.11)	0.83 (0.11)	0.38	0.77 (0.14)
Hip circ. (cm)	0.42 (0.06)	0.35 (0.05)	0.55	0.22 (0.04)	0.29 (0.04)	0.43	0.83 (0.12)
Thigh circ. (cm)	0.62 (0.08)	0.45 (0.06)	0.58	0.32 (0.07)	0.53 (0.06)	0.38	0.95 (0.12)
Waist:hip ratio	1.73 (0.26)	1.81 (0.22)	0.49	0.93 (0.21)	1.30 (0.19)	0.42	0.73 (0.13)
Waist:thigh ratio	2.63 (0.50)	5.19 (0.48)	0.34	2.02 (0.60)	4.98 (0.59)	0.29	0.77 (0.17)

Estimates were adjusted for age and inbreeding. Values are presented as variance (SE)
$\sigma^2 G$: genetic variance; $\sigma^2 E$: environmental variance; h^2: additive heritability; ρG : genetic correlation between women and men
BMI: body mass index; FMI: fat mass index; LMI: lean mass index

DISCUSSION

In this study we found evidence for a difference in genetic background between men and women for most of the body composition variables studied, namely for fat%, lean mass, LMI, and for measures of fat distribution by circumferences and by DXA. Specifically, the magnitude of the genetic correlations was significantly different from 1 for fat%, lean mass, LMI, android fat, android:gynoid fat ratio and WHR, indicating that different genes or different, although partly overlapping, subsets of genes contribute to variation of these traits in men and women. Furthermore, genetic variance was almost twice as large in women as in men for all circumferences and for WHR, suggesting that genetic determinants of fat distribution account for more variance in women. For height, BMI, gynoid fat mass and the WTR differences between the sexes were not significant while for weight, fat mass and FMI they were borderline significant. Evidence for sex-specific environment effects was only found for the android:gynoid fat ratio, with men showing significantly greater environmental variance than women, with borderline significant differences for lean mass, total fat mass and gynoid fat mass.

Table 5. Model estimates from sex-specific variance partitioning of body composition parameters in 2,506 ERF participants

		$\rho G = 1$			$\sigma^2 G_F = \sigma^2 G_M$			$\sigma^2 E_F = \sigma^2 E_M$		
	Log-likelihood (Full model)	Log-likelihood (Nested model)	$\chi 2$	*p* value	Log-likelihood (Nested model)	$\chi 2$	*p* value	Log-likelihood (Nested model)	$\chi 2$	*p* value
Height (m)	-6315.24	-6315.46	0.44	0.25	-6316.44	2.41	0.13	-6315.29	0.11	0.74
Weight (kg)	-3399.15	-3399.72	1.15	0.14	-3400.94	3.59	0.06	-3399.15	0.01	0.90
BMI (kg/m2)	-5844.98	-5845.61	1.26	0.13	-5846.09	2.20	0.14	-5845.59	1.21	0.27
Total fat mass (kg)	-1870.45	-1871.69	2.48	0.06	-1870.93	0.96	0.33	-1872.05	3.21	0.07
FMI (kg/m2)	-6300.35	-6301.56	2.42	0.06	-6301.32	1.94	0.16	-6300.51	0.32	0.57
Total fat mass (%)	-6981.80	-6983.97	4.35	0.02	-6983.01	2.43	0.12	-6982.02	0.45	0.50
Lean mass (kg)	272.44	270.96	2.96	0.04	271.08	2.73	0.10	270.49	3.90	0.05
LMI (kg/m2)	-3834.56	-3837.19	5.27	0.01	-3835.60	2.09	0.15	-3834.92	0.73	0.39
Android fat mass (kg)	-5282.68	-5284.87	4.39	0.02	-5283.27	1.19	0.28	-5283.33	1.31	0.25
Gynoid fat mass (kg)	-5045.57	-5046.23	1.32	0.13	-5046.25	1.36	0.24	-5047.20	3.27	0.07
Android:gynoid fat ratio	-3004.31	-3011.57	14.52	0.00	-3004.83	1.04	0.31	-3008.23	7.83	0.01
Waist circ. (cm)	-2639.99	-2641.26	2.55	0.06	-2642.76	5.54	0.02	-2640.05	0.12	0.73
Hip circ. (cm)	-1516.06	-1517.09	2.06	0.08	-1520.75	9.38	0.00	-1516.63	1.13	0.29
Thigh circ. (cm)	-2004.34	-2004.42	0.14	0.35	-2008.81	8.04	0.00	-2004.79	0.89	0.34
Waist:hip ratio	-3405.85	-3407.68	3.65	0.03	-3408.96	6.207	0.01	-3407.40	3.09	0.08
Waist:thigh ratio	-4561.15	-4561.96	1.631	0.10	-4561.44	0.59	0.44	-4561.18	0.07	0.79

ρG: genetic correlation between women and men; BMI: body mass index; FMI: fat mass index; LMI: lean mass index

$\sigma^2 G_F$: genetic variance females; $\sigma^2 G_M$: genetic variance males; $\sigma^2 E_F$: environmental variance females; $\sigma^2 E_M$: environmental variance males

p values based on a 1/2:1/2 mixture of a $\chi 2$ (1df) and a point mass of zero

The finding of small but significantly negative correlations between inbreeding and some of the traits, especially height, could point towards recessive alleles.

Our results of no sex-specific genetic effects for BMI are in agreement with some family studies [18, 19], but they are in contrast with twin studies that found higher [2, 3, 13, 14], or lower [15-17] heritability estimates in women than in men. Schousboe et al. [6] found slight differences between women and men in the extent of additive genetic and common environmental influences on BMI, anthropometric measures of fat distribution and fat% and LMI estimated by bioelectrical impedance in twins, but CIs were broad and overlapped for most estimates. In the Diabetes Heart Study [7] women appeared to have higher heritability of BMI and lean mass by DXA but differences were also not significant considering large SEs

Few of these studies used the same method of variance decomposition procedures to partition variation in body composition into genetic and environmental effects common to both sexes and to men and women separately and/or calculated genetic correlations between sexes. Several twin studies used self-reported data on height and body weight, potentially influencing outcomes. One study using the same approach as we did found evidence for sex-specific genetic effects for several anthropometric variables, including skinfold thicknesses, in a pedigree of 409 Mexican-Americans but no data were available on fat and lean mass [20]. Consistent with our findings, no significant sex-specific effect was found for weight and height. Aside from these studies, the issue of sex-specific genetic effects on body composition variables has not been formally addressed and especially data from lean and fat mass assessed by DXA are lacking.

The heritability estimates of the body composition variables in our study are well within the range of estimates reported in other family studies but are in general lower than those reported in twin studies. Possible explanations for higher estimates of heritability in twin than in family studies are increased variation in the apparent environmental influences in family studies due to generational differences between parents and their offspring as well as age differences between family members. Heritability in twin studies can, on the other hand, be inflated if the monozygotic twin pairs have been exposed to more similar environment than the dizygotic twin pairs [6].

Our findings of sex-specific genetic effects on body composition are important for several reasons. Both increased adiposity and android fat deposition are associated with increased risk of chronic disorders like cardiovascular disease and diabetes. An understanding of the genetic architecture of these risk factors and of their sexual dimorphism should lead to better insight into the relationship between these factors and complex diseases that may differ between sexes. This improved understanding may ultimately lead to better risk prediction and risk reduction. Also, in the search for genes that are associated with obesity, android fat deposition and muscularity, it is important to take into account sex differences by performing sex-stratified analyses.

There are some limitations to our study. As in many studies on heritability, we have not adjusted our analyses for covariates like smoking, diabetes status, dietary intake and physical activity. The latter two variables are not available in our study. In the Diabetes Heart Study, adjustments for these covariates resulted in only minor changes in the heritability estimates of body composition [7]. We studied individuals with a wide age range, including both pre- and postmenopausal women and we tried to overcome this limitation by adjusting all our analyses for age. Insufficient power precluded us to study sex-specific differences in different age groups. It is possible that the sex-specific effects we found may vary by age since gender differences in body composition are also not constant across the age range and most pronounced in adolescence and young adulthood [10]. Previous studies have examined possible age effects on genetic and en-

vironmental influences on BMI with unclear and inconsistent results [3, 6, 16]. The genetic make-up of genetically isolated populations may differ from a general population as a result of genetic drift and founder effects. Even though our population is a genetic isolate we found no evidence that it deviates much from the general population in its genetic composition [23, 34]. However, as was pointed out again recently [35], heritability estimates are by definition population specific and replication of our findings in other populations is necessary. We acknowledge that multiple testing may influence some of the significant findings in our study. Corrections (e.g., Bonferroni) would be too stringent since most body composition traits we studied are highly correlated. Considering the consistency of the results across sexes, we do not expect multiple testing to play a substantial role in the interpretation of our findings.

Strength of the study is the availability of a large population with extensive information on body composition and genealogy and the use of validated formal testing of sex-specific effects with variance decomposition procedures.

In conclusion, we found evidence for sex-specific genetic effects that may underlie sex differences in several body composition traits. The findings are relevant for the design of genetic association studies like Genome-wide Association and for studies on the relationship of body composition and fat distribution with common diseases such as cardiovascular disease and type 2 diabetes.

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Chapter 3

The role of body mass index, insulin and adiponectin in the relation between fat distribution and bone mineral density

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Submitted

ABSTRACT

Objective Despite the positive association between body mass index (BMI) and bone mineral density (BMD) and content (BMC), the role of fat distribution in BMD/BMC remains unclear. We examined relationships between BMD/BMC and various measurements of fat distribution and studied the role of BMI, insulin and adiponectin in these relations.

Methods Using a cross-sectional investigation of 2631 participants from the Erasmus Rucphen Family (ERF) study we studied associations between BMD (using dual-energy x-ray absorptiometry (DXA)) at the hip, lumbar spine and total body BMC and fat distribution by the waist-to-hip ratio (WHR), waist-to-thigh ratio (WTR) and DXA-based trunk-to-leg fat ratio and android-to-gynoid fat ratio. Analyses were stratified by gender and median age (48.0 years in women and 49.2 in men) and were performed with and without adjustment for BMI, fasting insulin and adiponectin.

Results Using linear regression (adjusting for age, height, smoking and use of alcohol) most relationships between fat distribution and BMD and BMC were positive, except for WTR. After BMI-adjustment, most correlations were negative except for trunk-to-leg fat-ratio in both genders. We did not find a consistent influence of age or menopausal status. Insulin and adiponectin levels did not explain either positive or negative associations.

Conclusions Positive associations between android fat distribution and BMD/BMC are explained by higher BMI but not by higher insulin and/or lower adiponectin levels. Inverse associations after adjustment for BMI suggest that android fat deposition as measured by the WHR, WTR and DXA-based android-to-gynoid fat ratio is not beneficial and possibly even deleterious for bone.

INTRODUCTION

Osteoporosis and obesity are important global health problems with increasing prevalence. A positive association between body weight or body mass index (BMI) and bone mineral density (BMD) has been clearly demonstrated as well as increased fracture risk with low BMI [1-3]. Possible explanations for higher BMD in heavier people include the weight-bearing effect of both fat and lean mass while lean mass is thought to influence bone through muscle mediated effects of physical exercise. Furthermore, adipose tissue might influence BMD through the production of hormones and adipokines by adipocytes (e.g., estrogen, leptin, adiponectin, resistin, interleukins) or through an effect on the secretion of bone-active hormones from the pancreas (e.g., insulin, amylin, preptin) [3]. There is ongoing controversy about the relative importance of the fat and lean components of the body to BMD [3, 4]. In postmenopausal women fat mass has been shown most consistently to be positively related to BMD, possibly mediated by higher estrogen levels. However, several discrepancies can be found in the literature, e.g., fat mass being important in young but not in old women and in old but not in young men [5] .

The effect of fat distribution on BMD is far from clear. Adipose tissue is metabolically heterogeneous, with differences between visceral and subcutaneous fat for example in the production of adipokines and in regulation of steroid hormone metabolism [6]. Android fat deposition (also named abdominal, central, visceral or upper body fat distribution) leads to increased risk of chronic diseases like cardiovascular disease and type 2 diabetes [7], while larger hip and thigh circumferences in gynoid fat deposition are associated with decreased risk of metabolic disease, independently of waist circumference [8, 9]. Circumference ratios, especially the waist-to-hip ratio (WHR) but also the waist-to-thigh ratio (WTR), have been consistently associated in epidemiological studies with metabolic and cardiovascular disease [7, 8]. Several physiological factors that are associated with fat distribution are also associated with BMD. These include age, gender, heredity, parity, menopausal status, physical activity, smoking and alcohol consumption and hormones such as sex-steroids, glucocorticoids, growth hormones, insulin, leptin and adiponectin [3, 10, 11]. Studies on the relationship between body fat distribution and BMD have yielded conflicting results. In late postmenopausal women from the Study of Osteoporotic Fractures (SOF), WHR was found to have no important relationship to BMD as compared to weight [12]. Heiss et al found that upper body obesity was associated with increased BMD [13], possibly due to higher levels of insulin, lower levels of sex-hormone binding globulin (SHBG) and higher free sex-steroid levels. Other studies also reported positive relations between android fat distribution and BMD [5, 14-18]. In contrast, two small studies found negative associations between android obesity and BMD or bone mineral content (BMC) [19, 20], and this was also seen in pre-pubertal children [21] and HIV infected patients [22]. Most studies included a small number of subjects and/or were not

population based and different techniques were used for measurement of BMD or BMC (DXA or peripheral Computed Tomography (CT)) and fat distribution (anthropometry, DXA, CT or MRI). Also, the effect of adiposity on the relationship between fat distribution and BMD was not always considered. Since, in general, increased obesity is associated with increased abdominal fatness, BMI might be a confounder in the relation between android obesity and BMD. Despite suggestions that android fat deposition is beneficial for bone through higher insulin and/or lower adiponectin levels [11, 13], their role in the relation between fat distribution and bone has not been fully explored.

The aim of the present study was to examine the relationship between various types of fat distribution assessment and BMD and BMC in a large number of Caucasian subjects from a genetically isolated population in the Netherlands and to explore potential sex and age differences in this relationship as well as the effect of BMI and plasma insulin and adiponectin.

METHODS

Study population

This study was carried out within the Erasmus Rucphen Family (ERF) study, a family-based cohort study that is embedded in the Genetic Research in Isolated Populations (GRIP) program in the South West of the Netherlands. The aim of this program was to identify genetic risk factors in the development of complex disorders [23-26].

For the ERF study, twenty-two families that had at least 5 children baptised in the community church between 1850-1900 were identified with the help of genealogical records. All living descendants of these couples and their spouses were invited to take part in the study. Data collection started in June 2002 and was finished in February 2005. In this study, we focused on the 2631 participants for whom complete phenotypic, and genealogical information was available. The Medical Ethics Committee of Erasmus Medical Center Rotterdam approved the study. Written informed consent was obtained from all participants.

Data collection

At the research center, located within the community, extensive clinical examinations were performed, including the collection of fasting blood samples, anthropometric measurements, DXA and personal interviews. A research physician obtained information on medical history, medication use, smoking and alcohol use in a personal interview.

Anthropometric measurements

Height and weight were measured with the participant dressed in light undercloth-ing. Body mass index (BMI) was calculated from these data (weight (kg)/ height^2 (m^2)). Circumferences of the waist, hip and thigh were measured using a tape measure with the participant in standing position without outer garments. Waist circumference was measured halfway between the rib cage and the pelvic bone. Hip circumference was measured at the maximal circumference of the hips. Thigh circumference was measured mid-way between the upper border of the patella and the inguinal fold on the right leg. The waist-to-hip ratio (WHR) and waist-to-thigh ratio (WTR) were calculated from these measurements.

Dual-energy x-ray absorptiometry (DXA) measurements

DXA scans were performed using a Prodigy total body fan-beam densitometer and analyzed with the enCORE 2005 software v.9.3 (DPX, Lunar corporation Madison, WI) as described previously [26]. BMD was measured at the femoral neck (mean value of left and right) and lumbar spine (L1-L4) and BMC at the total body. Total body scans were auto analyzed by the software, which employs an algorithm that divides body measurements into areas corresponding to head, trunk, arms and legs.

Total body fat mass (g), lean mass (g), and regional fat mass were obtained from total body scans. The trunk region was limited by vertical borders lateral to the ribs, and a lower border by the iliac crest and an upper horizontal border below the chin (neck cut), above which the head was defined. The arm region was limited by cuts that cross the arm sockets, as close to the body as possible and separate the arms and hands from the body. The leg region is limited above by the oblique lines passing through the hip joint, and cuts that separate the hands and forearms from the legs and a center leg cut

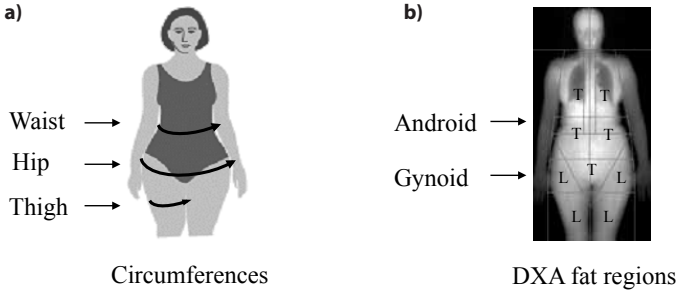

Figure 1.
1a): sites of the circumference measurements
1b): regions of trunk fat (T), leg fat (L), "android" and "gynoid" fat assessed by DXA.

which separates the right and left leg. Additional "android" and "gynoid" regions were defined using the software provided by the manufacturer. The "android region" has a lower boundary at the pelvis cut and the upper boundary above the pelvis cut by 20% of the distance between the pelvis and neck cuts. The lateral boundaries are the arm cuts. The "gynoid region" has an upper boundary between the upper part of the greater trochanters and a lower boundary defined at a distance equal to twice the height of the "android region". The lateral boundaries are the outer leg cuts. The android and gynoid fat mass and android-to-gynoid fat ratio were calculated from these measurements. A schematic representation of the trunk, leg, android and gynoid region is shown in Figure 1B. All analyzes were verified by a trained technician who performed adjustments when necessary. For 12 extremely obese subjects (BMI 43.5 – 61.8 kg/m^2), total body DXA-scans were considered inadequate, since they did not contain all body parts and they were therefore excluded from further analysis. Daily quality assurance tests were performed with a calibration block supplied by the manufacturer. Repeated measurements on the calibration block had coefficients of variation less than 0.5%. In addition a calibration aluminum phantom was measured weekly with coefficients of variation less than 0.5%.

In **Figure 1** the sites of circumference measurements and fat regions by DXA are shown

Laboratory examinations

Fasting plasma insulin was analyzed with the INS-Irma kit of Biosource (cat.#: KIP1254) and total plasma adiponectin was analyzed with the Human adiponectin RIA kit (ca.t#: HADP-61HK) of Linco Research (St. Charles, MO). All measurements were performed conform the manufactures protocol.

Statistical analysis

The associations between fat distribution parameters and BMI, BMD/BMC and plasma levels of insulin and adiponectin were studied as partial correlations with correction for age. Multivariate linear regression analyses were performed to evaluate the strength of the relationship between body fat distribution and BMD and BMC. In the regression models, BMD and BMC, which were normally distributed, were used as dependent variables. Fat distribution parameters with normal or near normal distribution were used untransformed as independent variables, while age, height and lifestyle factors (smoking and alcohol history) were also included because of their known association with bone and body composition. Smoking of cigarettes was categorized as never, past or current. Alcohol use was categorized as no drinking, 1 – 21 units a week or > 21 units a week. Additional adjustment for BMI was applied to evaluate its role as a possible confounder. To study the effect of plasma insulin and adiponectin, regression analyses were

performed with and without adjustment for these hormones. The results of multivariate regressions are expressed in standardized regression coefficients. A *p*-value of <0.05 was considered significant. To examine the effect of age, the study population was divided by median age (48.0 years for women and 49.2 for men), in order to have equal numbers of subjects per group. For women, analyses were repeated stratified by menopausal status (premenopausal vs. postmenopausal subjects). All of the statistical analysis was done using the statistical package SPSS for Windows, version 15.0.

Table 1. General and body composition characteristics of the study population

	Females	Males
Number	1467	1164
Age (years)	47.7 ± 14.2	48.6 ± 14.0
(range)	16.7 – 86.1	17.6 – 84.7
Postmenopausal	673 (45.9)	
Height (m)	1.62 ± 0.06	1.75 ± 0.07
Weight (kg)	69.2 ± 12.8	83.0 ± 13.3
Body mass index (kg/m^2)	26.4 ± 4.7	27.1 ± 3.9
Waist-to-hip ratio	0.80 ± 0.08	0.94 ± 0.08
Waist-to-thigh ratio	1.62 ± 0.20	1.85 ± 0.20
Android-to-gynoid fat ratio	0.45 ± 0.15	0.71 ± 0.20
Trunk-to-leg fat ratio	1.52 ± 0.46	2.42 ± 0.64
Femoral neck BMD (g/cm^2)	0.91 ± 0.13	0.97 ± 0.14
L1-L4 BMD (g/cm^2)	1.12 ± 0.16	1.18 ± 1.94
Total body BMC (kg)	2.32 ± 0.67	3.04 ± 0.44
Insulin (μU/ml) (n=2104)	12.8 ± 6.3	13.7 ± 8.8
Adiponectin (mg/L) (n=2104)	12.3 ± 5.8	8.0 ± 4.1
Smoking		
Never	420 (28.6)	398 (34.2)
Past	394 (26.9)	375 (32.2)
Current	653 (44.5)	391 (33.6)
Alcohol		
< 1 unit per week	681 (46.4)	221 (19.0)
1-21 units per week	770 (52.5)	834(71.6)
> 21 units per week	16 (1.1)	109 (9.4)

Values are presented as number (percentage) or mean ± standard deviation.

RESULTS

Table 1 shows general and body composition characteristics and BMD at the femoral neck and lumbar spine and total body BMC of the study population. Mean age was 47.7 years for women and 48.6 years for men. About 46% of women was postmenopausal. As expected, men were taller and had higher values for all fat distribution parameters, consistent with men having more android (apple shaped) and women more gynoid (pear shaped) fat distribution. Men also had higher values of BMD. Fasting plasma levels adiponectin levels were lower in men.

Table 2 presents partial correlations corrected for age between BMI, the four fat distribution parameters, BMD/BMC and fasting plasma levels of insulin and adiponectin. Regarding the relation between BMI and the fat distribution parameters, in women, the highest correlation was found between BMI and android-to-gynoid fat ratio (r = 0.59), followed by WHR (r = 0.44) while in men the highest correlation with BMI was found for WHR (r = 0.58), followed by android-to-gynoid fat ratio (r = 0.46). Both in women and in men, trunk-to-leg fat ratio had the lowest correlations with BMI (r = 0.26 and 0.20 for

Table 2: Partial correlations coefficients controlled for age between BMI, fat distribution, BMD and serum levels of insulin and adiponectin[a]

	BMI	Waist-to- hip ratio	Waist-to-thigh ratio	Android-to-gynoid fat ratio	Trunk-to-leg fat ratio	FN BMD	L1-L4 BMD	Total body BMC	Insulin	Adipo-nectin
Females* (n = 1467) →										
Males* (n=1164) ↓										
BMI	1	0,44	0,3	0,59	0,26	0,28	0,2	0,33	0,47	-0,27
Waist-to-hip ratio	0,58	1	0,84	0,72	0,64	0,1	0.03^{ns}	0.001^{ns}	0,37	-0,36
Waist-to-thigh ratio	0,28	0,74	1	0,67	0,68	0.02^{ns}	0.02^{ns}	-0,07	0,32	-0,3
Android-to-gynoid fat ratio	0,46	0,68	0,52	1	0,83	0,16	0,1	0,11	0,42	-0,4
Trunk-to-leg fat ratio	0,2	0,48	0,43	0,8	1	0,1	0,11	$0,01^{ns}$	0,28	-0,35
FN BMD	0,3	0.05^{ns}	-0,07	0,09	0,07	1	0,69	0,71	0.05^{ns}	-0,1
L1-L4 BMD	0,17	-0.03^{ns}	-0,09	0.05^{ns}	0,11	0,61	1	0,7	0.0^{ns}	-0,09
Total body BMC	0,25	-0.03^{ns}	-0,13	0.0^{ns}	$0,04^{ns}$	0,66	0,67	1	0.04^{ns}	-0,07
Insulin**	0,45	0,34	0,24	0,32	0,17	0,09	0.0^{ns}	0.0^{ns}	1	-0,29
Adiponectin**	-0,22	-0,26	-0,13	-0,34	-0,31	-0,13	-0,14	-0,11	-0,18	1

[a] All correlations are significant at the 0.05 level, except when indicated ns (not significant)
*Females in the upper right corner, males in the lower left corner
**For plasma levels of insulin and adiponectin n= 1207 for females;n=897 for males

women and men, respectively). Correlations between the fat distribution parameters themselves ranged between 0.43 and 0.84, with the highest correlation between the WHR and WTR and between the android-to-gynoid fat ratio and the trunk-to-leg fat ratio.

Fat distribution parameters were significantly correlated with BMD and BMC at several sites and correlations were mostly positive (highest r of 0.16 in females and 0.11 in males). Negative correlations were found for waist-to-thigh ratio (in females with total body BMC and in males with femoral neck BMD, lumbar spine and total body BMC).

Fasting levels of plasma insulin were positively correlated with BMI and fat distribution parameters in both genders and additionally with femoral neck BMD in males.

Fasting plasma levels of adiponectin showed negative correlations with BMI and fat distribution. There were also inverse correlations between adiponectin and all BMD/BMC measurements.

Table 3 shows the same relationship as in table 2 between the four parameters of fat distribution and BMD and/or BMC at three sites, expressed as standardized beta-coefficients after standard adjustment for age, height, smoking and alcohol intake (model1) and after additional adjustment for BMI and plasma insulin and adiponectin. Adjustment for plasma insulin (model 2), or plasma adiponectin (model 3) resulted in only minor changes in beta-coefficients and *p*-values. However, after adjustment for BMI (model 4) most relations changed and were now negative. Negative relations were most strong for the waist-to-thigh ratio and waist-to-hip ratio. For the android-to-gynoid fat ratio negative relations were significant only with total body BMC in both genders. For the trunk-to-leg fat ratio there was a small persisting positive relation with lumbar spine BMD after adjustment for BMI. Additional adjustment for either insulin (model 5) or adiponectin (model 6) with BMI in the models resulted again in only minor changes in beta coefficients. In general, they decreased after adjustment for insulin and increased after adjustment for adiponectin. The strongest negative relations were seen for the waist-to-thigh ration with total body BMC in males. Beta coefficients (*p*-values) were after BMI adjustment - 0.335 ($p=1.5\text{x}10^{-21}$**),** after BMI and insulin adjustment - 0.313 ($p=2.7\text{x}10^{-19}$) and after BMI and adiponectin adjustment - 0.339 ($p=3.5\text{x}10^{-22}$)

Overall, beta-coefficients were more negative in males than in females.

In **Table 4** the relationships between fat distribution and BMD are presented stratified by median age in both genders. There were no consistent differences between the age groups, although relations of fat distribution parameters with total body BMC appeared more positive without adjustment for BMI in younger compared to older women and more negative after BMI adjustment in older women.

Outcomes were essentially unchanged when we stratified women by postmenopausal status instead of median age and also when we adjusted our analyses in both genders for body weight instead of BMI (data not shown).

Table 3. Relationship between fat distribution and BMD and BMC with and without adjustment for BMI and plasma levels of insulin and adiponectin[†]

	Waist-to-hip ratio						**Waist-to-thigh ratio**					
	without BMI adjustment			with BMI adjustment			without BMI adjustment			with BMI adjustment		
Females (n=1207)	1[*]	2	3	4	5	6	1	2	3	4	5	6
Fem Neck BMD	**+ 0.112**[c]	**+ 0.106**[c]	**+ 0.078**[b]	- 0.018	+ 0.002	- 0.027	+ 0.028	+ 0.010	- 0.006	**- 0.070**[a]	- 0.050	**- 0.081**[a]
L1-L4 BMD	+ 0.040	+ 0.047	+ 0.010	**- 0.066**[a]	- 0.042	**- 0.081**[a]	- 0.019	- 0.020	- 0.052	**- 0.095**[b]	**- 0.070**[a]	**- 0.110**[b]
Total body BMC	+ 0.026	+ 0.030	+ 0.001	**- 0.161**[c]	**- 0.134**[c]	**- 0.165**[c]	**- 0.074**[b]	**- 0.097**[b]	**- 0.106**[c]	**- 0.204**[c]	**- 0.178**[c]	**- 0.209**[c]
Males (n=897)												
Fem Neck BMD	+ 0.053	+ 0.027	+ 0.019	**- 0.194**[c]	**- 0.195**[c]	**- 0.222**[c]	**- 0.103**[a]	**- 0.134**[a]	**- 0.125**[b]	**- 0.226**[c]	**- 0.219**[c]	**- 0.233**[c]
L1-L4 BMD	- 0.027	- 0.034	- 0.073	**- 0.222**[c]	**- 0.204**[c]	**- 0.245**[c]	**- 0.123**[b]	**- 0.132**[b]	**- 0.148**[b]	**- 0.203**[c]	**- 0.192**[c]	**- 0.214**[c]
Total body BMC	- 0.042	- 0.033	**- 0.073**[a]	**- 0.303**[c]	**- 0.286**[c]	**- 0.325**[c]	**- 0.216**[c]	**- 0.218**[c]	**- 0.235**[c]	**- 0.335**[c]	**- 0.313**[c]	**- 0.339**[c]
	Android-to-gynoid fat ratio						**Trunk-to-leg fat ratio**					
	without BMI adjustment			with BMI adjustment			without BMI adjustment			with BMI adjustment		
Females (n=1207)	1[*]	2	3	4	5	6	1	2	3	4	5	6
Fem Neck BMD	**+ 0.165**[c]	**+ 0.173**[c]	**+ 0.150**[c]	- 0.002	- 0.021	- 0.012	**+ 0.121**[c]	**+ 0.115**[c]	**+ 0.098**[b]	+ 0.052	**+ 0.072**[b]	+ 0.049
L1-L4 BMD	**+ 0.103**[c]	**+ 0.127**[c]	**+ 0.082**[a]	- 0.031	- 0.002	- 0.047	**+ 0.130**[c]	**+ 0.142**[c]	**+ 0.114**[c]	**+ 0.082**[b]	**+ 0.108**[c]	**+ 0.080**[a]
Total body BMC	**+ 0.115**[c]	**+ 0.120**[c]	**+ 0.105**[c]	**- 0.138**[c]	**- 0.108**[c]	**- 0.142**[c]	**+ 0.058**[a]	**+ 0.052**[a]	+ 0.040	- 0.032	- 0.005	- 0.027
Males (n=897)												
Fem Neck BMD	**+ 0.091**[a]	+ 0.071	+ 0.051	- 0.073	- 0.064	**- 0.102**[a]	**+ 0.076**[a]	+ 0.063	+ 0.039	+ 0.011	+ 0.017	- 0.009
L1-L4 BMD	+ 0.058	+ 0.062	+ 0.007	- 0.043	- 0.029	- 0.084	**+ 0.134**[c]	**+ 0.137**[c]	**+ 0.097**[a]	**+ 0.098**[a]	**+ 0.108**[c]	+ 0.072
Total body BMC	- 0.007	+ 0.066	- 0.045	**- 0.173**[c]	**- 0.147**[c]	**- 0.200**[c]	+ 0.053	**+ 0.061**[a]	+ 0.026	- 0.004	+ 0.013	- 0.017

[†]Effect size: standardised Beta coefficients from linear regression adjusted for age, height, smoking and alcohol intake.

* model 1: adjusted for age, height, smoking and alcohol; model 2: model 1 plus adjustment for insulin; model 3: model 1 plus adjustment for adiponectin model 4: model 1 plus adjustment for BMI; model 5: model 4 plus adjustment for insulin; model 6: model 4 plus adjustment for adiponectin

[a] $p < 0.05$; [b] $0.001 < p < 0.01$; [c] $p < 0.001$

Table 4. Relationship between fat distribution and BMD and BMC in males and females stratified by median age[†]

	Waist-to-hip ratio		waist-to-thigh ratio		android-to-gynoid fat ratio		Trunk-to-leg fat ratio	
A: Without adjustment for BMI								
	≤median age	>median age	≤median age	>median age	≤median age	>median age	≤median age	>median age
Females, n	734	733	734	733	734	733	734	733
Fem Neck BMD	**+ 0.146**[c]	**+ 0.101**[b]	+ 0.054	+ 0.024	**+ 0.138**[c]	**+ 0.155**[c]	**+ 0.127**[c]	**+ 0.115**[c]
L1-L4 BMD	+ 0.024	+ 0.049	- 0.046	+ 0.007	+ 0.059	**+ 0.103**[b]	**+ 0.128**[c]	**+ 0.131**[c]
Total BMC	**+ 0.082**[b]	- 0.003	- 0.032	**- 0.070**[a]	**+ 0.145**[c]	+ 0.058	**+ 0.112**[c]	+ 0.025
Males, n	582	582	582	582	582	582	582	582
Fem Neck BMD	+ 0.077	+ 0.078	**- 0.091**[a]	- 0.063	**+ 0.099**[a]	+ 0.070	+ 0.068	**+ 0.087**[a]
L1-L4 BMD	- 0.031	- 0.010	**- 0.164**[c]	- 0.066	- 0.025	+ 0.070	+ 0.072	**+ 0.154**[c]
Total BMC	- 0.019	- 0.064	**- 0.171**[c]	**- 0.179**[c]	- 0.019	- 0.038	+ 0.051	+ 0.036
B: with adjustment for BMI								
Females								
Fem Neck BMD	+ 0.045	- 0.048	- 0.010	**- 0.092**[a]	- 0.031	- 0.008	+ 0.052	+ 0.055
L1-L4 BMD	- 0.063	- 0.066	**- 0.095**[a]	**- 0.079**[a]	- 0.082	- 0.019	**+ 0.081**[a]	**+ 0.090**[a]
Total BMC	**- 0.099**[b]	**- 0.180**[c]	**- 0.133**[c]	**- 0.203**[c]	**- 0.146**[c]	**- 0.157**[c]	- 0.009	- 0.042
Males								
Fem Neck BMD	**- 0.157**[b]	**- 0.198**[c]	**- 0.177**[c]	**- 0.205**[c]	- 0.061	**- 0.098**[a]	+ 0.020	+ 0.012
L1-L4 BMD	**- 0.257**[c]	**- 0.179**[c]	**- 0.203**[c]	**- 0.155**[c]	**- 0.101**[a]	- 0.027	+ 0.061	**+ 0.114**[b]
Total BMC	**- 0.295**[c]	**- 0.287**[c]	**- 0.260**[c]	**- 0.280**[c]	**- 0.210**[c]	**- 0.156**[c]	+ 0.004	- 0.009

[†]Effect size: standardised Beta coefficients from linear regression adjusted for age, height, smoking and alcohol intake. Median age for women: 48.0 years; for men: 49.2 years
[a] $p < 0.05$; [b] $0.001 < p < 0.01$; [c] $p < 0.001$

The total variation of BMD explained by fat distribution parameters independent of age, height, BMI, smoking and alcohol intake was smaller than variation explained by BMI independent of age, height, smoking and alcohol intake.

Variation explained by BMI for femoral neck BMD, lumbar spine BMD and total body BMC was respectively, 5.7%, 2.9% and 9.0% in women and 7,8%, 2,4% and 5.0% in men.

Variation explained by the four fat distribution parameters independent of BMI at these sites was for waist-to-hip ratio: 0.0%, 0.2% and 1.3% in women and 1.6%, 2.1% and 4.0% in men; for the waist-to-thigh ratio: 0.0%, 0.6% and 2.3% in women and 1.8%, 1.8% and 4.9% in men; for the android-to-gynoid fat ratio: 0.0%, 0.1% and 1.3% in women and 0.5%, 0.1% and 1.8% in men. For the trunk-to-leg fat ratio the explained variance was: 0.2%, 0.5% and 0.0% in women and 0.0%, 0.7% and 0.0% in men, respectively.

DISCUSSION

In this study we show that positive associations between android fat distribution and BMD are explained by higher BMI. Once the effect of BMI is taken into account, android fat distribution has no or negative relationships with BMD. We also show that these relationships depend on the type of the fat distribution parameter used and the site of BMD/BMC measurement and we demonstrate that relations are independent of plasma levels of insulin and adiponectin. These observations may largely explain previous inconsistent findings of positive, negative or no associations between fat distribution and BMD [5, 13-18] and challenge the view that android fat deposition is beneficial for bone through higher insulin and/or lower adiponectin levels [11, 13].

Most of the positive associations we found between BMD and fat distribution are explained by increased BMI. After removing the effect of BMI by adjusting for it in the multiple regressions, no more positive associations were found except for small but significant positive relationships between trunk-to-leg fat ratio and lumbar spine BMD. In contrast, several associations became significantly negative with the lowest regression coefficients for circumference ratios WHR and WTR, followed by android-to-gynoid fat ratio, with no negative associations for the trunk-to-leg fat ratio. Thus, despite high correlations between the different parameters of fat distribution, there were clear differences in their relation with BMD but also with BMI. This shows that they do not measure the same aspect of fat distribution. Circumference measurements are not only related to the amount of adipose tissue but also to size of internal organs (waist circumference), and to size of bone and muscle (hip and thigh), the latter especially in men [8, 9]. This may influence the relation with BMD irrespective of fat distribution. The new DXA-based measurement of fat in the abdominal region is theoretically more closely related to visceral fat than total trunk fat mass, which also contains subcutaneous adipose tissue on the thorax, back and breasts. Another advantage of the android-to-gynoid fat ratio over the trunk-to-leg fat ratio is that in the latter assessment, gluteal and abdominal fat can not be perfectly separated [27]. However, neither anthropometry nor DXA can distinguish between visceral and subcutaneous fat in the android region, although waist circumference as well as trunk fat and abdominal fat in a sub region by DXA show high correlation with visceral fat measured by CT or MRI [28-33].

The only association that remained significantly positive in our study after BMI adjustment was between trunk-to-leg fat ratio and lumbar spine BMD, consistent with findings by Douchi et al [15]. It is unclear whether this is caused by the fact that the trunk-to-leg fat ratio is not a good estimate of fat distribution, or due to an artifact by the DXA measurement, as was observed after simulating changes in trunk fat with lard packets [34]. On the other hand, we cannot exclude that the observed relationship is real and that (subcutaneous) fat in the trunk, as opposed to the legs, produces factors that increase BMD.

There were small gender differences in our study, with males showing less positive (before BMI adjustment) and more negative relationships (after BMI adjustment) between fat distribution and BMD or BMC than women. This might be caused by a potentially stronger relation in males between hip and thigh circumference and lower body muscularity [8, 9], since these differences were most marked for the waist-to-hip and waist-to-thigh ratio's.

We found differences by the site of BMD/BMC measurement. Regression coefficients were in general lower for total body BMC than for femoral neck and lumbar spine BMD. To our surprise we found no consistent age differences in the relationship between fat distribution and BMD/BMC. Considering the strong effect of menopause and age on fat distribution and BMD [35, 36], the similarity in their relationships between younger and older women is remarkable.

There are several reasons why it is important to study the relationships between fat distribution and BMD and to try to explain previous controversial findings. It is important for fracture risk prediction, patient handling and understanding of biological mechanisms. Our study shows that measures of fat distribution explain more of the variation in BMD and BMC in men than in women but the effect is relatively small as compared to that of weight and BMI. In that respect our data are in agreement with those from the Study of Osteoporotic Fractures (SOF) in older women [12] but now extend these findings to males and younger subjects. For patient care, it is important to know that android obesity does not appear to be beneficial for bone, as was suggested by most previous studies. Instead, our data show that especially for males, gynoid fat distribution is better than android, possibly in part because it is a marker of greater physical activity with greater muscle mass on hips and thighs. Thus, our data comply with and underscore the importance of the advice for regular physical exercise that can potentially decrease android obesity and also prevents muscle wasting with ageing and increases mechanical loading on the skeleton. Other mediators than physical activity might be considered as a possible mechanistic explanation for our findings like glucocorticoids, growth- and sex- hormones, leptin and inflammatory adipokines. Our data show that it is unlikely that insulin and adiponectin mediate the association. Lastly, technical limitations of the DXA technique should be considered in the interpretation of these findings since the BMD measurement is influenced by the fat to lean ratio of soft tissues [37].

Given the cross-sectional nature of our study, it is not possible to make causal inferences from associations or to study the relationship of fat distribution with fractures. We acknowledge that multiple testing may influence some of the significant findings in our study. However, even after applying a Bonferroni correction, that might be too stringent considering the high correlation between the fat distribution traits, most correlations would remain significant. Considering the consistency of the results across genders, we don't expect multiple testing to play a substantial role in the interpretation of our find-

ings. Strength of our study is the large population which is not selected on disease and which includes both genders with a wide age range and the use of multiple bone sites and fat distribution parameters, including a new android-to-gynoid fat ratio by DXA and the availability of plasma levels of insulin and adiponectin.

We conclude that positive associations between android fat distribution and BMD are explained by higher BMI but not by higher insulin levels and/or lower adiponectin levels. Negative associations after adjustment for BMI suggest that android fat deposition as measured by the WHR, WTR and android-to-gynoid fat ratio is not beneficial and possibly even deleterious for bone health. The clinical relevance of these associations needs to be shown by studying their relationships with fractures.

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Chapter 4

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

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Bone 2007; 41:987-94

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 vitamins (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 ($ß$=0.09, p=1x10^{-8}) and riboflavin intake ($ß$=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 fractures.

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 pyridoxine [5-7].

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 cobalamin [8-11] or folate [12] are associated with increased BMD, decreased bone loss [13], 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 concentrations [15]. 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/cm^2).

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 antidiabetic 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 (μM/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 [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 encoded 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 humerus [25].

Vertebral fracture assessment

Both at baseline (1990-1993) and at the second follow-up visit (between 1997 and 1999) radiographs of the thoracolumbar 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 previously [26]. 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, and 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 method [19].

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 was the strongest predictor (*ß*=0.09, $p=1x10^{-8}$) and pyridoxine was a good predictor (*ß*=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 (*ß*=0.06, $p=1x10^{-5}$) and pyridoxine (*ß*=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

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
Dietary intakes	
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	
	ß†	*p*-value††	ß	*p*-value††	ß	*p*-value††	ß	*p*-value††
Riboflavin	0.082	$2.6x10^{-11}$	0.058	$7x10^{-6}$	0.10	$7x10^{-4}$	0.079	$6x10^{-5}$
Pyridoxine	0.061	$3x10^{-6}$	0.041	0.003	0.07	$4x10^{-4}$	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.
FN: femoral neck; LS: lumbar spine.
† Standardized beta coefficients,
†† 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	Starting model				Final Model			
	FN-BMD		LS-BMD		FN-BMD		LS-BMD	
	ß†	*p*-value ††	ß†	*p*-value††	ß†	*p*-value††	ß†	*p*-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	$4x10^{-4}$	-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 R^2 for final model; FN-BMD: (0.25, $p<0.001$); LS-BMD: (0.17, $p<0.001$).

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 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

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 (%)	ß †	Relative Risk (95% CI)	*p*-value††
Non-vertebral				
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
Fragility				
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
Vertebral				
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. General characteristics of age and energy-adjusted pyridoxine quartiles in 5304 men and women in the Rotterdam Study

	Quartile 1	*Quartile 2*	*Quartile 3*	*Quartile 4*	*p-value*[†]
	n=1326	n=1325	n=1327	n=1326	
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 ± .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

	Quartile 1	*Quartile 2*	*Quartile 3*	*Quartile 4*	*p-value*[†]
	n=1326	n=1325	n=1327	n=1326	
Mean pyridoxine intake (mg/day)	1.30	1.50	1.67	2.03	
Co-morbidity					
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)

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 adjustement for other nutritional factors.

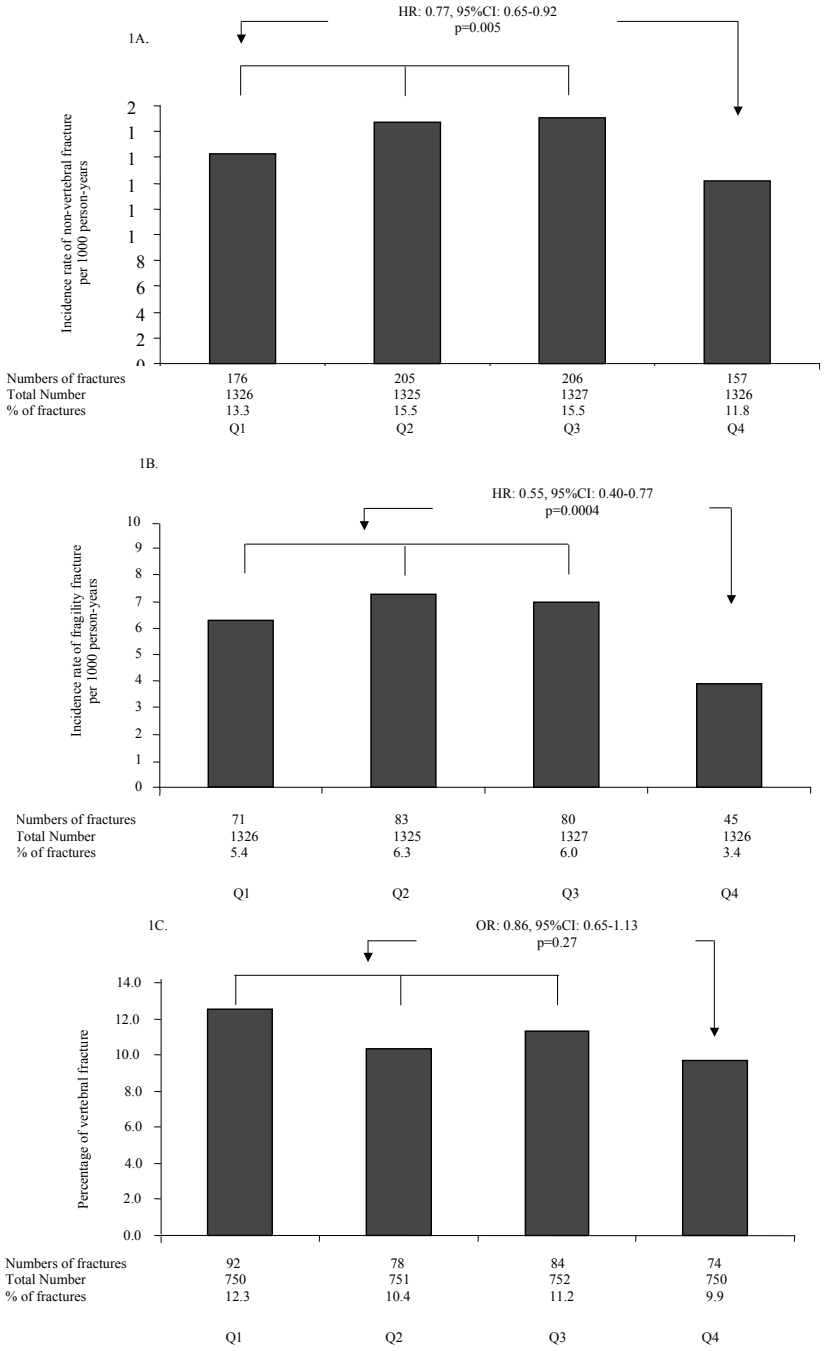

Figure 1. Incidence rate of (A) non-vertebral fracture and (B) fragility fracture and (C) percentage of vertebral fracture by quartiles of dietary pyridoxine intake.
HR: hazard ratio, CI: confidence interval, Q: quartile. Quartiles of dietary pyridoxine intake.

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. 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=4x10^{-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, 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).

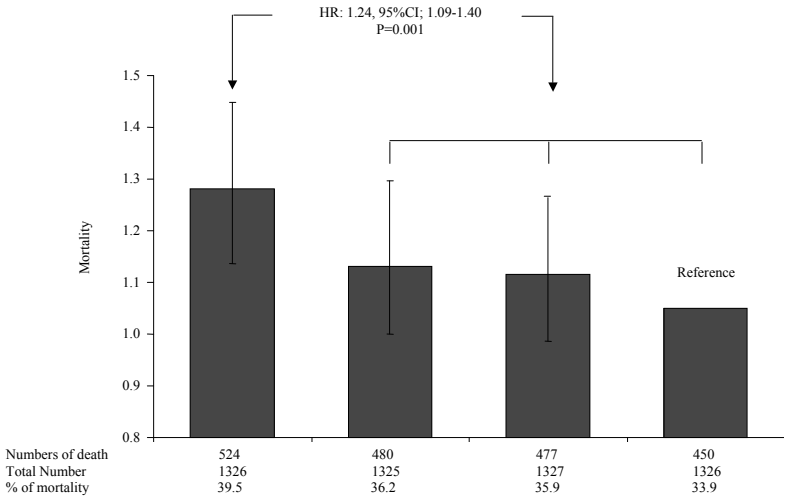

Figure 2. Risk of mortality by quartiles of dietary pyridoxine intake (highest quartile is reference), Gender and age adjusted

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.

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

Fractures	No.fractures/ Total no (%)	Relative risk	*p-value*†
Non-vertebral			
Model 1	101/739 (13.7)	0.56 (0.30-1.06)	0.08
Model 2		0.59 (0.31-1.13)	0.11
Fragility			
Model 1	42/739 (5.7)	0.37 (0.13-1.06)	0.06
Model 2		0.40 (0.13-1.16)	0.09
Vertebral			
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

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 levels [27]. 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 fracture [28] . 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 osteoclasts [29].

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 size-

able 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 folate [12] 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 vitamin 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. 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 bone [31]. Mice studies showed that pyridoxine deficiency results in a low amount of cross-link intermediates and impaired cross-link formation in bone [32]. In addition, a correlation was found between decreased circulating pyridoxine concentrations and impaired cross-link formation in bone of human individuals with fracture [33]. 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 elderly [35]. 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 5

SIRT1 genetic variation is related to body mass index and risk of obesity

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Diabetes (accepted)

ABSTRACT

Background SIRT1 has pleiotropic metabolic functions. We investigated whether *SIRT1* genetic variation is associated with obesity.

Methods In 6,251 elderly subjects from the prospective, population-based Rotterdam Study (RS), three Single Nucleotide Polymorphisms (SNPs) in the *SIRT1* gene were studied in relation with body mass index (BMI) and risk of obesity (BMI $\geq$ 30kg/m^2) and prospectively with BMI-change after 6.4 years follow-up. We used cross-sectional data from 2,347 participants from the Erasmus Rucphen Family (ERF) study for replication.

Results Minor alleles of rs7895833 (G=20.2%) and rs1467568 (A=36.8%) were associated with lower BMI in the RS (p=0.02 and 0.04) and in the replication cohort ERF (p=0.03 and 0.008) and in studies combined (p=0.002 for both SNPs) with 0.2-0.4 kg/m^2 decrease in BMI per allele copy. Carriers of these alleles had 13-18% decreased risk of obesity; odds ratio (95%CI) for rs7895833 the RS: 0.79 (0.67-0.94), p=0.007; in ERF: 0.93 (0.73-1.19), p=0.37 and in the studies combined 0.87 (0.77-0.97), p=0.02; for rs1467568 in the RS: 0.80 (0.68-0.94), p=0.007; in ERF: 0.85 (0.72-0.99), p=0.04 and in the studies combined: 0.82 (0.73-0.92), p=0.0009. In the RS, the two variants were also associated with lower BMI increase during 6.4 years of follow-up (p=0.01 and 0.08).

Conclusions Two common variants in *SIRT1* are associated with lower BMI in two independent Dutch populations. Carriers of these variants have 13-18% decreased risk of obesity and gain less weight over time. The availability of SIRT1 stimulators makes these findings relevant in light of the growing obesity epidemic.

INTRODUCTION

SIRT1 belongs to the Sirtuin protein family of nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetyleases, conserved in evolution from bacteria to humans. In lower organisms like yeast, flies, and worms, the silent information regulator Sir2 protein is related to longevity [1, 2] and to life span extension after caloric restriction [3-5]. Humans have 7 sirtuins (*SIRT1-7*) [6] of which SIRT1 has the highest homology to yeast Sir2. SIRT1 controls numerous physiological processes and protects cells against stress [2,7-13]. SIRT1 also has an important function in endocrine signaling, specifically in glucose and fat metabolism [14-18]. Increased hepatic SIRT1 activity enhances gluconeogenesis and inhibits glycolysis [15,16]. In the pancreas, SIRT1 stimulates insulin secretion in response to glucose [17,18]. In adipose tissue, SIRT1 interacts with PPARγ to repress its transcriptional activity, leading to inhibition of adipogenesis during fasting and activation of lipolysis[14]. This results in fat loss, which is an important component of the effect of caloric restriction on longevity in mammals[14]. It is not known whether caloric restriction can extend lifespan in humans, but clear beneficial effects on cardiovascular risk factors have been described [19,20]. Fasting leads to up-regulation of SIRT1 in adipose tissue of mice, pigs and humans [21-23]. Based on findings in lower organisms, it has been hypothesized that stimulation of SIRT1 by agonists may mimic the beneficial effects of caloric restriction in mammals. Resveratrol, a naturally occurring SIRT1 agonist present in red wine and grapes, was indeed shown recently to prevent diet-induced obesity and insulin resistance in mice and to improve their survival on a high-calorie diet [24,25]. If activation of SIRT1 can result in loss of body fat without decreasing caloric intake, this could open the door for novel treatment and prevention strategies for obesity and related diseases. However, in humans, effects of SIRT1 stimulation have not been investigated *in vivo* and there is concern that generalized SIRT1 activation may also have pro-ageing or adverse health effects, due to potential pleiotropic and tissue dependent physiological functions [26-29].

Based on the above findings we hypothesized that genetic variation in *SIRT1* may influence BMI and the risk of obesity in humans. Previous smaller studies, which have examined the relation between variants in *SIRT1* and obesity, have led to inconsistent findings. A recent case control study of 1.068 obese patients and 313 normal weight controls, found a *SIRT1* SNP associated to obesity risk. Unexpectedly, male but not female carriers of the allele associated with lower obesity risk had increased visceral adiposity on CT-scans. This was observed only after adjustment for BMI [30]. Another recent small study investigating associations of *SIRT1* SNPs with metabolic response to lifestyle intervention found no association of four *SIRT1* SNPs in 917 overweight subjects with baseline BMI or with BMI change after 9 months follow-up [31]. No population-based studies with large sample size and replication are available on the association between *SIRT1* genetic variation, BMI, BMI change in time and risk of obesity. We therefore assessed the

association of variation in the *SIRT1* gene with BMI and the risk of being overweight or obese in the large cohort of elderly subjects of the Rotterdam Study (RS). For replication we used subjects from a genetically isolated population in the Netherlands, participating in the Erasmus Rucphen Family (ERF) study. In the RS, we also assessed prospectively the relation of *SIRT1* variants with BMI change on follow-up.

METHODS

The Rotterdam study

The Rotterdam study is a prospective, population-based cohort study among 7,983 persons aged 55 years and older from a district of Rotterdam, The Netherlands. The study was designed to investigate the incidence and determinants of chronic disabling diseases. Rationale and design have been described previously [32, 33]. Informed consent was obtained from each participant, and the Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study.

At baseline (1990–1993), all participants were interviewed and underwent extensive physical examination. At the second follow-up (1997–1999) these examinations were performed according to the same protocol.

The Erasmus Rucphen Family (ERF) study

This study is a family-based cohort study that is embedded in the Genetic Research in Isolated Populations (GRIP) program in the South West of the Netherlands. The aim of this program was to identify genetic risk factors in the development of complex disorders [34-36].

For the ERF study, 22 families that had at least five children baptized in the community church between 1850-1900 were identified with the help of genealogical records. All living descendants of these couples and their spouses were invited to take part in the study. Data collection started in June 2002 and was finished in February 2005. In this study, we focused on 2,347 participants for whom complete phenotypic, genotypic and genealogical information was available.

The Medical Ethics Committee of Erasmus Medical Center Rotterdam approved of both studies and informed consent was obtained from all participants.

Measurements

For the two studies identical protocols were used for assembling phenotypic and genotypic information. Height (cm) and weight (kg) were measured at the initial examination and in the Rotterdam study also at follow-up examinations, 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).

Genotyping

Three tagging single nucleotide polymorphisms (SNPs) rs7895833, rs1467568 and rs497849 were selected from the HapMap database (http://www.hapmap.org) that, together with constructed haplotypes, covered 100% of the common (minor allele frequency >10%) variation of the *SIRT1*gene in Caucasians. Genotyping of the *SIRT1* SNPs was performed by Taqman on genomic DNA isolated from peripheral leucocytes by standard salting-out procedures. 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. All used primers and probes are available on request.

Statistical Analyses

Hardy-Weinberg equilibrium of the three *SIRT1* SNPs was tested with the GENEPOP-package [37]. Subjects were grouped according to genotype for individual SNP alleles, and by allele copy number of haplotype alleles. We inferred multimarker haplotypes in the Rotterdam study only from these SNPs using the program Phase [38]. In the ERF study haplotypes were not determined because they could not be inferred with high certainty due to the complex pedigree structure.

Haplotype alleles were numbered in order of decreasing frequency in the population (Figure1). Subjects were grouped according to genotype. Genotype groups were based on allele copy number (0, 1 and 2, corresponding to non-carriers, heterozygote carriers and homozygote carriers, respectively, of the most common haplotype alleles).

The relation between BMI and *SIRT1* genotypes was assessed using linear regression analysis, assuming an additive model. The odds ratio of being overweight (BMI $\geq$ 25 kg/m^2) or obese (BMI $\geq$ 30 kg/m^2) compared to normal weight (BMI = 18.5-25 kg/m^2) was assessed using logistic regression. The 95% confidence intervals (CIs) of the odds ratios (ORs) were calculated as the exponent of the regression coefficient and its standard error. For the assessment of an association between *SIRT1* genotypes and BMI at follow-up in the RS we used BMI data of the second follow-up measurement, which was 6.4 $\pm$ 0.4

(mean ± SD) years after baseline. Linear regression analysis was also used for the association between *SIRT1* genotypes and BMI change from baseline to second follow-up.

In the Rotterdam study, genetic outliers as identified by the IBS clustering analysis clustering > 3 standard deviations away from the population mean were removed prior to the analyses. Therefore, this population is ethnically homogenous. *p*-values were corrected by the inflation factor using genomic control method [39]. In the Rotterdam study, lambda's (obtained after running genomewide association (GWA) analysis for these traits) were small: 1.049, 1.045 and 1.036 for BMI, the risk of overweight and the risk of obesity, respectively.

Since genetic association may be influenced by family relationships we performed the analyses in the ERF study with a polygenic model using a variance component approach with the pedigree information in SOLAR (Sequential Oligogenic Linkage Analysis Routine) (http://solar.sfbrgenetics.org). The program takes into account the familial relationships by estimating heritability from the pedigree data. Subsequently, analyses were corrected for residual genomic inflation, which was very small (lambda's 1.02, values obtained from GWAS).

We performed a meta-analysis using fixed effects on the results of the two cohorts using the software Review Manager (www.cc-ims.net/RevMan/RevMan5). We also performed meta-analysis for the risk of obesity using results from the Rotterdam and ERF studies, together with the published data from the study by Peeters [30] et al. We used the rs1467568 and rs7069102 SNPs which are in almost perfect LD (D'=0.99 and r^2=0.96) to meta-analyse results across studies. All analyses were adjusted for age and sex. The statistical analyses were performed using SPSS software, version 15.0 in the RS and with SOLAR software, package 2.1.2 in ERF.

RESULTS

General characteristics

In **Table 1**, baseline characteristics of the two study populations are presented. In both studies a slight majority of participants were females. BMI was 1.1% higher in subjects from ERF than in the RS at baseline.

SIRT1 genotype

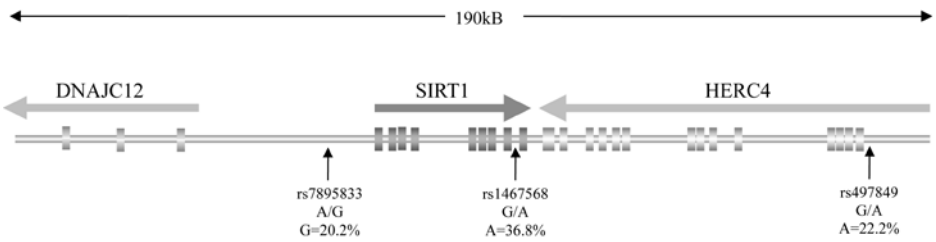
Figure 1 shows the schematic representation of the *SIRT1* locus with the localization of the three tagging SNPs and an LD plot of the SNPs (D' and r^2) as well as haplotype construction and observed frequencies of the haplotypes in the RS. In the RS, allele and

Table 1. General characteristics of the 2 study populations

	Rotterdam study	ERF study
Number	6251	2347
Women (n, %)	3665 (59 %)	1385 (55%)
Age (years)	69.1 ± 8.8	47.5 ± 13.8
range	55.1 – 99.0	17.6 - 85.3
Height (m)	1.67 ± 0.09	1.68 ± 0.09
Weight (kg)	73.2 ± 12.0	75.3 ± 15.4
BMI (kg/m^2) baseline	26.3 ± 3.7	26.6 ± 4.4
BMI (kg/m^2) follow-up (n ~ 3700)	26.8 ± 3.9	
BMI change (kg/m^2) follow-up - baseline	0.55 ± 1.8	
range	- 10.0 - +11.0	

Values are presented as number (percentage) or mean ± standard deviation.
BMI: body mass index

genotype distributions of the three tagging SNPs of *SIRT1* follow Hardy Weinberg equilibrium proportions (P>0.10). LD between the SNPs was high (D′> 0.8) which enabled us to infer multimarker haplotypes in the RS with high confidence. The haplotype frequencies of the four most common haplotypes were 40.9 %, 22.1 %, 20.0% and 16.7%. As shown in Figure 1, rs497849 fully tags haplotype 2 and rs7895833 fully tags haplotype 3. In the ERF study we genotyped the two SNPs showing association with obesity in the



A)

D' \ r^2	rs7895833	rs1467568	rs497849
rs7895833		0.41	0.07
rs1467568	0.98		0.16
rs497849	0.98	0.99	

D' 1 0 r^2 1 0

B)

Haplotype	rs7895833	rs1467568	rs497849	Frequency (%)
1	A	G	G	40.9
2	A	G	A	22.1
3	G	A	G	20.0
4	A	A	G	16.7

Figure 1. Schematic representation of the SIRT1 gene with the localization of the 3 tagging SNPs.
A) LD plot of the SNPs with estimates of r′ in the upper right corner and D′ in the left lower corner.
B) Haplotype construction and observed frequencies of the haplotypes in the Rotterdam study.

RS. These SNPs showed slightly different frequencies between the two studies but were also in Hardy Weinberg equilibrium proportions in ERF.

Relation of *SIRT1* variants with BMI

Table 2 shows the relationship of *SIRT1* SNPs with BMI in the RS at baseline and at the second follow-up examination and the replication data of two *SIRT1* SNPs in the ERF study. In the RS, two of the three SNPs, rs7895833 and rs1467568, had a significant association with BMI at baseline p=0.02 and 0.04 for the two SNPs at baseline, respectively, and p=0.02 for both SNPs at the follow-up examination with an allele-dose effect. Carriers of the minor alleles of the two SNPS had, on average, 0.2-0.3 kg/m^2 decreased BMI per allele copy. The third SNP (rs497849) that tags haplotype no. 2 showed no association with BMI. Haplotype analysis offered little additional information. For haplotype 1, which contains the major alleles for the two SNPs associated with BMI (rs7895833 and rs1467568), we observed a significant positive association with BMI that was stronger at the second follow-up (Beta=0.273, p=0.008) than at baseline (Beta=0.130, p=0.07). Haplotype 4 showed no association with BMI (data not shown).

Table 2. Associations of *SIRT1* genetic variants with BMI in the Rotterdam study (RS) and the ERF study

	MAF*	Difference in BMI per alle copy (kg/m^2,SE)	*p* trend	*p* combined**
rs7895833				
RS Baseline	0.20	-0.190 (0.08)	0.02	0.002
RS Follow-up	0.20	-0.291 (0.12)	0.02	
ERF study	0.23	-0.350 (0.16)	0.03	
rs1467568				
RS Baseline	0.37	-0.149 (0.07)	0.04	0.002
RS Follow-up	0.37	-0.238 (0.10)	0.02	
ERF study	0.41	-0.372 (0.14)	0.008	
rs497849				
RS Baseline	0.22	+0.030 (0.08)	0.70	
RS Follow-up	0.22	- 0.054 (0.11)	0.62	
Haplotype 1				
RS Baseline	0.41	+0.130 (0.07)	0.07	
RS Follow-up	0.41	+0.273 (0.10)	0.008	

* MAF: minor allele frequency. Minor allele rs 7895833: G; rs1467568: A; rs497849: A.
** *p* combined Rotterdam study baseline and ERF study
Analyses adjusted for age, sex, genomic control and in ERF also for family structure

Similar to the findings in the RS, carriers of the minor alleles of rs7895833 and rs1467568 had a lower BMI in the ERF study (p=0.03 and 0.008 for the two SNPs, respectively).

To exclude that associations between the SNPs and BMI were explained by a difference in body height we also analyzed the relation between the SNPs and height. No differences in height were seen between the genotypes (data not shown).

Combined analysis of relation of *SIRT1* variants with BMI in two studies

The combined analysis (Table 2) on the outcomes of the two studies RS baseline and ERF showed a highly significant association with BMI (p=0.002 for both SNPs).

SIRT1 variants and risk of overweight/obesity

Table 3 A presents the odds ratios (ORs) and 95% CI for the risk of being overweight (BMI $\geq$ 25 kg/m^2) compared to normal weight (BMI = 18.5-25 kg/m^2) for the two SNPs in the *SIRT1* gene in the RS at baseline and in the replication cohort of the ERF study. In the RS, carriers the minor alleles of rs7895833 had 12% decreased OR of being overweight compared to non-carriers of 0.88 (95%CI 0.79 - 0.99), p=0.03. For rs1467568, a similar, yet not significant, trend was seen with an OR of 0.91 (95%CI 0.82 - 1.02). Similar results were observed in the ERF study, with the strongest and most significant effect for rs1467568. At the second follow-up measurement of the RS (n=3,630) the risk of overweight in relation to *SIRT1* was similar to the risk at baseline but did not reach statistical significance (for rs7895833: 0.91 (95%CI 0.79-1.05), p=0.21; for rs1467568: 0.90 (95%CI 0.78-1.04), p=0.14, data not shown)

In **table 3B** the odds ratios and 95%CI for the risk of being obese (BMI $\geq$ 30 kg/m^2) compared to normal weight (BMI = 18.5-25 kg/m^2) are presented for 2 SNPS in the *SIRT1* gene in the two studies. In the RS, both SNPs were associated with the risk of being obese. Carriers of minor alleles of the two SNPs had a 20-21% decreased risk with an OR for rs7895833 of 0.79 (95%CI 0.67-0.94), p=0.007, and for rs1467568 of 0.80 (95%CI 0.68-0.94), p=0.007. Similar, trends were seen in the ERF population, only statistically significant for rs1467568. At the second follow-up measurement of the RS the association with risk of obesity was similar for carriers of the 2 SNPs (OR for rs7895833: 0.78 (95%CI 0.64-095), p=0.01; for rs1467568: 0.69 (95%CI 0.57-0.84), p=0.0002) (data not shown)

Combined analysis of the risk of overweight/obesity in two studies

Table 3 also presents the data from the meta-analysis of the risk of overweight or obesity for the two SNPs in the *SIRT1* gene in the Rotterdam study at baseline and the ERF study. Carriers of the minor alleles of two SNPs had 9-11% decreased risk of being overweight

Table 3. Risk of being overweight (A) or obese (B) in the Rotterdam study at baseline, ERF study and studies combined by *SIRT1* genotype

A: Overweight (BMI>25 kg/m²)

	genotype	no. cases/ controls	OR (95% CI)	*p* -d*	*p*-a	*p*-r	genotype	no. cases/ controls	OR (95% CI)	*p*-d	*p*-a	*p*-r
	rs7895833						rs1467568					
Rotterdam study	AA	2389/1390	1 (reference)				GG	1497/863	1 (reference)			
	AG+GG	1311/869	0.88 (0.79 - 0.99)	0.03	0.03	0.42	GA+AA	2207/1398	0.91 (0.82 - 1.02)	0.09	0.27	0.85
ERF study	AA	854/487	1 (reference)				GG	509/259	1 (reference)			
	AG+GG	559/359	0.95 (0.84 - 1.06)	0.35	0.08	0.01	GA+AA	910/593	0.88 (0.84 - 0.91)	0.02	$1.9x10^{-6}$	0.02
Studies combined			0.91 (0.84 - 0.99)	0.02	0.006	0.02			0.89 (0.83 - 0.97)	0.005	$3.5x10^{-6}$	0.06
B: Obese (BMI > 30kg/m^2)												
	rs7895833						rs1467568					
Rotterdam study	AA	577/1390	1 (reference)				GG	377/863	1 (reference)			
	AG+GG	282/869	0.79(0.67 - 0.94)	0.007	0.008	0.20	GA+AA	483/1398	0.80 (0.68- 0.94)	0.007	0.01	0.34
ERF study	AA	276/487	1 (reference)				GG	164/259	1 (reference)			
	AG+GG	161/359	0.93 (0.73 - 1.19)	0.37	0.23	0.25	GA+AA	277/593	0.85(0.72 - 0.99)	0.04	0.05	0.35
Studies combined			0.87 (0.77 - 0.97)	0.02	0.009	0.10			0.82 (0.73 - 0.92)	0.0009	0.002	0.20

*p-d: p value dominant model; p-a: p value additive model; p-r: p value recessive model

Analyses adjusted for age, sex, genomic control and in ERF also for family structure. BMI of controls 18.5-25 kg/m^2

compared to non-carriers, with OR of 0.91 (95%CI 0.84–0.99), *p*=0.02 (rs7895833) and OR=0.89 (95%CI 0.83-0.97), *p*=0.005 (rs1467568) with evidence for allele dose-effects (*p* additive model= 0.006 and $3{,}5x10^{-6}$ for rs7895833 and rs1467568, respectively). The risk for obesity was decreased by 13-18% (for rs7895833 OR 0.87 (95%CI 0.77-0.97), *p*=0.02 and for rs1467568 with OR= 0.82 (95%CI 0.73-0.92), *p*=0.0009, with allele dose effects (*p* values additive models 0.009 and 0.002 or rs7895833 and rs1467568, respectively). The *p* values for the recessive model were always lower than for the dominant or additive models.

Meta analysis with published data

Figure 2 shows a forest plot from the meta-analysis of risk of obesity for carriers compared to non-carriers of the A allele of rs1467568 in the Rotterdam and ERF study and the C allele of rs7069102 from the study by Peeters et al.[30] The A and C alleles of these SNPs are in high LD (r^2=0.96). The combined odds ratio for the three studies was 0.81 (95%CI 0.73-0.90), *p*=0.00007.

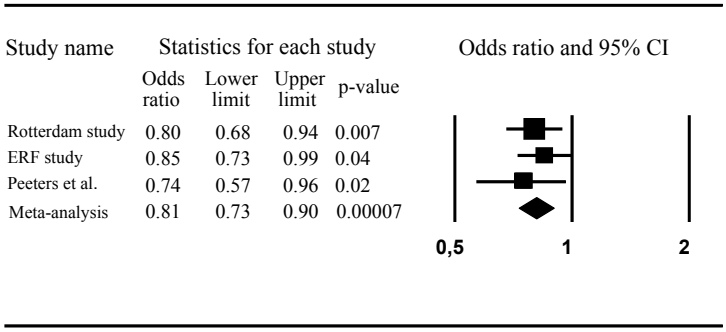


Figure 2.
Meta-analysis on the risk of obesity from three studies by *SIRT1* genotype for carriers compared to noncarriers of the A allele of rs1467568 in the Rotterdam and ERF study and the C allele of rs7069102 from the study by Peeters et al [30]

Relation of *SIRT1* variants with BMI change during follow-up in the Rotterdam study

Table 4 presents data from linear regression analysis of change in BMI or body weight from baseline to second follow-up for three SNPs in the *SIRT1* gene in the RS over a period of 6.4 years on average. The two SNPs rs7895833 and rs1467568 that were associated with BMI at baseline and follow-up and with the risk of overweight and obesity were also associated with BMI change at the second follow-up examination. After adjustment

for age and sex the *p* value for trend was 0.01 for rs7895833, and *p*=0.08 for rs1467568. Adjustment for BMI at baseline did not change these results (data not shown). Findings were similar when change of body weight instead of BMI was assessed.

Table 4. Linear regression of change in BMI or weight in the Rotterdam study for 3 SNPs in the *SIRT1* gene .

	n	beta (SE)	*p* trend*	*p* trend†
rs7895833	3585	-0.126 (0.05)	0.01	0.03
rs1467568	3598	-0.075 (0.04)	0.08	0.13
rs 497849	3622	-0.020 (0.05)	0.69	0.81

*: P trend for BMI change adjusted for age and sex

†: P trend for weight change adjusted for age and sex

DISCUSSION

In this study we show in two large and independent Dutch Caucasian populations that the minor alleles of two common *SIRT1* variants are associated with a decreased BMI. Carriers of these two common genetic variants had 9-11% decreased risk of being overweight and 13-18% decreased risk of being obese compared to non-carriers. In line with these findings, we also observed in the Rotterdam study, that carriers of these *SIRT1* variants had a smaller increase of BMI over a 6.4 year follow up period.

Our study is hypothesis driven and based on the known functions of SIRT1 in cell cultures and animal studies such as an inhibitory effect of SIRT1 on PPAR gamma in adipose tissue[14] and a stimulatory effect on peroxisome proliferator-activated receptor coactivator-1alpha (PGC-1α) [24, 25]. This powerful transcriptional co-activator is regarded as a key mediator of many of the known beneficial effects of physical activity on skeletal muscle physiology [40]. Treatment of mice with resveratrol leads to increased mitochondrial biogenesis and increased energy expenditure in mice, possibly by SIRT1 mediated increase in PGC-1α activity [25]. It is also possible that the effects on BMI are caused by an influence of SIRT1 on appetite and energy intake, since *SIRT1* is highly expressed in brain [41].

Interestingly, SIRT1 has recently been shown to modulate CLOCK-gene expression [42-44] and it may thus form an intriguing link between sensing of cellular metabolism and the circadian clock, which merits further study.

Inhibitory effects of SIRT1 on differentiation of skeletal myoblasts have been shown under glucose-restricted conditions in relation with activation of the AMP-activated protein kinase (AMPK) [45]. Therefore, we cannot exclude that effects on lean mass as well as on fat mass explain the relation with BMI that we observed. Further studies are needed to investigate the relation between *SIRT1* genetic variants and human body composition

traits and muscle strength. Yet, the strong association with the risk of obesity that we found makes a predominant effect on lean mass unlikely since obesity is associated more strongly with excess adipose tissue than with excess muscle mass.

This is the first large population-based study reporting an association between *SIRT1* genetic variation and BMI and obesity in humans with validation in an independent population-based cohort. Two recently published genetic studies in humans corroborate our findings. In a Belgian case-control study with 1.068 obese patients and 313 normal weight controls, carriers of the minor allele of the SNP rs7069102 (which is in high LD (r^2=0.96) with our SNP rs1467568) had a reduced obesity risk with an OR of 0.74 (CI 0.57-0.96, P 0.025) [30]. After including their results in a meta-analysis with our results the association became more significant and the combined *p*-value decreased from $9x10^{-4}$ to $7x10^{-5}$. In their study, the variant that was protective against obesity was associated with increased visceral obesity as measured by CT-scanning after adjusting for BMI in obese male but not female subjects. This unexpected finding may be caused by over-adjustment when the decrease in BMI is caused by a decrease in lean as well as in fat mass or represent a real sex-specific effect, which needs further investigation. A second recent study in 917 overweight German Caucasian subjects found no significant change in BMI by *SIRT1* genotype, yet we discovered in their data a similar decrease in BMI as we found for two SNPs in high LD with ours [31]. For carriers of the minor variants of rs7069102 (r^2=0.96 with rs1467568), BMI was 2.4% lower and for rs730821 (r^2=1.0 with rs7895833) BMI was 6.1% lower, compared to non-carriers. These differences were not significant, possibly because of low power. Recent genome-wide association studies (GWAS) have shown that common SNPs contributing to common complex diseases have modest effects and require large sample sizes to be discovered [46]. The absence of *SIRT1* among the genome-wide significant new findings in recent GWAS for BMI may be explained by the stringent criteria used to correct for multiple testing in GWAS and shows the added value of candidate gene analyses.

The strength of our study is the use of two large and independent population based cohorts with consistency of findings between studies and between cross-sectional and longitudinal analyses. The findings robustly show that *SIRT1* variants influence human obesity. A limitation of our study is the use of tagging SNPs. The SNPs we selected are non-coding and we therefore assume these two variants to be linked with one or more functional variants within the *SIRT1* gene or its regulatory regions. This will require more in depth molecular studies. Future fine-mapping and re-sequencing of the SIRT1 gene may detect such functional variants. Further studies into underlying mechanisms and body composition are also needed as well as prospective studies on a relation with obesity-associated diseases.

The results of our study may have important clinical implications. Obesity has become a global epidemic and represents an important risk factor for type 2 diabetes mellitus,

hypertension, cardiovascular disease, stroke, some types of cancer and disability. Few, if any, effective options for treatment and prevention are available. The findings of our study suggest that stimulators of SIRT1 activity may decrease BMI and the risk of becoming overweight and obese and may thus provide a valuable new strategy for treatment and prevention of obesity and its related diseases. An attractive aspect of our findings is that, in contrast to novel genetic findings from GWAS, many data exists on SIRT1 biological functions, while in addition several SIRT1 modifiers have already been identified. The protective effects of the SIRT1 stimulating flavenoid resveratrol against diet-induced obesity in mice may potentially apply to humans as well [25] but its effects are not only SIRT1 mediated [47, 48] and low bioavailability is a concern [49]. More research on pharmacology and possible side effects in humans is necessary for this compound as well as for recently developed more potent SIRT1 activators [50]. Alternatively, SIRT1 inhibitors might be of use in cachexia.

In summary, we found that carriers of two common variants in the *SIRT1* gene have lower BMI, a 13-18% decreased risk of being obese and less BMI gain in time. Together with results from recent studies these consistent findings warrant research into a potential role for SIRT1 activators in prevention and treatment of human obesity.

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Chapter 6

SIRT1 genetic variation and mortality in type 2 diabetes: interaction with smoking and dietary niacin

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Free Radic Biol Med. 2009;46:836-41

ABSTRACT

SIRT1 protects cells against oxidative stress and ageing. Its activity may be modulated by dietary niacin (vitamin B3) intake. We studied the association of *SIRT1* genetic variation with mortality in subjects with increased oxidative stress (type 2 diabetes and smokers) in relation with dietary niacin. In 4573 participants from the Rotterdam Study, including 413 subjects with prevalent and 378 with incident type 2 diabetes, 3 *SIRT1* tagging SNPs were genotyped and all-cause mortality was studied (average follow-up12 years). We found no association between *SIRT1* variation and mortality in the total population and smokers. In subjects with prevalent type 2 diabetes, homozygous carriers of the most common *SIRT1* haplotype 1 had 1.5 times (95%CI 1.1-2.1) increased mortality risk compared to noncarriers. This risk further increased among smokers and with low niacin intake. In the lowest tertile of niacin intake, mortality risk was increased 2.3 (1.1-4.9) and 5.7 (2.5-13.1) times for heterozygous and homozygous carriers of haplotype 1. Subjects with incident diabetes showed similar findings but only when they smoked. We conclude that in subjects with type 2 diabetes, *SIRT1* genetic variation influences survival in interaction with dietary niacin and smoking. Correction of niacin deficiency and SIRT1 modulators may prolong life of patients with diabetes.

INTRODUCTION

The silent information regulator Sir2 (an NAD^+-dependent histone deacetylase) protein is related to longevity in lower organisms like yeast, flies, and worms [1, 2]. Sir2 has also been implicated in life span extension during caloric restriction in these organisms [3-5]. The protein is highly conserved across species and humans have 7 sirtuins (*SIRT1-7*) [6]. Mammalian SIRT1 is most homologous to yeast Sir2 and has been studied most extensively. SIRT1 may protect cells against oxidative and genotoxic stress by binding to and deacetylating a large number of substrates, such as tumor suppressor p53 and forkhead transcription factors FOXO's [2]. Accordingly, SIRT1 plays a protective role in many cell types under various conditions of increased stress [7-13]. By regulating other transcription factors, SIRT1 is also involved in glucose and fat metabolism [14-18].

During the deacetylation reaction, SIRT1 consumes NAD^+ and produces nicotinamide that is a potent SIRT1 inhibitor [7, 19]. SIRT1 activity can thus be modulated by pathways that influence NAD^+, and/or nicotinamide levels [9, 20-22]. Nicotinamide and its acid form, nicotinic acid, are together known as vitamin B3 or niacin. Both serve as a precursor for the generation of cellular NAD^+, while only nicotinamide inhibits *SIRT1* activity.

Only a few human genetic association studies on *SIRT1* have been published so far. Variation in the *SIRT1* gene was not associated with longevity in a case-control study comparing long-lived individuals with younger subjects [23] and all-cause mortality was not influenced by *SIRT1* genetic variation in the Leiden 85-Plus study [24]. Among healthy non-diabetic offspring of type 2 diabetic patients, carriers of *SIRT1* polymorphisms had increased whole body energy expenditure [25].

In light of the role of *SIRT1* in protection against oxidative stress, we hypothesized that a chronic increase in oxidative stress is required to observe an association of genetic variation of *SIRT1*. Diabetes and smoking are well-known conditions with increased oxidative stress. Moreover, hyperglycemia in diabetes leads to activation of the DNA repair enzyme Poly (ADP-ribose) polymerase-1 (PARP-1), which also uses NAD^+ and produces nicotinamide and thus could reduce SIRT1 activity [12, 26-28]. SIRT1 expression in diabetes may also be decreased due to redox imbalance leading to a lower NAD^+ to NADH ratio[29]. In endothelial progenitor cells (EPCs), SIRT1 expression and activity was reduced by treatment with high glucose and SIRT1 was found to be a critical modulator of EPC dysfunction during alteration of glucose metabolism [30]. Cigarette smoke was shown to decrease SIRT1 levels and activity in rat lungs, possibly by oxidative stress [31] and SIRT1 levels are reduced in macrophages and lungs of smokers and patients with chronic obstructive pulmonary disease (COPD) [32]. Hence, in subjects with diabetes and in smokers SIRT1 expression and/or activity may be decreased while there is a high need for SIRT1 protection against oxidative stress.

In the large cohort of elderly subjects in the Rotterdam study we assessed the association of variation in the *SIRT1* gene with mortality in persons with type 2 diabetes mellitus and in cigarette smokers, according to dietary niacin intake.

RESEARCH DESIGN AND METHODS

The Rotterdam Study

The Rotterdam Study is a prospective, population-based cohort study of 7,983 persons aged 55 years and older from a district of Rotterdam, The Netherlands. The study was designed to investigate the incidence and determinants of chronic disabling diseases. Rationale and design have been described previously [33, 34]. Informed consent was obtained from each participant, and the Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study.

At baseline (1990–1993), all participants were interviewed and subsequently underwent extensive physical examination. Three follow-up examinations following to the same protocol took place in 1993–1994, 1997–1999, and 2002–2004. In addition, the cohort has been continuously monitored for major disease outcomes and mortality through computerized linkage of the study database to general practitioners' medical files. Procedures on follow-up of all-cause mortality and coding of cardiovascular disease (CVD) mortality was described previously [35]. Follow up data on overall mortality were available until September 1, 2006. Cardiovascular disease-specific mortality was based on data until January 1, 2004.

Assessment of diabetes mellitus type 2

Prevalent diabetes was defined as use of anti-diabetic medication, and/or abnormal non-fasting glucose, and/or an abnormal oral glucose tolerance test (OGTT). A Non-fasting or post-load glucose level of 11.1 mmol/L or over was considered abnormal.

During follow-up, incident cases of diabetes were diagnosed by use of information from the general practitioners, the pharmacies' databases, and our follow-up examinations. Based on guidelines of the American Diabetes Association and WHO we defined incident diabetes as follows: fasting plasma glucose level >= 7.0 mmol/l and/or random (non-fasting) plasma glucose level >= 11.1 mmol/l and/or use of oral anti-diabetic medication and/or use of insulin (but not type 1 diabetes) and/or treatment by diet and registered by a general practitioner as having diabetes.

Assessment of dietary variables and covariates

At baseline, dietary intake was assessed through an interview by a trained dietician, using an extensive, validated semi-quantitative food-frequency questionnaire (SFFQ) [36].

The amounts of food and drink intake indicated on the SFFQ were converted to energy intake and nutrient intake by means of the computerized Dutch Food Composition Table. For the current study, we used data on dietary intake of niacin (vitamin B3) (in mg/day), pyridoxine (vitamin B6), riboflavin (vitamin B2), vitamin C and E and total energy intake (in kcal/day), total protein intake (g/day), as well as data on use of B vitamin and multivitamin supplements. Dietary niacin intake was analyzed using the residual method to obtain sex-specific age- and energy-adjusted tertiles [37]. At baseline, smoking status was assessed during the interview and subjects were classified as current smokers or non-smokers of cigarettes.

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/m^2). Two standardized blood pressure measurements were taken by using a random zero sphygmomanometer, with the participant in sitting position, and averaged. Serum total cholesterol level was determined by an enzymatic procedure [38].

Genotyping

Three tagging single nucleotide polymorphisms (SNPs) were selected from the HapMap database (http://www.hapmap.org) that covered most of the common (minor allele frequency >10%) variation of the *SIRT1* gene in Caucasians: rs7895833, rs1467568 and rs497849. Genotyping of the *SIRT1* SNPs was performed by Taqman on genomic DNA isolated from peripheral leucocytes by standard salting-out procedures. The PCR reaction mixture included 2 ng of genomic DNA in a 2 μl volume and the following reagents: FAM and VIC probes (200 nM), primers (0.9 uM), 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 were performed in 384 wells 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. All used primers and probes are available on request.

Population for analysis

The study sample for the current study comprised 4573 independently living participants, with complete dietary data and genotyping information. A total of 1067 of these subjects were smokers at baseline, while 413 subjects had diabetes mellitus at baseline (prevalent diabetes) and 378 subjects developed diabetes mellitus at follow up (incident diabetes). Only subjects with minimal follow-up time of one year after the diagnosis of type 2 diabetes were studied to avoid interference from mortality due to underlying co-morbid conditions. Of the subjects with prevalent diabetes 97 were smokers at baseline and of those with incident diabetes 96.

Statistical Analyses

Hardy-Weinberg equilibrium of the *SIRT1* SNPs was tested with the GENEPOP-package [39]. We inferred 4 common (frequencies > 10%) multimarker haplotypes from these SNPs using the program Phase [40]. Haplotype alleles were numbered in order of decreasing frequency in the population (Figure 1). Subjects were grouped according to genotype. Groups were based on allele copy number (0, 1 and 2, corresponding to non-carriers, heterozygote carriers and homozygote carriers, respectively, of the most common haplotype alleles).

Cumulative survival was determined with Cox proportional hazards analyses. The 95% confidence intervals (CIs) of the hazard ratios (HRs) were calculated as the exponent of the regression coefficient and its standard error. The analyses were adjusted for age and sex and BMI. We performed separate analyses in subjects with prevalent and incident diabetes to test for consistency. Additional analyses were performed in tertiles of age- and energy adjusted intake of niacin. A relationship between intake of niacin and intake of protein and vitamin B2, B6, C and E was tested using partial correlations with adjustment for age, gender and energy intake. The data were reanalyzed after exclusion of users of B vitamin and multivitamin supplements (n = 540). We tested for statistical interaction between *SIRT1* haplotypes and smoking status and between *SIRT1* haplotypes and niacin intake in tertiles by adding multiplicative interaction terms to the model. A *p*-value of 0.05 was considered significant in all of the analyses. We chose not to make any corrections for multiple testing since the main hypothesis of increased oxidative stress being necessary for an effect of SIRT1 on mortality was tested in several subgroups of increased oxidative stress with the objective of showing consistency. The statistical analyses were performed using SPSS software, version 11.0.

RESULTS

General characteristics

In **Table 1**, baseline characteristics of the study population are presented for the total population and for subjects with prevalent and incident type 2 diabetes. The subjects with prevalent diabetes were 3 years older at baseline and had a higher prevalence of myocardial infarction compared to the total population and subjects with incident diabetes. Mean (SD) follow up time was 12.0 (3.8), 10.5 (4.3) and 13.0 (2.5) for the total population and the subjects with prevalent and incident diabetes, respectively. The diagnosis of incident type 2 diabetes was made after a mean (SD) follow up time of 6.5 (2.8) years.

Table 1. Baseline characteristics of the study population

	Total population	*Prevalent diabetes*[a]	*Incident diabetes*[b]
n	4573	413	378
Age, years	67.6 (7.7)	70.7 (7.6)	67.0 (6.8)
Women, (n, %)	2665 (58)	242 (58.5)	207 (54.8)
BMI, kg/m^2	26.3 (3.6)	27.0 (4.0)	28.3 (3.8)
Intake of energy, kcal/day	1976 (504)	1944 (465)	1933 (473)
Intake of proteins, g/day	81.4 (19.4)	83.4 (19.9)	81.7 (18.1)
Intake of niacin, mg/day[c]	16.1 (4.7)	16.0 (4.6)	16.0 (4.3)
Use of B vitamin or multivitamin (n, %)	540 (11.8)	49 (11.9)	41 (10.8)
Current smoking (n, %)	1067 (23.3)	97 (23.5)	87 (23.0)
Prevalent myocardial infarction (n, %)	556 (12.2)	76 (18.4)	49 (13.0)

data are mean (SD) or number (percentage)

[a] Type 2 diabetes at baseline;

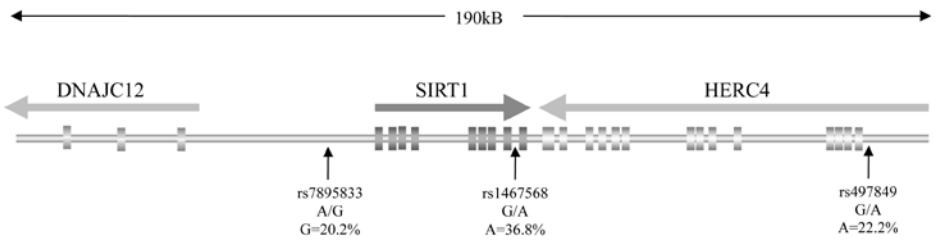
[b] Type 2 diabetes on follow-up

[c] The U.S. RDA for niacin is 20 milligrams per day.

Dietary niacin (vitamin B3) intake was not different between subjects with and without type 2 diabetes. Since it was significantly associated with age, gender, and energy intake we adjusted niacin intake for these variables. Niacin intake was also significantly correlated with other B-vitamins, especially with vitamin B6 (partial r = 0.57, $p < 0.001$) as well as with intake of protein, vitamin C and BMI (partial r = 0.52, 0.16 and 0.12, respectively, p values < 0.001), but not with the intake of vitamin E or with smoking (data not shown). We performed all analyses of niacin intake in sex-specific age- and energy-adjusted tertiles. Mean ± SD intake of niacin in the lowest, middle and highest tertile was 12.8 $\pm$ 3.1, 15.5 $\pm$ 2.8 and 19.8 $\pm$ 5.0 mg per day, respectively.

SIRT1 genotype

Allele and genotype distributions of the three tagging SNPs of *SIRT1* follow Hardy Weinberg equilibrium proportions in all groups (p>0.10). LD between the SNPs was high (D' > 0.8) which enabled us to infer multimarker haplotypes with high confidence. The haplotype frequencies of the four most common haplotypes were 40.9 %, 22.1 %, 20.0% and 16.7% (Figure 1). As shown in **Figure 1**, rs497849 fully tags haplotype 2 and rs7895833 fully tags haplotype 3. Haplotype 1 represents the major alleles of the three SNPs. Figure 1 shows the schematic representation of the *SIRT1* gene with the localization of the 3 tagging SNPs and an LD plot of the SNPs (D' and r') as well as haplotype construction and observed frequencies of the haplotypes.



A)

D'	rs7895833	rs1467568	rs497849
rs7895833		0.41	0.07
rs1467568	0.98		0.16
rs497849	0.98	0.99	

D' 1 0 r^2 1 0

B)

Haplotype	rs7895833	rs1467568	rs497849	Frequency (%)
1	A	G	G	40.9
2	A	G	A	22.1
3	G	A	G	20.0
4	A	A	G	16.7

Figure 1.
Schematic representation of the SIRT1 gene with the localisation of the 3 tagging SNPs.
A) LD plot of the SNPs with estimates of r' in the upper right corner and D' in the left lower corner.
B) Haplotype construction and observed frequencies of the haplotypes in the current study.

All-cause mortality by *SIRT1* genotype

No association was found between any of the four *SIRT1* haplotypes and all-cause mortality in the total population or in the group of smokers (data not shown). The mortality risk according to the number of copies of the most frequent haplotype 1 is shown in **Table 2**. Homozygosity for haplotype 1 increased all-cause mortality by 50% in subjects with prevalent type 2 diabetes (diabetes at baseline) (HR=1.5, 95% CI, 1.1-2.1). The smokers

among them had a higher risk estimate of 2.2 (95% CI, 1.0-4.7), but the interaction term between *SIRT1* haplotype 1 and smoking was not significant. In subjects with incident diabetes (diabetes on follow-up), significant excess mortality was also present among those who smoked (HR for heterozygotes: 2.2 (95% CI, 1.0-4.9); for homozygotes: 3.0 (95% CI, 1.1-8.2)) with a significant interaction between *SIRT1* haplotypes 1 and smoking (p=0.008). All analyses were adjusted for age, gender, and BMI at baseline.

Table 2. Risk of mortality by SIRT1 haplotype 1 in the total population, in smokers and in subjects with diabetes

	No. of Person-years	No. of Deaths	HR (95%CI)	No. of Person-years	No. of Deaths	HR (95%CI)
	Total population (n:4573)			*Current smokers (n:1067)*		
0[a]	18,984	611	1 (Reference)	4,490	151	1 (Reference)
1	27,152	862	1.0 (0.9-1.1)	6,445	240	1.1 (0.9-1.4)
2	8,815	276	1.0 (0.9-1.2)	2,083	73	1.1 (0.8-1.5)
	Prevalent diabetes[b] (n:413)			*Incident diabetes[c] (n:378)*		
0[a]	1,548	79	1 (Reference)	1,585	40	1 (Reference)
1	2,001	109	1.1 (0.8-1.5)	2,600	79	1.0 (0.7-1.4)
2	806	56	1.5 (1.1-2.2)*	735	16	0.8 (0.4-1.4)
	Prevalent diabetes non-smoking (316)			*Prevalent diabetes smoking (n:97)*		
0[a]	1,220	64	1 (Reference)	331	15	1 (Reference)
1	1,534	82	1.0 (0.7-1.4)	459	27	1.7 (0.9-3.3)
2	592	40	1.4 (0.9-2.0)	214	16	2.2 (1.0-4.7)*
	Incident diabetes non-smoking (291)			*Incident diabetes smoking (n: 87)*		
0[a]	1,192	31	1 (Reference)	393	9	1 (Reference)
1	2,086	53	0.8 (0.5-1.2)	508	26	2.2 (1.0-4.9)
2	559	9	0.5 (0.2-1.1)	176	7	3.0 (1.1-8.2)*

All analyses adjusted for sex and age and BMI

[a] 0, 1 and 2 denote number of copies of haplotype 1

[b] Type 2 diabetes at baseline

[c] Type 2 diabetes on follow-up

* P trend: $p< 0.05$

Effect of dietary niacin intake

We repeated the analyses in sex-specific age- and energy-adjusted tertiles of niacin intake. In the total population, *SIRT1* haplotype 1 was not associated with mortality in any of the 3 tertiles of niacin intake. **Table 3** summarizes the results for the lowest tertile of dietary niacin. Looking at the smokers in general, we found 50% increase in the mortality risk (95% CI, 1.1-2.1) in the lowest tertile of niacin intake that was significant for heterozygotes and for carriers versus noncarriers of haplotype 1**.**

Table 3. Risk of mortality by *SIRT1* haplotype 1 in the lowest tertile of niacin intake in the total population, in smokers, and in subjects with diabetes

	No. of Person Years	No. of Deaths	HR (95%CI)	No. of Person Years	No. of Deaths	HR (95%CI)
	Total population (n:1524)			*Current smokers (n:367)*		
0[a]	6,324	193	1 (Reference)	1,648	52	1 (Reference)
1	9,068	306	1.1 (0.9-1.3)	2,189	95	1.5 (1.1-2.1)
2	2,844	82	1.1 (0.8-1.4)	666	24	1.5 (0.9-2.5) *
	Prevalent diabetes[b]: (n: 110)			*Incident diabetes[c] (n:121)*		
0[a]	413	9	1 (Reference)	529	14	1 (Reference)
1	583	30	2.3 (1.1- 4.9)	800	26	1.1 (0.8-2.2)
2	199	19	5.7 (2.5-13.1) ***	204	8	1.9 (0.8-4.7)
	Prevalent diabetes non-smoking (n:84)			*Prevalent diabetes smoking (n:26)*		
0[a]	313	8	1 (Reference)	102	1	1 (Reference)
1	456	24	1.8 (0.8-4.0)	124	6	10.7 (1.0-111)
2	118	14	5.6 (2.2-14.2) **	80	5	9.9 (0.7-133)
	Incident diabetes non-smoking (n:90)			*Incident diabetes smoking (n:31)*		
0[a]	403	11	1 (Reference)	127	3	1 (Reference)
1	616	13	0.7 (0.3-1.7)	181	13	4.9 (1.3-19.4)
2	145	5	1.5 (0.5-4.6)	59	3	3.1 (0.6-16.3)

All analyses adjusted for sex and age and BMI

[a] 0, 1 and 2 denote number of copies of haplotype 1

[b] Type 2 diabetes at baseline

[c] Type 2 diabetes on follow-up

* P trend: $p < 0.05$, ** P trend < 0.001, *** P trend: <0.0001

In subjects with prevalent diabetes, a significant interaction was observed between niacin intake in tertiles and *SIRT1* haplotype 1 (p=0.003). In the lowest tertile of niacin intake, the subjects with prevalent diabetes had a 2.3 (95% CI, 1.1- 4.9) times increased risk of mortality when they were heterozygous for haplotype 1 and a 5.7 (95% CI, 2.5-13.1) times increased risk when they were homozygous (p for trend = 0.00002). The smokers among them tended to have a higher point estimate than the non-smokers but confidence intervals were very wide as a result of the low numbers (HR:10.7, 95% CI,1.0-110 for heterozygous carriers who smoke).

When we examined the three SNPs individually, we observed decreased mortality risk for carriers of the minor alleles for all three SNPs in subjects with prevalent diabetes and low niacin intake, most marked for rs1467568 and rs7895833 (data not shown). The effects were stronger (and in opposite direction) for carriers of haplotype 1.

Figure 2 shows a Kaplan-Meier curve with the mortality of the persons with prevalent type 2 diabetes in the lowest tertile of niacin intake according to *SIRT1* haplotype 1

genotype. In the middle and highest tertile of niacin intake no excess mortality was found in relation to *SIRT1* haplotype 1 (**Figure 3**).

Figure 2.
Kaplan-Meier curve of mortality in subjects with prevalent diabetes in the lowest tertile of niacin intake by *SIRT1* haplotype 1 genotype.

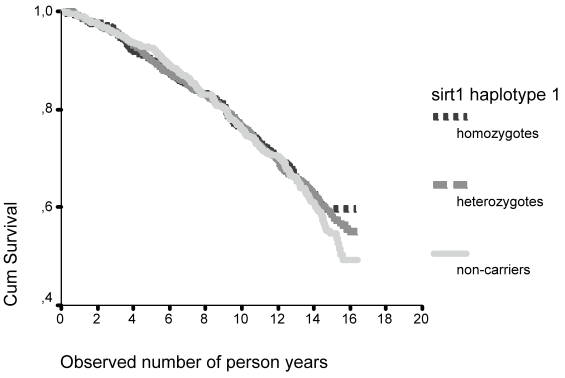


Figure 3.
Kaplan-Meier curve of mortality in subjects with prevalent diabetes in the middle and highest tertile of niacin intake by *SIRT1* haplotype 1 genotype.

In subjects with incident diabetes, the risk of mortality was not significantly higher for carriers of *SIRT1* haplotype 1 in the lowest niacin tertile, although there was a trend in the same direction as for the subjects with prevalent diabetes **(Table 3).** However, when they smoked the risk of mortality was also significantly higher in the lowest tertile of niacin intake (HR for heterozygotes: 4.9 (95% CI,1.3-19.4), for homozygotes: 3.1 (95% CI, 0.6 –16.3), for carriers compared to noncarriers: 4.3 (95% CI, 1.2-15.6)).

The results were not substantially different after adjusting for other risk factors of cardiovascular disease (blood pressure and cholesterol level) and for intake of protein and other vitamins (B6, E and C) and when niacin containing supplement users were excluded from the analyses (data not shown).

Cardiovascular mortality

The homozygous carriers of *SIRT1* haplotype 1 with prevalent diabetes had 1.9 times higher cardiovascular disease mortality risk than noncarriers (95% CI, 1.1-3.2). We also found a significant interaction (p=0.02) in the persons with prevalent type 2 diabetes between niacin intake in tertiles and *SIRT1* haplotype 1 for cardiovascular mortality. In the lowest tertile of niacin intake, the HR was 2.0 for heterozygous carriers (95% CI, 0.4-9.9) and 9.7 for homozygous carriers (95% CI, 2.8-45) compared with noncarriers, p trend =0.0004.

DISCUSSION

In this prospective population-based study, subjects with prevalent type 2 diabetes had a 50% higher risk of all-cause mortality and in particular cardiovascular mortality when they were homozygous carriers of the most common *SIRT1* haplotype 1 compared to noncarriers. This risk further increased among smokers and in those with low dietary niacin intake. In the lowest tertile of niacin intake, they had a 2.3 and 5.7 times increased risk of death when they were heterozygous or homozygous for *SIRT1* haplotype 1, respectively. In the subjects with incident type 2 diabetes, we also found higher mortality for carriers of this haplotype but only for the smokers among them, with a further increase of the risk in the lowest tertile of niacin intake. The absence of an association with mortality in the total population is in line with data from two previous studies [23, 24] and supports our hypothesis that increased oxidative stress may be necessary to show an effect of genetic variation in *SIRT1* on mortality. Diabetes duration can strongly influence the outcome of genetic studies of complications of diabetes [41]. Therefore, we decided to study prevalent and incident cases separately and use incident cases to show consistency of our findings. The results suggest that even in incident cases, with shorter duration of diabetes by definition, a detectable effect from the *SIRT1* haplotype 1 risk allele can be observed when they are exposed to an additional source of oxidative stress like smoking of cigarettes. From our data we cannot conclude whether differences in mortality by the *SIRT1* haplotype 1 were associated with increased or decreased SIRT1 activity. Based on the known cell-protective data in animals and *in vitro* studies, our results would predict that carriers of the *SIRT1* haplotype 1 had reduced SIRT1 activity,

influencing survival only under adverse circumstances. In the Leiden 85-Plus study (24) heterozygous carriers of the rs3758391 minor allele had lower cardiovascular mortality risk, although this was considered not significant because multiple end-points were tested in this study. Since this SNP is in high LD with SNP rs1467568 (r' 0.96) this finding is in line with the decreased mortality risk that we found in the subjects with diabetes and low niacin intake carrying the minor allele of rs1467568 and the increased mortality risk for carriers of haplotype 1.

We studied the effect of dietary niacin intake because of its potential involvement in SIRT1 enzymatic activity, as a precursor of NAD^+ that SIRT1 needs, while nicotinamide is a strong inhibitor of SIRT1 activity. We found effects of *SIRT1* genotype on mortality only in the lowest tertile of niacin intake. In theory, this could be explained by a decreased inhibition of SIRT1 by low nicotinamide intake. A more likely explanation is that low niacin intake further reduced SIRT1 activity due to lower NAD^+ production. This might be particularly relevant when NAD^+ is used by the DNA repair enzyme Poly (ADP-ribose) polymerase-1 (PARP-1). Increased activation of this enzyme due to high oxidative stress in diabetes is considered an important factor in the pathogenesis of endothelial dysfunction and other diabetic complications [27].

Alternatively, an overall lower dietary anti-oxidant content in the lowest tertile of niacin intake might explain the effects we found, although our findings did not disappear after adjustment for the vitamins with anti-oxidant properties associated with niacin intake (vitamin B6 and C). Good sources of niacin are meat (especially liver and heart), fish, nuts and some fruits and vegetables and coffee. Although protein intake was associated with niacin intake, adjustment for it did not influence the mortality data.

Methodological strengths of this study include the prospective design with validated dietary assessments and virtually complete follow up with a large number of person-years. Among the methodological constraints of our study are the limited statistical power to perform stratified analyses and the possibility of misclassification of nutrient intake inherent to food frequency questionnaires. This might however only be expected to weaken a true interaction between dietary intake and genotype. Only baseline dietary intakes were available and dietary intake may not necessarily correspond to actual levels of B vitamins. Unfortunately current methods of assessing niacin status by measurement of blood nicotinamide or urinary excretion of niacin metabolites are considered unsatisfactory [42]. Nevertheless, dietary intakes are quite stable in Europe, especially among elderly [43] and so a single validated food frequency test is considered as a good measure for long-term assessment of nutrient intake.

Although our results are very consistent, we cannot exclude population specific factors that were unaccounted for and this makes replication in other diabetes populations necessary. The findings may have important clinical implications. First, that niacin deficiency appears undesirable in subjects with type 2 diabetes and may require correction,

especially in carriers of *SIRT1* haplotype 1. Second, that smoking seems extra deleterious for carriers of *SIRT1* haplotype 1 with type 2 diabetes. Last, the findings suggest that high mortality in a disease that has reached epidemic proportions worldwide may potentially be modified by actions that interfere with SIRT1 activity and do justify further investigations into SIRT1 stimulators like resveratrol on morbidity and mortality in patients with diabetes. This flavenoid, present in red wine and grapes, was recently shown to prevent diet-induced obesity and insulin resistance in mice and improve survival in mice on a high-calorie diet [25, 44]. It also protected endothelial cells in rats against cigarette smoking-induced oxidative effects, most likely in a SIRT1 dependent way [45]. Resveratrol, however, also has many SIRT1 independent effects and it has low bioavailability [46]. Novel and more potent activators of SIRT1 were recently identified, that improve glucose homeostasis of insulin-resistant animals [47]. Whether these compounds have therapeutic potential in situations with reduced SIRT1 expression and/or activity and increased oxidative stress like diabetes and smoking can be studied once data on pharmacology and safety in humans are available.

In summary, in subjects with type 2 diabetes *SIRT1* genetic variation influences survival in interaction with dietary niacin intake and cigarette smoking, most likely through an effect on cardiovascular mortality. Correction of niacin deficiency and modulators of SIRT1 activity may increase survival of patients with diabetes.

ABBREVIATIONS

BMI, Body mass index; LD, Linkage Disequilibrium; (PARP-1), Poly (ADP-ribose) polymerase-1; NAD^+, Nicotinamide Adenine Dinucleotide; SIRT, Silent Information Regulator Two; SNP, Single Nucleotide Polymorphism.

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Chapter 7

Interactions between diet and *SIRT1* genetic variation influence body mass index

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Submitted

ABSTRACT

Background Variation in the *SIRT1* gene has been associated with body mass index (BMI) and risk of obesity. Since dietary factors may influence SIRT1 activity we studied gene-diet interaction on BMI at the *SIRT1* locus.

Methods In 4,575 elderly men and women of the prospective, population-based Rotterdam Study (RS), the effect of *SIRT1* genetic variants was studied on BMI in relation with dietary intake of energy, fat, calcium, milk, antioxidant vitamins and niacin as precursor for NAD^+. We used cross-sectional data from 2,566 participants from the Erasmus Ruchphen Family (ERF) study for replication of findings with milk intake.

Results There was no difference in energy and fat intake by *SIRT1* genotypes. Significant associations of the *SIRT1* genetic variants with BMI were found in the lowest tertiles of intake of energy, fat and vitamins and in the highest tertile of calcium and milk intake. Interaction terms with the *SIRT1*variants were significant for intake of vitamin E (p=0.06, 0.005 and 0.0005 for rs7895833, rs1467568 and haplotype 1, respectively) and calcium (p=0.02, 0.01 and 0.13 for rs7895833, rs1467568 and haplotype 1, respectively). In ERF, we found associations between the two SNPs rs7895833, rs1467568 and BMI in the highest tertiles of milk intake.

Conclusions Dietary intake of anti-oxidants, especially Vitamin E, and of calcium and milk modify the associations of *SIRT1* variants with BMI. These data support that gene-diet interactions influence BMI. Additional replication of our findings and further in depth studies of specific dietary patterns that modify SIRT1 may lead to clinical studies with dietary modification of SIRT1 to influence obesity.

INTRODUCTION

Obesity has become a global epidemic and represents an important risk factor for many diseases such as type 2 diabetes mellitus, hypertension, cardiovascular disease, stroke, some types of cancer and disability. The growing prevalence of obesity is most likely driven by increasing caloric intake and decreasing energy expenditure by physical activity, but individual susceptibility varies widely and is strongly influenced by genetic factors. Twin, adoption and family studies have shown that between 40 and 80% of inter-individual variation of body mass index (BMI) is heritable [1-3]. A lot of genetic association studies on obesity traits have been published but only a few have been consistently replicated [4, 5]. Early genome-wide association studies (GWAS), identified both FTO and *MC4R* as genes related to BMI [6, 7]. Several new loci have been identified in three recent obesity related GWAS studies [8-10]. Collectively, these variants explain only a small proportion of the genetic influence of obesity [8-10]. It is possible that interactions between genes and between genes and environmental factors such as diet and physical activity explain part of the so far unexplained genetic variance of complex traits like BMI. We recently demonstrated that genetic variation in the *SIRT1* gene is associated with BMI and risk of obesity (Zillikens et al, manuscript under review). SIRT1 belongs to the Sirtuin protein family of nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetyleases. In lower organisms like yeast, flies, and worms, the silent information regulator Sir2 protein is related to longevity [11, 12] and to life span extension after caloric restriction [13-15]. The closest Sir2 human homologue SIRT1 controls numerous physiological processes, protects cells against stress, such as oxidative stress [16-18] and has an important function in endocrine signaling, specifically in glucose and fat metabolism [19-22]. The NAD^+ dependence of SIRT1 links it activity to the metabolic state of an organism. Thus we hypothesized that dietary factors that regulate SIRT1 could modify the association of *SIRT1* genetic variants with BMI. Fasting leads to up-regulation of SIRT1 in adipose tissue of mice, pigs and humans [23-25] and the SIRT1 stimulator resveratrol protects mice against a high-fat diet [26, 27]. NAD^+ is synthesized from the precursors nicotinic acid and nicotinamide, collectively known as niacin or vitamin B3 while a small part of cellular niacin is formed *de novo* from the amino-acid tryptophan [28]. Recently, a newly discovered NAD^+ precursor in milk called nicotinamide riboside was found to stimulate SIRT1 activity, [29, 30]. Thus, intake of precursors of NAD^+ may influence SIRT1 activity.

Moreover, since SIRT1 protects cells under conditions of oxidative stress and is also regulated itself by oxidative stress [31], we hypothesized that SIRT1 function is influenced by dietary intake of anti-oxidants. Based on these potential influences of dietary factors on SIRT1, we investigated in the large cohort of elderly subjects of the Rotterdam study (RS) whether interaction between *SIRT1* genetic variation and diet influences

BMI. Specifically, we studied a relation with dietary intake of energy, fat, calcium, milk, antioxidant vitamins (beta-carotene, vitamin C and E) and niacin.

To replicate findings of milk consumption we used data from subjects from a genetically isolated population in the Netherlands, participating in the Erasmus Rucphen Family (ERF) study.

METHODS

The Rotterdam Study

The Rotterdam study is a prospective, population-based cohort study among 7,983 persons aged 55 years and older from a district of Rotterdam, The Netherlands. The study was designed to investigate the incidence and determinants of chronic disabling diseases. Rationale and design have been described previously [32, 33]. Informed consent was obtained from each participant, and the Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study. At baseline (1990–1993), all participants were interviewed and subsequently underwent extensive physical examination. Three follow-up examinations following to the same protocol took place in 1993–1994, 1997–1999, and 2002–2004

The Erasmus Rucphen Family (ERF) study

This study is a family-based cohort study that is embedded in the Genetic Research in Isolated Populations (GRIP) program in the South West of the Netherlands. The aim of this program was to identify genetic risk factors in the development of complex disorders [34-36].

For the ERF study, 22 families that had at least five children baptized in the community church between 1850-1900 were identified with the help of genealogical records. All living descendants of these couples and their spouses were invited to take part in the study. Data collection started in June 2002 and was finished in February 2005. In this study, we focused on 2,566 participants for whom complete phenotypic, genotypic and genealogical information was available.

The Medical Ethics Committee of Erasmus Medical Center Rotterdam approved of both studies and informed consent was obtained from all participants.

Assessment of dietary variables

In the Rotterdam study dietary intake was assessed at baseline, through an interview by a trained dietician, using an extensive, validated semi-quantitative food-frequency questionnaire (SFFQ) [37].

The amounts of food and drink intake indicated on the SFFQ were converted to energy intake and nutrient intake by means of the computerized Dutch Food Composition Table. For the current study, we used data on dietary intake of energy (in kcal/day), macronutrients (fat, protein and carbohydrates in g/day), calcium, milk and the vitamins beta-carotene (vitamin A), vitamin C and vitamin E and niacin (vitamin B3) (in mg/day). Also at baseline, we recorded data on the use of vitamin supplements. Dietary intake was analyzed using the residual method to obtain sex-specific energy-adjusted tertiles and when intake was associated with age we used age- and energy-adjusted tertiles [38]. Tertiles of sex-specific energy intake were created with and without adjustment for body weight. In ERF, participating subjects were asked in a home questionnaire for the current average amount of glasses of milk they drink per day. Since no information was obtained on total caloric intake, sex-specific tertiles of milk intake were constructed after adjustment for body weight.

Measurements

For the two studies identical protocols were used for assembling phenotypic and genotypic information. Height (cm) and weight (kg) were measured 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).

Genotyping

Three tagging single nucleotide polymorphisms (SNPs) rs7895833, rs1467568 and rs497849 were selected from the HapMap database (http://www.hapmap.org) as described previously [39]. Together with constructed haplotypes, they covered 100% of the common (minor allele frequency >10%) variation of the *SIRT1*gene in Caucasians. Genotyping of the *SIRT1* SNPs was performed by Taqman as described earlier [39]. For the current study, data were analysed for the 2 SNPs (rs7895833, rs1467568) and the most common haplotype 1, which were associated previously with BMI and risk of obesity (Zillikens et al, Manuscript under review).

Statistical Analyses

Hardy-Weinberg equilibrium of the two *SIRT1* SNPs was tested with the GENEPOP-package [40]. Subjects were grouped by genotype for individual SNP alleles, and by allele copy number of haplotype 1. In the Rotterdam study, multimarker haplotypes were inferred from the three SNPs using the program Phase [41]. **Figure 1** (chapter 6 page 100) shows the schematic representation of the SIRT1 locus with the localization of the three tagging SNPs and an LD plot of the SNPs (r' and R^2) as well as haplotype construction and observed frequencies of the haplotypes in the RS. As described earlier (Zillikens et al, manuscript under review), in the Rotterdam study, allele and genotype distributions of the three tagging SNPs of SIRT1 follow Hardy Weinberg equilibrium proportions (p>0.10). LD between the SNPs was high (D' > 0.8) which enabled us to infer multimarker haplotypes in the Rotterdam study with high confidence. The haplotype frequencies of the four most common haplotypes were 40.9 %, 22.1 %, 20.0% and 16.7%. In the ERF study only the two SNPs (rs7895833 and rs1467568) were genotyped that previously showed association with BMI in the RS (Zillikens et al, manuscript under review). These SNPs showed slightly different frequencies between the two studies but were also in Hardy Weinberg equilibrium proportions in ERF. In the ERF study haplotypes were not determined because they could not be inferred with high certainty due to the complex pedigree structure.

The relation between BMI and *SIRT1* genotypes was assessed using linear regression analysis, assuming an additive model. In the ERF study, the *p* values were corrected by the inflation factor using genomic control method because of the familial relationships [42]. We tested for statistical interaction between SIRT1 variants and nutrient intake in tertiles by adding multiplicative interaction terms to the model. All analyses were adjusted for age and sex. The statistical analyses were performed using SPSS software, version 15.0. A p value of < 0.05 was considered statistically significant, while we considered Bonferroni correction for multiple testing of 8 independent dietary variables and 3 genetic variants (significant p values below 0.002). This correction might be too stringent for the current study since our genetic and dietary variables are not totally independent.

RESULTS

General characteristics

In **Table 1**, baseline characteristics of the two study populations are presented. In both studies a slight majority of participants was female. BMI was 1.5% higher in subjects from ERF than in the RS at baseline. For the subjects of the Rotterdam study, dietary

intakes of energy and fat and micronutrients are presented. For the subjects from the ERF study only intake of milk is presented.

Table 1. General and body composition characteristics of the 2 study populations

	Rotterdam study	ERF study
Number	4,575	2,566
Women (n, %)	3191 (59.1 %)	1321 (55.5%)
Age (years)	67.7 ± 7.8	48.3 ± 14.2
range	55.0 – 94.5	16.7 - 86.1
Height (m)	1.67 ± 9.2	1.68 ± 9.4
Weight (kg)	73.6 ± 11.7	75.3 ± 14.8
BMI (kg/m2)	26.3 ± 3.7	26.7 ± 4.4
Users of vitamin supplements (n,%)	775 (17%)	NA
Energy intake (kJ/day)	8259 ± 2110	NA
Energy intake (kcal/day)	1974 ± 504	NA
Fat intake (g/day)	80.6 ± 27.4	NA
Betacarotene intake(mg/day)	2.71 ± 1.66	NA
Vitamin C intake(mg/day)	120.7 ± 54.2	NA
Vitamin E intake(mg/day)	13.8 ± 6.2	NA
Niacin intake (mg/day)	16.3 ± 4.7	NA
Calcium intake (mg/day)	1127 ± 395	NA
Milk intake (ml/day)	397 ± 254	254 ± 278

Data are mean + SD or numbers (percentage).

Effect of intake of vitamin supplements on the association of *SIRT1* variants with BMI

Table 2 shows the relationship between the two *SIRT1* SNPs rs7895833 and rs1467568 and haplotype 1 and BMI in the Rotterdam study. For the current sample of 4,575 subjects with complete dietary and genotype information the association between *SIRT1* genetic variation and BMI was significant only for haplotype 1 (p= 0.03) with carriers having higher BMI. For rs7895833 and rs1467568 the relations were of borderline significance in this smaller sample of the original study with carriers of the minor alleles having lower BMI than non-carriers. Effect sizes were stronger and p values were lower for all three *SIRT1* variants when the analysis was limited to the non-users of vitamin supplements. Among the users of vitamin supplements (n = 775) there was no association between *SIRT1* variants and BMI.

Table 2. BMI by *SIRT1* genotype in all subjects and stratified by use of vitamin supplements in the Rotterdam study

		genotype			beta (SE)	*p* trend
rs7895833		AA	AG	GG		
all subjects	No.	2,898	1,489	188		
	BMI	26.4 (0.07)	26.2 (0.09)	26.2 (0.26)	-0.126 (0.09)	0.18
non-users of supplements	No.	2414	1219	167		
	BMI	26.5(0.07)	26.2 (0.10)	26.2 (0.27))	-0.206 (0.10)	0.04
users of supplements	No.	484	270	21		
	BMI	26.0 (0.17)	26.3 (0.23)	26.6 (0.83)	+0.312 (0.25)	0.22
rs1467568		GG	GA	AA		
all subjects	No.	1,826	2,230	619		
	BMI	26.5 (0.08)	26.2 (0.08)	26.3(0.14)	-0.122(0.08)	0.12
non-users of supplements	No.	1,525	1,754	521		
	BMI	26.5 (0.08)	26.2 (0.09)	26.3(0.15)	-0.156 (0.08)	0.06
users of supplements	No.	309	392	102		
	BMI	26.1 (0.22)	26.1 (0.20)	26.3 (0.39)	+0.053 (0.20)	0.80
Haplotype 1		0 copies	1 copy	2 copies		
all subjects	No.	1,587	2,242	746		
	BMI	26.3 (0.09)	26.3 (0.08)	26.7 (0.13)	+0.167 (0.08)	0.03
non-users of supplements	No.	1308	1863	746		
	BMI	26.2 (1.0)	26.3 (0.08)	26.7 (0.14)	+0.205 (0.08)	0.01
users of supplements	No.	279	379	117		
	BMI	26.2 (0.23)	26.1 (0.20)	26.2 (0.35)	-0.039 (0.20)	0.85

BMI: mean (SE), adjusted for age and sex

Intake of energy and macronutrients by *SIRT1* variants

Table 3 shows mean intake of energy and of macronutrients by *SIRT1* genotype in the Rotterdam study. There were no significant differences in dietary intake of energy, fat, proteins and carbohydrates by *SIRT1* genotype. Most importantly, carriers of the genotype with the lowest BMI (carriers of the minor alleles of rs7895833 and rs1467568 and non-carriers of haplotype 1) did not have decreased intake of energy or fat. Also, when intakes of macronutrients were adjusted for energy intake (and when energy intake was adjusted for body weight) there were no significant differences in intake between the *SIRT1* variants.

Table 3. Intake of macronutrients by *SIRT1* genotype in the Rotterdam study

	genotype			*p* model 1	*p* model 2
rs7895833	AA	AG	GG		
No.	2,898	1,489	188		
Energy intake (KJ/day)	8259 (35.0)	8254 (48.8)	8294 (137)	0.96	
Fat intake (Grams/day)	80.5 (0.47)	80.6 (0.67)	80.1 (1.88)	0.96	0.59
Protein intake (Grams/day)	81.4 (0.34)	81.4(0.47)	80.3 (1.33)	0.72	0.39
Carbohydrate intake (Grams/day)	212.0 (1.08)	212.4 (1.51)	218.5 (4.25)	0.34	0.10
rs1467568	GG	GA	AA		
No.	1,826	2,230	619		
Energy intake (Kcal/day)	8271 (44.0)	8234 (40.8)	8310(75.6)	0.63	
Fat intake (Grams/day)	80.6 (0.60)	80.2 (0.56)	81.3 (1.04)	0.63	0.93
Protein intake (Grams/day)	81.7 (0.42)	81.0 (0.39)	81.6 (0.73)	0.71	0.60
Carbohydrate intake (Grams/day)	212.6 (1.36)	211.8 (1.26)	214.2 (2.34)	0.65	0.93
Haplotype 1	0 copies	1 copy	2 copies		
No.	1,587	2,242	746		
Energy intake (Kcal/day)	8265(47.2)	8228 (39.7)	8339 (68.9)	0.37	
Fat intake (Grams/day)	80.6 (0.65)	80.2 (0.54)	81.5 (0.94)	0.51	1.00
Protein intake (Grams/day)	81.5 (0.46)	80.8 (0.38)	82.5 (0.69)	0.05	0.08
Carbohydrate intake (Grams/day)	213.7 (1.46)	211.2 (1.23)	213.6 (2.13)	0.35	0.30

data are mean (SE)
model 1: adjusted for age and sex
model 2: adjusted for age, sex and energy intake; energy intake adjusted for age, sex and weight

Correlations between dietary components

In table 4 the spearman correlation coefficients are presented between the different dietary components under investigation in the current study.

As expected, intake of energy correlated strongly with intake of carbohydrates, fat and proteins (spearman's *rho* $\geq$ 0.73). Energy intake correlated significantly with intake of all vitamins. There were modest correlations between intake of the 4 vitamins, with the highest correlation coefficient between intake of niacin and vitamin E (*rho* = 0.33) and the lowest between vitamin C and niacin (*rho* = 0.17) and between vitamin C and vitamin E (rho = 0.17). After adjustment for energy intake, vitamin intakes were also correlated with each other except for intake of vitamin E with niacin intake (data not shown). Intake of total calcium and milk were highly correlated (*rho* = 0.80).

Table 4. Spearman's correlations between intake of dietary components in 4,575 subjects from the Rotterdam study

	Energy	Carbo-hydrates	Fat	Protein	Beta-carotene	Vitamin C	Vitamin E	Niacin	Calcium	Milk
Energy	1,00	0,80	0,86	0,70	0,14	0,11	0,54	0,59	0,36	0,22
Carbohydrates		1,00	0,52	0,51	0,12	0,18	0,40	0,36	0,33	0,25
Fat			1,00	0,57	0,08	0,00^{ns}	0,57	0,48	0,25	0,12
Protein				1,00	0,25	0,20	0,36	0,74	0,67	0,45
Betacarotene					1,00	0,35	0,19	0,25	0,22	0,10
Vitamin C						1,00	0,17	0,17	0,23	0,11
Vitamin E							1,00	0,33	0,19	0,07
Niacin								1,00	0,22	0,05
Calcium									1,00	0,80
Milk										1,00

All correlations significant at the p= 0.01 level except for ^{ns}: not significant

Effect of dietary intake on the relation between *SIRT1* variants and BMI

Table 5 shows the relationship in the Rotterdam study between the *SIRT1* haplotype1 and BMI in tertiles of intake of energy, fat, vitamins and calcium and milk. Results for the two SNPs, rs7895833 and rs1467568, were similar but less pronounced and are presented in Supplementary Table 1.

For sex-specific tertiles of energy intake (adjusted for age and body weight) we observed an association between *SIRT1* haplotype 1 and BMI only in the lowest tertile (p trend = 0.03) In the highest two tertiles there was no association between *SIRT1* variants and BMI. For fat intake (adjusted for age and energy intake), we also observed an association between haplotype 1 and BMI only in the lowest tertile (p trend = 0.03). Again, no associations were seen in the highest two tertiles. For all four vitamins (beta-carotene, vitamin C, vitamin E and niacin) similar trends were seen with significant associations only in the lowest sex-specific tertiles of age- and energy adjusted intake. These effects were most pronounced for vitamin E with the strongest effect for haplotype 1 (beta 0.685 $\pm$ 0.14, p trend = 1x 10^{-6}). In this lowest tertile, BMI was 26.1, 26.2 and 27.7 kg/m^2 for non-carriers, heterozygous and homozygous carriers of haplotype 1, respectively, while in the highest tertile of vitamin E intake BMI was similar across genotype groups. In contrast, for calcium and milk intake significant associations between *SIRT 1* haplotype 1 and BMI were seen only in the highest tertiles. In the highest tertile of calcium intake BMI was and 26.2, 26.5 and 27.2 kg/m^2 for non-carriers, heterozygous and homozygous carriers of haplotype 1, respectively (beta 0.460 $\pm$ 0.13, p trend = 5 x 10^{-4}) while in the lowest tertile BMI was not significantly different. Interaction terms between the *SIRT1* haplotype 1 and tertiles of nutrient intake were significant for vitamin E only (p= 0.0005).

Table 5. BMI by *SIRT1* haplotype 1 in tertiles of dietary intake in the Rotterdam study

	BMI*		beta (SE)	*p*
	Overall	0/1/2&		
Energy adjusted for age and weight				
Tertile 1	26.7 (0.09)	26.5/26.8/27.1	**+ 0.269 (0.14)**	**0.03**
Tertile 2	26.3 (0.09)	26.4/26.1/26.6	+ 0.017(0.14)	0.19
Tertile 3	26.0 (0.09)	25.9/25.9/26.4	+ 0.197 (0.12)	0.11
Fat adjusted for age and energy intake				
Tertile 1	26.1 (0.09)	25.9/26.1/26.6	**+ 0.312 (0.13)**	**0.02**
Tertile 2	26.4 (0.09)	26.3/26.4/26.6	+ 0.152 (0.13)	0.25
Tertile 3	26.5 (0.09)	26.6/26.3/26.8	+ 0.031 (0.14)	0.82
Betacarotene adjusted for age and energy intake				
Tertile 1	26.1 (0.09)	25.9/26.0/26.7	**+ 0.355 (0.13)**	**0.007**
Tertile 2	26.3 (0.09)	26.2/26.4/26.5	+ 0.164 (0.14)	0.23
Tertile 3	26.6 (0.09)	26.7/26.4/26.8	- 0.01 (0.13)	0.97
Vitamine C adjusted for age and energy intake				
Tertile 1	26.1 (0.09)	25.9/26.0/26.5	**+ 0.293 (0.14)**	**0.03**
Tertile 2	26.3 (0.09)	26.3/26.2/26.9	+ 0.199 (0.14)	0.14
Tertile 3	26.6 (0.09)	26.5/26.6/26.6	+ 0.028 (0.13)	0.82
Vitamin E adjusted for energy intake				
Tertile 1	26.4 (0.09)	26.1/26.2/27.7	**+ 0.685 (0.14)**	**0.000001**
Tertile 2	26.4 (0.09)	26.6/26.3/26.2	- 0.184 (0.13)	0.16
Tertile 3	26.2 (0.09)	26.1/26.2/26.1	+ 0.017 (0.13)	0.90
Niacin adjusted age and energy intake				
Tertile 1	25.8 (0.09)	25.7/25.8/26.2	+ 0.221 (0.13)	0.10
Tertile 2	26.4 (0.09)	26.2/26.3/26.7	+ 0.236 (0.13)	0.07
Tertile 3	26.8 (0.09)	26.8/26.6/27.0	+ 0.038 (0.14)	0.81
Calcium adjusted for age and energy				
Tertile 1	26.1 (0.09)	26.0/26.0/26.4	+ 0.178 (0.13)	0.18
Tertile 2	26.4 (0.09)	26.5/26.3/26.4	- 0.125 (0.14)	0.36
Tertile 3	26.5 (0.09)	26.2/26.5/27.2	**+ 0.460 (0.13)**	**0.0005**
Milk adjusted for age and energy intake				
Tertile 1	26.2 (0.09)	26.2/26.1/26.4	- 0.072 (0.13)	0.59
Tertile 2	26.5 (0.09)	26.6/26.5/26.6	+ 0.000 (0.14)	1.00
Tertile 3	26.2 (0.09)	26.0/26.2/26.9	**+ 0.430 (0.13)**	**0.001**

*BMI mean $\pm$ SE, data adjusted for age and sex. & 0/1/2 copies of haplotype 1

Interaction terms between the *SIRT1* genetic variants and calcium intake were significant only for the two SNPs (p= 0.02, 0.01 and 0.13 for rs7895833, rs1467568 and haplotype 1, respectively, data not shown).

Irrespective of genotypes, BMI in tertiles of intake were not significantly different for vitamin E and milk. For energy intake BMI decreased with increasing tertiles while for fat and the other vitamins BMI increased.

Effect of milk intake on the relation between *SIRT1* variants and BMI in the Rotterdam and ERF study

Relationships between *SIRT1* genetic variants and BMI in tertiles of milk intake for the two SNPS rs7895833, rs1467568 in the Rotterdam study and in ERF are shown in **table 6**. In ERF, sex-specific tertiles for milk intake were created with adjustment for age and weight, since no data on caloric intake were available. In the Rotterdam study, associations between rs7895833, rs1467568 and BMI were significantly negative in the third tertile of milk intake only (p= 0.01 and 0.007 for the two SNPs, respectively). In ERF, negative associations were observed for rs7895833 in relation with BMI in the second and third tertile that was significant in the second tertile only (p trend = 0.008). For rs1467568 the same pattern was seen with significant inverse associations in the second (p trend = 0.002) and third (p trend = 0.03) tertile.

Table 6. BMI by two *SIRT1* SNPs in tertiles of milk intake in the Rotterdam study and the ERF study

		BMI	beta (SE)	*p*	BMI	beta (SE)	*p*
		rs7895833			rs1467568		
	Overall*	AA/AG/GG			GG/GA/AA		
Rotterdam study: Milk adjusted for age and energy intake							
Tertile 1	26.2 (0.09)	26.2/26.1/26.0	- 0.082 (0.16)	0.61	26.1/26.2/26.2	- 0.100 (0.13)	0.46
Tertile 2	26.5 (0.09)	26.5/26.6/26.7	- 0.083 (0.16)	0.61	26.7/26.4/26.5	- 0.116 (0.14)	0.40
Tertile 3	26.2 (0.09)	26.4/25.9/26.0	**- 0.408 (0.16)**	**0.01**	26.6/26.0/26.1	**- 0.360 (0.13)**	**0.007**
ERF: Milk adjusted for age and weight							
Tertile 1	26.9 (0.16)	26.7/26.9/28.1	+ 0.421 (0.27)	0.12	26.8/26.7/27.5	+ 0.286 (0.29)	0.21
Tertile 2	26.5 (0.16)	26.8/26.5/24.7	**- 0.690 (0.26)**	**0.008**	26.9/26.7/25.4	**- 0.667 (0.22)**	**0.002**
Tertile 3	26.7 (0.16)	26.8/26.8/25.7	- 0.250 (0.27)	0.36	27.3/26.5/26.4	**- 0.494 (0.23)**	**0.03**

*Data are mean $\pm$ SE, adjusted for age and sex

DISCUSSION

SIRT1 is a plausible candidate gene for obesity, based on numerous functions in metabolism. We recently demonstrated an association of two *SIRT1* SNPs and the most common haplotype 1 with BMI in two independent Dutch populations. In the current study we investigated a potential gene-diet interaction, based on known regulators of SIRT1. In the Rotterdam study, we found that the effects of the *SIRT1* variants on BMI were only seen in the lowest tertiles of intake of energy, fat and the three antioxidant vitamins and niacin, while, in contrast, for calcium and milk intake effects were only seen in the highest tertile.

At first sight it appears exciting that we found an effect of *SIRT1* haplotype 1 on BMI with low intake of energy per kg body weight, as Sirtuins are regarded as potential mediators of caloric restriction on increased life span in lower organisms [13-15]. However, other explanations for this finding are possible. We did not find that subjects with the lowest intake of energy per kg body weight had the lowest BMI. In fact, BMI decreased with increasing caloric intake. This could suggest that subjects with low energy intake for body weight may represent those with low physical activity. Moreover, it is known that there may be selective underreporting of dietary intake in persons with higher BMI [43]. Even though subjects were excluded when dieticians considered dietary information unreliable, e.g. with unrealistic values of caloric intake for body weight, we cannot exclude that selective underreporting in subjects in the lowest tertile of energy intake plays a role in our findings. For the effects of fat, vitamins, calcium and milk, underreporting is less likely a confounder since we adjusted intake of these nutrients for energy intake.

The largest effects were seen with vitamin E and calcium intake and interaction terms for these nutrients with haplotype 1 were the only significant ones. This suggests that intake of vitamin E or calcium or other compounds in milk are the true modifiers of SIRT1 but other associated dietary or lifestyle factors may very well underlie these interactions. It is also possible that the findings are chance findings. After applying a Bonferroni correction for testing three genetic variants and 8 dietary traits, which may be too stringent because the traits are not totally independent, only the interaction between haplotype 1 and vitamin E intake remained significant.

Interestingly, we observed that the strength of the relationship between *SIRT1* genetic variants and BMI increased after excluding subjects reporting use of supplements with the vitamins under investigation. In users of supplements there was not even a trend for a similar effect on BMI for the genetic variants. This supports that our findings of an association in the lowest tertiles of vitamin intakes are genuine.

No studies have so far found, an interaction between vitamin E and SIRT1 except for one, in which vitamin E prevented a decrease in *SIRT1* expression in the hippocampus and cerebral cortex in rodents caused by a high fat diet [44]. The interaction between *SIRT1*

and vitamin E, that we found, needs further study. It could indeed be due to antioxidant function of vitamin E although there is debate about its anti-oxidant effects *in vivo* [45]. Recent discoveries of vitamin E, as a regulator of enzymes and gene activity [45] are also important to consider as an explanation for its interaction with *SIRT1*. A recent paper found that vitamin C and E supplements inhibited exercise induced beneficial effects on insulin sensitivity in healthy young men, by preventing the promotion of endogenous antioxidant defense capacity trough peroxisome proliferator-activated receptor-gamma (*PPARg*) and *PPARg*- co-activators *PGC1α* and *PGC1β* [46]. Since *SIRT1* expression in muscle is increased by physical exercise [47] and SIRT1 influences PPARγ and PGC1α, a potential role of SIRT1 on these exercise induced metabolic effects should be explored. Vitamin E is abundant in foods such as nuts, seeds, vegetable oils and green leafy vegetables. High intake of vitamin E may thus also represent a healthy diet or an otherwise healthy lifestyle.

We also found effects of *SIRT1* with high intake of calcium and milk. This observation also needs further validation, although similar findings in the ERF study in the highest tertiles of self-reported milk intake adjusted for body weight lend support to the findings in the Rotterdam study. It is possible that these findings are related to nicotinamide riboside, a SIRT1 stimulator found in milk [29, 30]. There is evidence for a role of dietary calcium in human body weight regulation possibly by suppression of 1,25 $(OH)_2$ vitamin D (calcitriol) [48, 49], although there is debate about the underlying mechanisms and the clinical relevance [50]. We did not observe decreased BMI independent of *SIRT1* genotype with higher intake of milk in the Rotterdam study nor in the ERF study but the interaction of *SIRT1* genetic variants with calcium and milk intake on BMI merits further studies on potential role for dairy products or nicotinamide riboside supplementation on body weight through *SIRT1* stimulation.

It is not yet clear how SIRT1 influences BMI but potential mechanisms include a repressive effect on PPARγ, central effects through satiety, increased energy expenditure or effects by modification of Clock genes. Our finding that energy intake did not differ between genotypes makes it unlikely that appetite plays a role in the association between *SIRT1* and BMI. However, we cannot exclude that small differences in caloric intake can be found in large-scale studies. Our results are not in line with findings of decreased food intake in transgenic mice with moderate over expression of *SIRT1* [51].

Limitations of our study are the possibility of misclassification of nutrient intake inherent to food frequency questionnaires, the lack of data on functionality of the *SIRT1* SNPS and the lack of replication in other cohorts with validated information of dietary intake. The results of this study, while preliminary, are important because they highlight that gene-diet interaction may influence BMI and the risk of obesity and strongly support further research on *SIRT1* gene-diet interaction in larger populations. This will require meta-analysis of data from multiple studies within international consortia after

standardization of different methods used to assess dietary intake. Gene-environment interactions may explain part of the so-called "dark matter" in heritability where so far variation in genes explain only a few percent of the heritability of traits and diseases [52]. They may also underlie heterogeneity in large-scale meta-analyses of genome-wide association studies if dietary habits differ between study populations. It is of great importance to uncover such interactions in the growing epidemic of obesity. Knowledge on gene-diet interaction may modify dietary advice, potentially in the future personalized and based on genotype. This is preferable to new and costly medication with potential side effects, especially when used on large scale. However, more research is needed before any personalized dietary advice can be used in clinical practice.

We conclude that associations between *SIRT1* genetic variants and BMI are not explained by differences in caloric intake. Dietary intake of antioxidants, especially vitamin E, and of calcium and milk modify the associations of *SIRT1* variants with BMI. These data further support evidence that gene-diet interactions influence complex traits like BMI. Replication of our findings and further study of specific dietary factors that can modify SIRT1 can pave the way for prospective studies on dietary modification of SIRT1 to influence obesity.

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Chapter 8

NRXN3 is a novel locus for waist circumference: a genome-wide association study from the CHARGE Consortium

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PLoS Genet. 2009 Jun;5(6):e1000539. Epub 2009 Jun 26

ABSTRACT

Central abdominal fat is a strong risk factor for diabetes and cardiovascular disease. To identify common variants influencing central abdominal fat, we conducted a two-stage genome-wide association analysis for waist circumference (WC). In total, three loci reached genome-wide significance. In stage 1, 31,373 individuals of Caucasian descent from eight cohort studies confirmed the role of *FTO* and *MC4R* and identified one novel locus associated with WC in the neurexin 3 gene (*NRXN3* [rs10146997, $p = 6.4 \times 10^{-7}$]). The association with *NRXN3* was confirmed in Stage 2 by combining stage 1 results with those from 38,641 participants in the GIANT consortium ($p = 0.009$ in GIANT only, $p = 5.3 \times 10^{-8}$ for combined analysis, n=70,014). Mean WC increase per copy of G allele was 0.0498 z-score units (0.65 cm). This SNP was also associated with body mass index (BMI) ($p = 7.4 \times 10^{-6}$, 0.024 z-score units [0.10 kg/m^2] per copy of the G allele) and the risk of obesity (odds ratio 1.13, 95% CI 1.07-1.19; $p = 3.2 \times 10^{-5}$ per copy of the G allele). The *NRXN3* gene has been previously implicated in addiction and reward behavior, lending further evidence that common forms of obesity may be a central nervous system-mediated disorder. Our findings establish that common variants in *NRXN3* are associated with WC, BMI, and obesity.

INTRODUCTION

Body mass index (BMI) is a commonly used measure of overall adiposity. However, specific fat depots may confer differential metabolic risk. In particular, central abdominal fat, as measured by waist circumference (WC), may be more strongly associated with the development of metabolic risk factors and cardiovascular disease as compared with BMI [1-4]. Therefore, understanding the pathogenesis of central fat distribution may provide further insight into the relationship between adiposity, cardiometabolic risk, and cardiovascular disease.

Both genetic and environmental factors have been linked to obesity [5]. Heritability estimates for BMI and WC range from 30 to 70% in family and twin studies [6], and multiple quantitative trait loci and candidate genes have been mapped to genes for central adiposity [5]. Despite strong evidence for an underlying genetic component, genes for obesity-related traits, particularly central obesity, have been difficult to identify and replicate.

Early genome-wide association studies (GWAS) identified both *FTO* and *MC4R* as genes related to BMI and WC [7-10]. Many new loci have been identified in recent obesity related GWAS studies [11-13]. However, collectively these variants explain only a small proportion of the variation in adiposity [7-13]. In addition, no GWAS exist exclusively to identify genes for central fat. Thus, to identify new variants, we carried out a large-scale meta-analysis of GWAS from eight studies to detect variants associated with central body fat distribution.

METHODS

Study Samples

Participants for the current analysis were drawn from 8 cohort studies, including the Age, Gene/Environment Susceptibility-Reykjavik Study (AGES- Reykjavik Study), the Atherosclerosis Risk in Communities Study (ARIC), the Cardiovascular Health Study (CHS), the European Special Population Network consortium (EUROSPAN), the Family Heart Study, the Framingham Heart Study, Old Order Amish (OOA), and the Rotterdam Study (RS). These groups comprise the CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. All participants provided informed consent. Local ethical committees at each institution approved the individual study protocols. The online supplement contains details regarding all participating cohorts.

Imputation and Statistical Analysis

Common to all analyses were use of the raw WC measures and the assumption of an additive model; study specific details follow. Each study reported an effect allele, which was meta-analyzed consistently across all studies. Results are currently presented relative to the minor G allele for the *NRXN3* SNP. In all studies except CHS, MACH (version 1.0.15 in Family Heart, Framingham, EUROSPAN and RS; version 1.0.16 in ARIC and AGES) was used to impute all autosomal SNPs on the HapMap, using the publicly available phased haplotypes (release 22, build 36, CEU population) as a reference panel. In CHS, the program BIMBAM was used [14]. Details are provided in Supplementary Table S1 regarding covariates and trait creation.

In ARIC, Framingham, and RS, sex- and either cohort-specific or study center-specific residuals were created after adjustment for age, age-squared, and smoking status. In CHS and Family Heart, linear regression models were used to adjust for age, age-squared, sex, smoking, and study center. In AGES, linear regression models using PLINK v1.04 [15] were used to adjust for age, age-squared, sex, and smoking. In the OOA the measured genotype mixed effects model was used adjusting for age, age-squared, sex and family structure based on the complete 14-generation pedigree as implemented in ITSNBN [16]. Framingham employed the linear mixed effect model for continuous traits and the generalized estimating equations for dichotomous traits in R [17] to account for family relatedness. In RS, linear regression models were run using MACH2QTL [18]. In ARIC and EUROSPAN, all regression models were run using the ProbABEL package from the ABEL set of programs [19] and in EUROSPAN genomic control [20] was used to correct standard errors of the effect estimates for relatedness among individuals. The Family Heart Study determined the effect of each SNP using linear mixed effects models to account for the siblings present in the data using SAS®.

Principal components calculated using EIGENSTRAT [21] were adjusted for in the individual studies when significant in order to account for population substructure.

Meta-analysis

A weighted z-score approach was used to conduct meta-analyses with METAL (www.sph.umich.edu/csg/abecasis/metal/). Genomic control correction was applied to each study prior to the full meta-analysis. *p*-values less than 4.4×10^{-7} were considered genome-wide significant [22].

In Silico Exchange with the GIANT Consortium

In stage 2 of our study, we conducted an *in silico* exchange of the results from 48 SNPs with the GIANT consortium. To create our list of SNPs to exchange, we first selected the top 34 SNPs from independent loci (defined as SNPs with R^2<0.2) from our meta-analysis of WC, excluding SNPs in known loci for adiposity. An additional 14 SNPs of independent loci with a *p*-value<1.0 x 10^{-5} from a secondary list that focused on SNPs for WC with corresponding BMI *p*-values > 0.01 were also included in an attempt to isolate genes that might be specifically associated with central fat deposition. Our *a priori* threshold for replication was a *p*-value<0.001 (0.05/48 SNPs) and/or reaching genome-wide significance in a combined meta-analysis. CHARGE and GIANT results were then meta-analyzed using METAL.

RESULTS

Table 1 presents descriptive statistics across the 8 cohorts providing data for the meta-analysis. We had a total sample size of 31,373 individuals of Caucasian descent. Participants were mostly middle-aged with ages ranging from a mean of 45 to 76 years of age.

Table 1. Descriptive statistics across the eight cohorts.

Cohort	N	Age (years)	% Women	Current smokers (%)	Waist Circ (cm)	BMI (kg/m^2)
AGES	3172	76.4 (5.4)	58.0 (1840)	12.7 (402)	100.7 (12.1)*	27.1 (4.4)
ARIC	8097	54.3 (5.7)	52.8 (4276)	25.2 (2036)	96.2 (13.4)	27.0 (4.9)
CHS	3213	72.3 (5.4)	60.0 (1942)	11.0 (354)	93.6 (12.6)	26.4 (4.3)
Family Heart Study	855	55.6 (11.0)	51.5 (440)	11.9 (101)	98.6 (13.6)	27.8 (5.1)
Framingham Heart Study	7115	45.2 (10.9)	52.7 (3750)	18.8 (1338)	91.4 (15.0)	26.0 (5.1)
Old Order Amish	1134	49.6 (16.8)	48.4 (549)	9.4 (106)	88.5 (11.4)	27.0 (4.7)
Rotterdam Study	5471	69.0 (8.8)	58.6 (3205)	23.0 (1258)	90.6 (11.2)	26.3 (3.7)
EUROSPAN Consortium						
ERF (Dutch)	1239	48.3 (14.7)	60.1 (744)	43.6 (540)	87.0 (13.7)	26.7 (4.7)
CROATIAN	784	56.5 (15.3)	58.6 (459)	27.7 (217)	95.9 (11.8)	27.3 (4.3)
MICROS (South Tyrolean)	293	46.3 (15.6)	59.7 (175)	45.3 (125)	88.5 (13.3)	25.4 (5.4)

Data provided as mean (standard deviation) for continuous and % (n) for dichotomous data.
*N=3,167 for WC by tape measure; mean (SD) of WC measured by computed tomography is 125.9(14.0) cm

Supplementary Figure S1 (Supplementary material available at the PLoS Genetics website) shows the genome-wide association results for WC in the stage 1 CHARGE only analysis. The top SNPs for WC were in the *FTO* and *MC4R* genes (Supplementary Table S3). Supplementary Figure S2 shows the QQ plot for our results excluding SNPs in *FTO* and *MC4R*. For *FTO*, the top SNP was rs1558902 (p= 4.6 x 10^{-19}). For *MC4R*, the top SNP was rs489693 (p= 3.5 x 10^{-7}). The top results excluding SNPs in *FTO* and *MC4R* from our stage 1 meta-analysis are shown in **Table 2** along with the stage 2 *in silico* replication results from the GIANT consortium; additional meta-analysis results from CHARGE are presented in Supplementary Table S3. The lowest p-value on our list, for SNP rs10146997 in the *NRXN3* gene, had a stage 1 meta-analysis p-value of 6.4 x10^{-7} and was confirmed in 38,641 participants from the GIANT consortium with a p-value of 0.009 and a combined p-value of 5.3 x 10^{-8}. The *NRXN3* SNP was derived from the list of SNPs associated with WC irrespective of association with BMI. None of the other SNPs that were exchanged were confirmed in GIANT. We do note that while rs10857809 (proxy for rs10857810) in the *FAM40A* gene had a p-value of 0.003 in GIANT, the results were not direction-consistent with CHARGE and therefore did not replicate in the combined analysis.

Table 2. Top 48 SNPs exchanged with the GIANT Consortium, GIANT p-values, and the combined results

Marker	Chromosome	Position	CHARGE pvalue	GIANT pvalue*	COMBINED pvalue	Nearest Gene**
rs10146997	14	79014915	6.4E-07	0.009	5.3E-08	***NRXN3***
rs981113	5	75556684	9.8E-07	0.55	3.4E-03	***SV2C***
rs7338657	13	62299289	1.1E-06	0.75	4.4E-04	*PCDH20*
rs6714750	2	136499639	1.9E-06	0.48	2.9E-03	*DARS*
rs1555967	6	51267954	1.9E-06	0.07	3.3E-06	*PKHD1*
rs4701252	5	21814911	2.5E-06	0.45	2.3E-06	***CDH12***
rs4420638	19	50114786	3.6E-06	0.80	3.8E-04	*APOC1*
rs2365642	1	199501709	4.1E-06	0.79	3.4E-03	*PKP1*
rs17008958	3	71838178	4.5E-06	0.18	5.7E-05	***EIF4E3***
rs7932813	11	7664857	4.6E-06	0.09	5.0E-06	*OVCH2*
rs569406	9	77219165	4.7E-06	0.54	3.7E-04	*OSTF1*
rs6837818	4	168112	5.2E-06	0.81	1.1E-03	*ZNF718*
rs17537900	13	42593449	7.3E-06	0.07	2.9E-03	*DNAJC15*
rs17476669	2	50579975	7.9E-06	0.27	1.1E-04	***NRXN1***
rs11857639	15	71424825	8.0E-06	0.94	3.8E-04	***HCN4***
rs3758063	8	87754664	1.2E-05	0.76	5.4E-03	***CNGB3***
rs804569	20	22099652	1.4E-05	0.29	1.7E-04	*FOXA2*
rs13002346	2	133761936	1.6E-05	0.78	1.9E-03	***NAP5***
rs7138803	12	48533735	1.6E-05	0.01	8.0E-07	*BCDIN3D*
rs17201502	12	48571829	1.7E-05	0.02	4.2E-06	***FAIM2***
rs154168	5	107078981	1.7E-05	0.86	2.0E-03	*EFNA5*
rs1324618	9	121107783	1.8E-05	0.62	0.01	***DBC1***
rs1553754	17	43918706	2.0E-05	0.05	1.2E-05	*HOXB1*

Table 2. continued

Marker	Chromosome	Position	CHARGE pvalue	GIANT pvalue*	COMBINED pvalue	Nearest Gene**
rs12971184	18	32134683	2.1E-05	0.43	0.03	***FHOD3***
rs253414	5	74992273	2.3E-05	0.47	8.0E-04	*C5orf37*
rs309193	19	52317155	2.4E-05	0.20	1.8E-04	*C19orf7*
rs12457723	18	27981438	2.4E-05	0.14	0.08	*RNF138*
rs8006194	14	88980606	2.5E-05	0.63	0.01	***FOXN3***
rs10172766	2	205587746	3.0E-05	0.30	0.01	***PARD3B***
rs11096633	2	20067535	3.1E-05	0.47	5.2E-04	***MATN3***
rs8049894	16	75371885	3.1E-05	0.67	1.9E-03	*CNTNAP4*
rs12148445	15	34703950	3.1E-05	0.60	0.01	***C15orf41***
rs9829637	3	135638752	3.5E-05	0.10	4.9E-05	*ANAPC13*
rs7666149	4	41017949	3.7E-05	0.06	2.1E-05	*LIMCH1*
rs13421140	2	1753016	4.2E-05	0.97	6.1E-03	*MYT1L*
rs4238692	16	82149934	5.8E-05	0.14	1.4E-04	***CDH13***
rs17833967	12	13846345	6.0E-05	0.46	1.2E-03	***GRIN2B***
rs1532206	3	99153367	6.2E-05	0.89	9.2E-03	***MINA***
rs6723108	2	135196450	6.2E-05	0.27	4.4E-04	*TMEM163*
rs12704232	7	85640166	7.4E-05	0.61	0.05	*GRM3*
rs12377679	9	128437576	8.0E-05	0.12	1.1E-04	***LMX1B***
rs1017643	6	156835825	9.5E-05	0.04	2.6E-05	*ARID1B*
rs6485438	11	43643194	1.3E-04	0.09	9.7E-05	*HSD17B12*
rs7116632	11	129452949	1.9E-04	0.74	0.04	***APLP2***
rs422988	1	4718977	2.4E-04	0.62	3.5E-03	***AJAP1***
rs5771623	22	47415000	2.9E-04	0.07	0.28	***FAM19A5***
rs6728666	2	216894986	5.3E-04	0.76	0.02	***MARCH4***
rs10857810***	1	110403320	1.8E-04	.003	0.97	***FAM40A***

*GIANT sample size is 38,641.

**Nearest reference is bolded if SNP is within the reference gene

***GIANT SNP is proxy rs10857809 (r^2=0.92)

Figure 1 presents the genomic region for SNP rs10146997 (intronic) in *NRXN3*. **Table 3** shows detailed results of rs10146997 in the *NRXN3* gene by contributing CHARGE study and corresponding results appear in the forest plot in Supplementary Figure S3; there was no evidence for heterogeneity across the Stage 1 studies (p=0.64). The minor allele (G) frequency (MAF) for rs10146997 in our sample ranged from 0.14 in the OOA to 0.24 in the Croatians; the frequency of the *NRXN3* SNP G allele is 0.275, 1.0, 1.0, and 0.35, in Hapmap CEPH, Han Chinese, Japanese, and Yoruba populations, respectively. This SNP was genotyped in AGES, CHS, Family Heart Study, Rotterdam and all EUROSPAN studies, and imputation scores for the other studies indicated very high quality. Overall, per copy of the G allele, mean WC was increased 0.0498 z-score units (0.65 cm). Beta coefficients (in z-score units) were consistently positive in all samples except the ERF study (β=-0.0098; p= 0.86), which is most likely due to chance. Due to overlap in participants

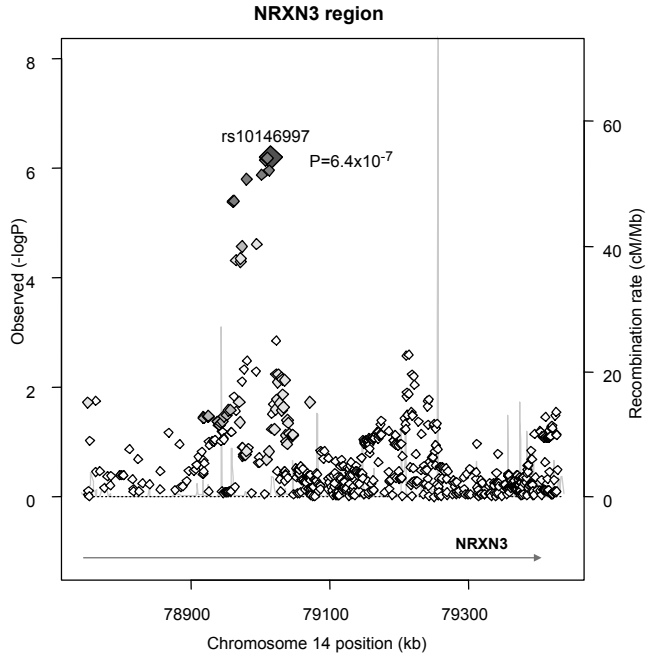

Figure 1. Regional Association Plot for rs10146997 on chromosome 14 in the stage 1 CHARGE-only analysis. The scheme is black for strong linkage disequilibrium (LD; $r^2 \geq 0.8$), dark grey for moderate LD ($r^2 \geq 0.5$ and < 0.8), light grey for weak LD ($r^2 \geq 0.2$ and < 0.5) and white for limited or no LD ($r^2 < 0.2$).

Table 3. Results per copy of the G allele for rs10146997 by contributing study; beta coefficients expressed as z-scores

Cohort	N	MAF (G)	Imputation Quality Score	Beta Coefficient	SE	*p*-value
AGES	3170	0.21	Genotyped	0.058	0.031	0.06
ARIC	8097	0.22	0.98	0.032	0.019	0.12
CHS	3213	0.21	Genotyped	0.103	0.030	0.00048
Family Heart Study	855	0.21	Genotyped	0.003	0.055	0.65
Framingham Heart Study	7115	0.20	1.00	0.068	0.022	0.0019
Old Order Amish	1097*	0.14	0.87	0.049	0.073	0.33
Rotterdam Study	5471	0.21	Genotyped	0.042	0.024	0.08
EUROSPAN Consortium						
ERF (Dutch)	1241	0.20	Genotyped	-0.010	0.052	0.86
Croatia	784	0.24	Genotyped	0.039	0.059	0.52
MICROS (South Tyrolean)	293	0.17	Genotyped	0.057	0.101	0.60
Meta-analysis results	31373	0.21	N/A	0.0498	0.010	6.4×10^{-7}

SE=standard error; MAF=minor allele frequency

*Sample size reduced from 1134 because smokers excluded due to the low smoking prevalence

from the Framingham Heart Study and ARIC with those from the Family Heart Study, the CHARGE meta-analysis was re-run for the *NRXN3* SNP without the Family Heart Study; results were essentially unchanged (p= 6.6 x 10^{-7}). Individual study-specific results for rs10146997 from the studies comprising the GIANT consortium can be found in Supplementary Table S2.

Within CHARGE we also observed an association of rs10146997 with BMI (p=7.4 x 10^{-6}). Overall, per copy of the G allele, mean BMI was increased 0.024 z-score units per G allele (0.10 kg/m^2). When WC was additionally adjusted for BMI, the signal was completely attenuated (0.0065 z-score units per G allele; p= 0.32). The association of rs10146997 with WC was similar in women and men and in older and younger individuals (**Table 4**). After excluding smoking from the covariate adjustment list, results were essentially similar. Per copy of the G allele, the odds ratio of having high WC (≥88 cm in women; ≥102 cm in men) was 1.07 (95% CI 1.02-1.11; Table 4). Similarly, the odds ratio of obesity was 1.13 (95% CI 1.07-1.19).

We calculated a risk score of *FTO* (rs9939609), *MC4R* (rs17782313), and *NRXN3* with possible scores ranging from 0-6 risk alleles (**Figure 2**). Across this range, mean WC increased from 92.4 cm among those with 0 risk alleles, to 95.7 cm among those with 4

Table 4. CHARGE consortium secondary analysis results per copy of the G allele for rs10146997 in 31373 individuals; beta coefficients expressed as z-scores

	Beta Coefficient	**SE**	***p*-value**
Overall	0.0498	0.010	6.4 x10^{-7}
Overall without adjusting for smoking	0.0460	0.010	5.6 x10^{-6}
Sex stratification			
Women	0.0500	0.014	4.7 x10^{-4}
Men	0.0427	0.013	0.001
Age stratification			
<55 years	0.0520	0.017	0.002
55+ years	0.0560	0.013	7.4 x10^{-6}
	Odds Ratio	**95% CI**	***p*-value**
WC category*			
High WC (women ≥88 cm, men ≥102 cm)	1.07	1.02-1.11	0.003
BMI categories**			
Overweight (BMI 25 to <30)	1.03	0.98-1.07	0.250
Obese (BMI ≥ 30)	1.13	1.07-1.19	3.2 x10^{-5}

*Referent = normal WC category (women <88 cm; men <102 cm)
**Referent = normal weight category (BMI 18.5-<25 kg/m^2)

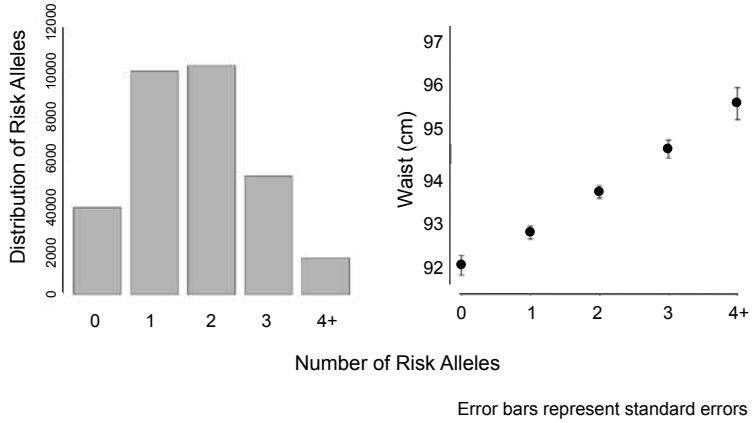

Figure 2. Mean waist circumference by number of risk alleles for *FTO*, *MC4R*, and *NRXN3*. Bars represent standard errors. The panel on the left represents the distribution of risk alleles in the overall sample.

or more risk alleles. To put our findings in perspective, per copy of the effect allele the *NRXN3* SNP resulted in a WC difference of 0.65 cm; *FTO* 0.73 cm, and *MC4R* 0.37 cm.

CHARGE consortium meta-analysis results for BMI can be found in Supplementary Table S4; Manhattan and QQ plots can be found in Supplementary Figure S4 and Supplementary Figure S5**,** respectively.

DISCUSSION

In a discovery sample of more than 30,000 individuals from several cohort studies, we identified a novel locus in the *NRXN3* gene associated with WC. In combination with data from the GIANT consortium, the *p*-value for this finding exceeded our pre-defined threshold for genome-wide statistical significance. This SNP was also significantly associated with BMI and obesity. This gene has previously been associated with addiction and reward behavior, and is a compelling biologic candidate for obesity. We also confirmed the significant associations with *FTO* and *MC4R* that have previously been reported with BMI and WC.

Although our genome-wide scan was performed for WC, the *NRXN3* SNP was also significantly associated with BMI. In secondary analyses, the signal for WC was attenuated after additionally adjusting for BMI, suggesting that this locus is most likely involved in overall adiposity and not specific to central fat deposition. Similar observations have been made for *FTO* [10] and *MC4R* [7], highlighting the inter-dependence between different measures of adiposity and the importance of performing GWAS on multiple adiposity-related traits.

The small magnitude of the effect size of the *NRXN3* variant on WC is consistent with what has previously been reported for *FTO* and *MC4R*. These findings highlight the need for large sample sizes in order to facilitate continued gene discovery for obesity-related traits. In particular, genes that emerge for waist circumference will most likely be genes for overall adiposity because of the strong correlation between the two measurements [22]. More specific measures of visceral abdominal fat depots may make it possible to isolate genes involved in regional body composition.

NRXN3 is part of a family of central nervous adhesion molecules and is highly expressed in the central nervous system. Prior studies of *NRNX3* point towards an important role in alcohol dependence, cocaine addiction, and illegal substance abuse [23-26]. In addition, opioid dependence has been linked to the chromosome 14q region [23]. In mice, *NRXN3* beta expression was observed in the globus pallidus when exposed to cocaine [24]. Many of the neuronal pathways in these sub-cortical regions of the brain in which *NRXN3* is expressed are involved with learning and reward training [25].

Obesity and addiction may share common neurologic underpinnings [26]. Other well-replicated obesity loci, including *MC4R*, have also been shown to be associated with centrally-mediated phenomena including binge eating behavior [11,12,27]. Studies in mice indicate that FTO expression is particularly pronounced in regions of the brain known to regulate energy balance [28], and recent data suggest that variants in the *FTO* gene may regulate food intake and selection [29].

Additional research is needed to understand the association of rs10146997 with the *NRXN3* gene and to identify a causal variant. Since there are no other genes within a distance of more than several hundred kilobases of this SNP, it is unlikely that a different gene accounts for this finding. A search of publically available databases [30-31] did not identify an association between SNPs in *NRXN3* and gene expression.

A relationship between WC and causal variants in the *NRXN3* gene may have clinical implications. Obesity is a multifactorial trait that results from a complex interaction between genes and environment. The identification of an association between obesity and variants in a gene that has been associated with substance abuse suggests that further exploration of the role of this gene in vulnerability to addiction to food substances should be undertaken.

The strengths of this work include the large discovery sample size. The effect size was small, and achieving conventional levels of genome-wide significance required combining data from more than 70,000 participants in two large consortia. Although the confirmation with the GIANT consortium is promising, the joint *p*-value based on more than 70,000 participants achieved only borderline genome-wide significance. Our findings warrant the need for further replication in other ethnic groups.

We identified a SNP at a novel locus in the *NRXN3* gene associated with WC. This gene has previously been implicated in addiction and reward behavior, lending further support to the concept that obesity, in part, is a centrally-mediated disorder.

ACKNOWLEDGEMENTS

The authors acknowledge the essential role of the CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium in development and support of this manuscript. CHARGE members include the Netherland's Rotterdam Study (RS), the NHLBI's Framingham Heart Study (FHS), Cardiovascular Health Study (CHS), the NHLBI's Atherosclerosis Risk in Communities (ARIC) Study, and the Icelandic Heart Association's and NIA's Iceland Age, Gene/Environment Susceptibility (AGES) Reykjavik Study and the European Special Population Network (EUROSPAN).

We are indebted to the staff and participants of the AGES Reykjavik Study, the ARIC Study, the CHS Study, the FHS Study, the Rotterdam Study, and EUROSPAN for their important contributions. A full list of principal CHS investigators and institutions can be found at http://www.chs-nhlbi.org/pi.htm. We acknowledge the National Heart, Lung, and Blood Institute, who has made the SHARe (SNP Health Association Resource) project possible. We thank Pascal Arp, Mila Jhamai, Dr Michael Moorhouse, Marijn Verkerk and Sander Bervoets for their help in creating the Rotterdam database and Maxim Struchalin for his contributions to the imputations of the Rotterdam data.

NOTE: Supporting Information can be found on the PLoS Genetics website at: www.plosgenetics.org

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Chapter 9

Twenty bone mineral density loci identified by large-scale meta-analysis of genome-wide association studies

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Nature Genet. (2009, in press)

ABSTRACT

Bone mineral density (BMD) is a heritable complex trait used in the clinical diagnosis of osteoporosis and the assessment of fracture risk. We performed meta-analysis of five genome-wide association studies of femoral neck and lumbar spine BMD in 19,195 subjects of Northern European descent. We identified 20 loci reaching genome-wide significance (GWS; $p<5x10^{-8}$), of which 13 map to new regions including 1p31.3 (*GPR177*), 2p21 (*SPTBN1*), 3p22 (*CTNNB1*), 4q21.1 (*MEPE*), 5q14 (*MEF2C*), 7p14 (*STARD3NL*), 7q21.3 (*FLJ42280*), 11p11.2 (*LRP4*; *ARHGAP1*; *F2*), 11p14.1 (*DCDC5*), 11p15 (*SOX6*), 16q24 (*FOXL1*), 17q21 (*HDAC5*) and 17q12 (*CRHR1*). The meta-analysis also confirmed at GWS level, seven known BMD loci on 1p36 (*ZBTB40*), 6q25 (*ESR1*), 8q24 (*TNFRSF11B*), 11q13.4 (*LRP5*), 12q13 (*SP7*), 13q14 (*TNFSF11*), and 18q21 (*TNFRSF11A*). The numerous SNPs associated with BMD map to genes in signaling pathways with relevance to bone metabolism, and highlight the complex genetic architecture underlying osteoporosis and BMD variation.

INTRODUCTION

Osteoporosis is a condition characterized by reduced bone mass and microarchitectural deterioration of bone tissue, leading to loss of bone strength and increased risk of fracture. Osteoporosis increases in incidence with age, and is a major health threat to hundreds of millions of elderly individuals worldwide. Linkage analysis in monogenic bone disorders like Osteoporosis-Pseudoglioma syndrome, sclerosteosis, high bone mass syndrome and Paget's disease, have yielded major advances in recent years and highlighted the importance of the *Wnt* signaling[1] and the *RANK/RANKL/Opg* pathways in the regulation of bone mass and bone turnover[2]. Linkage studies for the common form of osteoporosis have had limited success however[3]. As with other complex diseases, most of the candidate gene association studies in osteoporosis have produced conflicting results with limited replication[4] mostly due to small sample sizes[5]. However, large-studies of major candidate gene polymorphisms within the sufficiently powered setting of the GENOMOS consortium, have been successful in obtaining consistent evidence of association between some genetic variants, BMD and fracture[6-10]. Concurrently, genome-wide association studies (GWAS), which perform a hypothesis-free search for genetic determinants,[11] have already been successful in identifying 10 loci associated at a genome-wide significance (GWS) level with BMD[12,13]. Four of these loci involve members of the *Wnt* and *RANK*/*RANKL*/*Opg* signaling pathways highlighting their role in monogenic forms as well as the common form of osteoporosis.

BMD is used in clinical practice for the diagnosis of osteoporosis and in the assessment of fracture risk[14]. BMD is usually measured at the hip (femoral neck) and lumbar spine, which are common sites of fracture. However, BMD measurements at different skeletal sites are predictive of fracture in general because of their high correlation (r^2~0.60).[15] From a genetic perspective, BMD at both spine and hip is a complex, but highly heritable trait (h^2~0.60-0.80)[16]. As shown for human height[17-19], dozens and possibly hundreds of loci with small effects can be expected to influence the variation in BMD. Detection and reliable documentation of these loci of weak effects requires studies with comprehensive coverage of the genome and very large sample sizes. Here, we report the findings of a large-scale meta-analysis of 19,195 adult individuals from five GWAS on BMD of the lumbar spine (LS) and the femoral neck (FN), within the setting of the *GEnetic Factors of OSteoporosis* (GEFOS) consortium.

METHODS

Study population

The GEnetic Factors of Osteoporosis (GEFOS) consortium is a coalition of teams of investigators working on the genetics of osteoporosis. The current meta-analysis incorporated 19,195 individuals of Northern European ancestry derived from five GWAS on BMD of the lumbar spine (LS-BMD) and the femoral neck (FN-BMD) including: the *Rotterdam Study* (RS, n=4,987), *Erasmus Rucphen Family Study* (ERF, n=1,228), *Twins UK Study* (TUK, n=2,734), *deCODE Genetics Study* (dCG, n=6,743) and the *Framingham Osteoporosis Study* (FOS, n=3503). All studies were approved by institutional ethics review committees at the relevant organizations and all participants provided written informed consent. The Rotterdam Study (RS) is a prospective population-based cohort study of chronic disabling conditions in Dutch elderly individuals age 55 years and over (http://www.epib.nl/ergo.htm).[50,51] The Erasmus Rucphen Family (ERF) study is a family-based study of a genetic isolate in the South West Netherlands to identify genetic risk factors for complex disorders.[52] The Twins UK (TUK) study is a population-based sample of twins from the UK studying the hereditary basis of a wide variety of age-related traits and diseases (http:/www.twinsUK.ac.uk).[53] The Icelandic deCODE Genetics (dCG) study comprises a population-based sample to identify the genetic basis of complex diseases.[13] The Framingham Osteoporosis Study (FOS) is embedded in the Framingham Heart Study (FHS), a community-based, longitudinal, prospective cohort comprising three generations of individuals in multigenerational pedigrees and additional unrelated individuals (http://www.framinghamheartstudy.org/). Individuals of "Generation 1" include those first examined in 1948[54], "Generation 2" includes those examined at the first cycle from 1971 to 1975[55], and "Generation 3" includes those examined at the first cycle beginning in 2002-2005.[56] For these analyses, 812 members of the Generation 1 cohort (22.6% of the sample) and 2783 (77.4%) of the Generation 2 cohort who had BMD measured as part of the FOS were included.

Bone mineral density and anthropometric measurements

BMD was measured in all cohorts at the lumbar spine (either at L1–L4 or L2-L4) and femoral neck using dual-energy X-ray absorptiometry following standard manufacturer protocols (GE-Lunar Corporation, Madison, WI or Hologic Incorporated, Bedford, MA) see Supplementary Table 1 for details. All DXA and anthropometric measurements were performed in the RS at the baseline visit baseline between 1991-1992, in ERF between 2002-2003, in TUK at the latest follow-up, dCG at the baseline visit and in FOS Generation 1 between 1992-1997, and Generation 2 between 1996-2001.

Phenotype modeling

The overall strategy involved linear regression to adjust BMD measurements for effects of age, weight, sex and study using standardized residuals with mean 0 and standard deviation 1 in the genotype-phenotype association testing. Such residuals were obtained by regressing within each study the raw BMD measurements on age and weight (and principal components in FOS to adjust for population substructure using Eigenstrat[57]) in sex-specific models. Thus, in studies including both men and women the data for each gender are included as separate estimates in the meta-analysis.

Genotyping

The five GWAS were genotyped using the Illumina Infinium HumanHap550 Beadchip (RS), the Illumina Infinium HumanHap300 or HumanCNV370 Beadchip (ERF, TUK & dCG) or the Affymetrix Dual NspI/StyI GeneChip 2x250K with 50K gene-centered MIP set (FOS), all according to manufacturer's protocols and quality control standards. The exclusion/filtering criteria for individuals and SNPs are described in Supplementary Tables 2-3.

Genotype imputation

Imputation was used to evaluate associations for the same SNPs across study populations using scans from different genotyping platforms. Genotypes were imputed for all polymorphic SNPs oriented to the positive strand from phased autosomal chromosomes of the HapMap CEU Phase II panel (release 22, build 36)[58]. Hidden Markov Model-based algorithms were used to infer unobserved genotypes probabilistically as implemented in either MACH[59] or IMPUTE[60]. Imputation quality control metrics from MACH and IMPUTE were used. Detailed descriptions of quality control and imputation procedures are summarized for all studies in Supplementary Table 3. We performed technical validation of the imputed genotypes in an independent set of 880 individuals of Icelandic origin using Centaurus (Nanogen)[61] discrimination assays, for the top associated hits that reached GWS for the first time in this study (Supplementary Table 10).

Genotype-phenotype association testing [62]

Each study performed genome-wide association for BMD using sex-specific, age- and weight-adjusted standardized residuals analyzed under an additive (per allele) genetic model. Analysis of imputed genotype data accounted for uncertainty in each genotype prediction by utilizing either the dosage information from MACH or the genotype probabilities from IMPUTE. Studies used MACH2QTL[59], which uses genotype dosage value

(0 – 2, continuous) as a predictor in a linear regression framework, SNPTEST[60], Merlin[63] or the linear mixed effects model of the Kinship[64] and ProABEL[65] packages in R[66] to account for relatedness (Supplementary Table 3). The genomic control method[20] was used to correct the standard error by the square root of the genomic inflation factor (lambda): $SE_{corrected} = SE * \sqrt{\lambda}$, which is equivalent to the proposed correction of the Chi^2-statistic by lambda. Genomic inflation factors for the studies are presented on Supplementary Table 3. Overall meta-analysis genomic control inflation factors were calculated as described previously.[67] Genomic inflation factors scaled to a standard size (1000 individuals) to calibrate for the effect of sample size on λ[67], showed residual genomic inflation was negligible ($\lambda_{LSBMD1000}$=1.005 and $\lambda_{FN\text{-}BMD1000}$=1.004).

Meta-analysis

The minor allele from HapMap CEU genotypes was used to define the coded allele in all analyses, regardless of frequency in individual cohorts. All meta-analysis calculations were done using the METAL[21] software package applying inverse-variance methodology assuming fixed effects with Cochran's Q and I^2 metrics used to quantify between-study heterogeneity. We also calculated the summary results by random effects using STATA software[68] for those markers associated at GWS level with Cochran's Q *p*-value < 0.05 and/or I^2 estimates > 50%, reflecting large heterogeneity beyond chance. Random effects models also incorporate in the calculations the between-study heterogeneity, and estimate the average genetic effect from the population of genetic effects that may differ in different studies. In the absence of between-study heterogeneity fixed and random effects calculations give identical results. We declared results genome-wide significant at $\alpha=5x10^{-8}$ after adjusting for all common variant tests in the human genome[22,23]. To test for BMD site specificity we estimated the effect difference ($\Delta\beta$) as $\beta_{femoralneck} - \beta_{lumbarspine}$, the SE of the mean difference (ΔSEM) as $sqrt(SE_{femoralneck}^2 + SE_{lumbarspine}^2)$ and the *Z*-statistic as $\Delta\beta/\Delta SEM$ from which the *p*-values were computed.

eQTL analysis in human osteoblast (HOb)

SNPs from new loci associated with BMD at the genome-wide significance (GWS) level were tested for association with *cis*-allelic expression of neighboring gene transcripts, in primary human osteoblasts (HOb) derived from 95 unrelated Swedish donors. Detailed cell culture methods have been described previously[69]. Expression profiling was performed using the Illumina HumRef-8 BeadChips according to the manufacturer protocol. Genotyping for genotype-expression association was performed using the Illumina HumanHap 550k Duo chip. Individuals with low genotyping rate and SNPs showing significant deviation from Hardy-Weinberg equilibrium ($P < 0.05$) were ex-

cluded. Similarly low frequency (MAF<0.05) SNPs and SNPs with high rates of missing data were excluded. The association of the expression levels was focused on *cis*-acting genetic variants, defined as being within 250kb window flanking the gene, using a linear regression model implemented in the PLINK[70] software package with age and sex as covariates. SNPs included on the Illumina 550K chip were assessed for expression *cis*-associations directly. In addition, all genotyped SNPs included on the Illumina chip that were in strong LD (defined as D´≥0.8) and mapping ± 50kb from the GWS hit were included in the association study. To test for a significant enrichment of functional SNPs (i.e., SNPs associated with gene expression in HObs at $p<0.05$) among the candidate SNPs, a Chi^2- statistic was obtained to test whether observed associations were different from expected associations in the expression data set beyond chance. Expected values (7.1%) were based on the proportion of SNPs with $p<0.05$ seen in the HOb gene expression data set and in a random selection of 1200 SNPs associated with both LS- and FN-BMD at P>0.90, MAF>0.20, and present in the Illumina HumanHap550 array (assessed as negative controls for association with HOb expression).

Combined effect of associated loci

Within the setting of the prospective population-based Rotterdam Study the combined effect of all 20 BMD loci was studied by classifying subjects according to the number of BMD decreasing (risk) alleles. This was based on 15 lumbar spine and 10 femoral neck BMD loci as follows:

Lumbar spine (15 SNPs) => rs7524102 [*ZBTB40*], rs1430742 [*GPR177*], rs11898505 [*SPTBN1*], rs1471403 [*MEPE*], rs2504063 [*ESR1*], rs1524058 [*STARD3NL*], rs4729260 [*FLJ42280*], rs2062377 [*TNFRSF11B*], rs16921914 [*DCDC5*], rs599083 [*LRP5*], rs2016266 [*SP7*], rs9533090 [*TNFSF11*], rs10048146 [*FOXC2*], rs9303521 [*CRHR1*] and rs884205 [*TNFRSF11A*].

Femoral neck (10 SNPs) => (rs6426749 [*ZBTB40*], rs2566755 [*GPR177*], rs87938 [*CTNNB1*], rs1366594 [*MEF2C*], rs2941740 [*ESR1*], rs7781370 [*FLJ42280*], rs11995824 [*TNFRSF11B*], rs7117858 [*SOX6*], rs7932354 [*ARHGAP1*], and rs228769 [*HDAC5*]). The mean BMD for each risk allele-count group was determined, and at the extremes of the distribution counts were pooled into the nearest risk allele-count group of size >100 individuals. The approximate BMD difference in g/cm^2 was obtained by multiplying in each group the mean Z-score LS-BMD by 0.18 g/cm^2 and the mean Z-score FN-BMD by 0.13 g/cm^2 (the SDs of BMD in the Rotterdam study). The allele score was obtained by dividing the number of "BMD decreasing alleles" by the total number of alleles. Also within the setting of the prospective population-based Rotterdam Study**,** we determined the risk for vertebral and non-vertebral fracture for the combined allelic scores constructed for all the top hits associated at a genome-wide significantly level with BMD. Risk ratio

(RR) estimates were obtained from logistic regression (vertebral fractures) and Cox-proportional hazards (incident non-vertebral fractures) models adjusted for sex, age and weight. To determine the fraction of fracture risk explained by BMD, we applied the following formula: $[\ln RR_{unadjusted} - \ln RR_{BMDadjusted}] / \ln RR_{unadjusted}$. Methods describing the fracture datasets have been published previously[71,72]. In summary, thoracolumbar radiographs of the spine were obtained in 3308 (genotyped) individuals who survived on average 6.4 ± 0.4 (SD) years and were scored for presence of vertebral fractures (n=329) using the McCloskey/Kanis method[73]. Record of the incident non-vertebral fractures (n=900) occurring between the baseline visit from 1990 through 1993 until January 1, 2002, was obtained from the computerized records of general practitioners and hospital registries for 5974 genotyped individuals followed on average 8.2 ± 2.7 (SD) years after the baseline visit.

RESULTS

Samples, genotyping and (meta) analysis of genome-wide scans

The five study populations included the Rotterdam Study (RS, n=4,987), Erasmus Rucphen Family Study (ERF, n=1,228), Twins UK Study (TUK, n=2,734), deCODE Genetics Study (dCG, n=6,743) and the Framingham Osteoporosis Study (FOS, n=3,503). The age of participants ranged from 18 to 96 years. All studies had a majority of women (range 57-88%) in their samples with TUK including women only. Additional characteristics of the study populations and subject exclusion criteria are presented in Supplementary Tables 1 and 2, respectively (www.nature.com/ng/index.html).

BMD loci identification

Association results (corrected by the genomic control method[20]) of all HapMap CEU imputed SNPs passing quality-control (QC) criteria in each study (Supplementary Table 3), were meta-analyzed using METAL[21]. We declared results genome-wide significant at $\alpha=5x10^{-8}$ after adjusting for all common variant tests in the human genome[22,23]. We investigated if there was an excess of significant associations by comparing the test statistics to those expected under the null distribution using inter quantile-quantile (QQ) plots (corrected for overall meta-analysis genomic control $\lambda_{LS\text{-}BMD}=1.09$ and $\lambda_{FN\text{-}BMD}=1.08$). As observed in **Figure 1**, strong (and not early) deviation of the observed statistics from the null distribution was observed for both BMD traits, corresponding to an excess of significant and likely true associations. Excluding all SNPs within 500 Kb of the SNPs associated at a GWS level and correction for overall meta-analysis genomic control, still

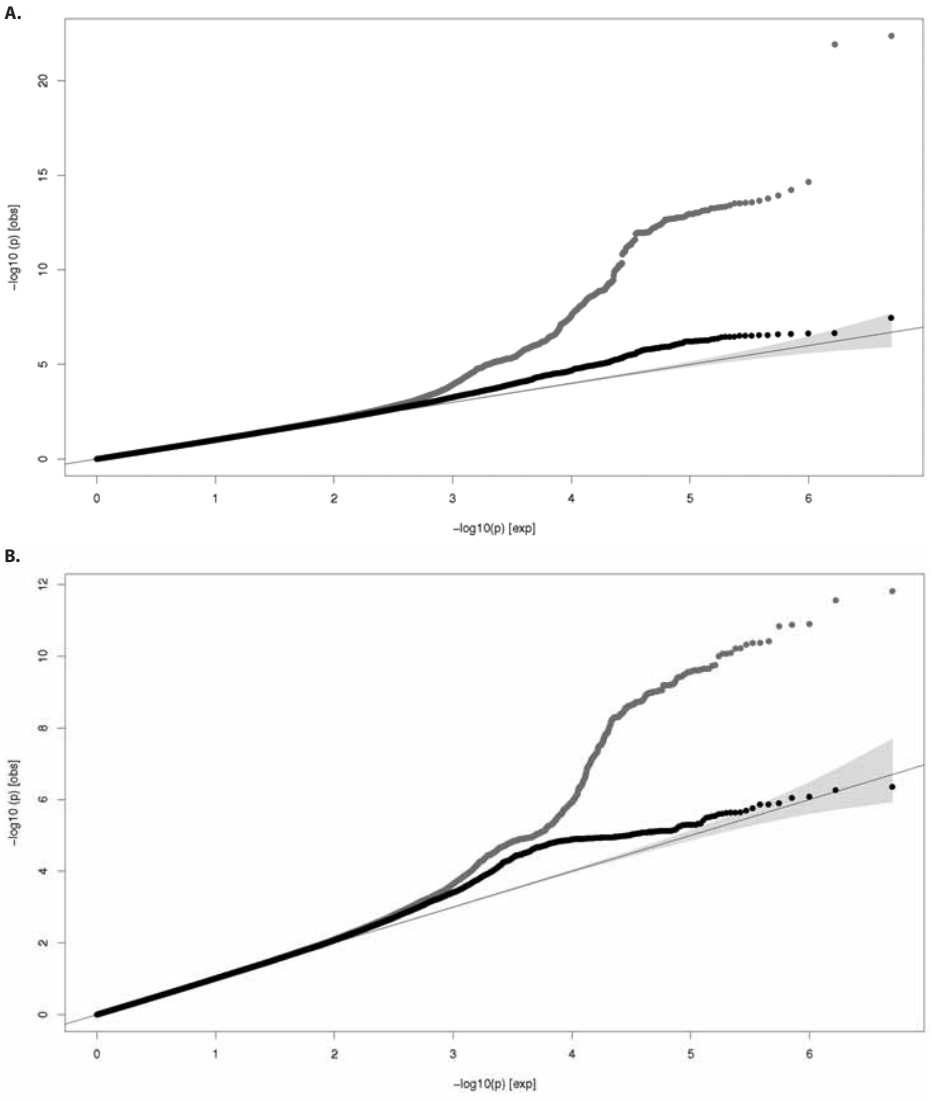

Figure 1. Quantile-Quantile (Q-Q) plots for: **A.** Lumbar Spine BMD and **B.** Femoral Neck BMD; comparing additive model statistics to those expected under the null distribution using fixed-effects for all analyzed HapMap CEU imputed SNPs passing quality-control (QC) criteria in the studies (upper lines), and after exclusion of all genome-wide significant and correlated (r^2>0.1) SNPs (lower lines).

left many SNPs associated with BMD more than expected by chance alone. This suggests that among many false positives appearing at less stringent statistical thresholds, additional truly associated BMD variants may exist.

The meta-analysis identified 467 SNPs from 20 genomic loci exceeding the GWS threshold of association with the BMD traits (**Figure 2**). Of these, 15 loci associated with LS-BMD (Supplementary Tables 4A and 5A) and 10 with FN-BMD (Supplementary

Table 1. Top genome-wide significant markers of the 20 loci associated at GWS with lumbar spine and/or femoral neck BMD

	Marker Information						Lumbar spine BMD		
							Effect estimate (in SD)		
Locus	**SNP**	**A1*/A2**	**FREQ***	**Type**	**Closest Gene**	**Distance (kb)**	**Beta**	**SE**	**P-value**
Novel loci associated with BMD at GWS level									
1p31.3	rs1430742	T/C	0.79	I	GPR177	44.0	0.105	0.014	**2.6E-13**
	rs2566755	T/C	0.79	G	GPR177	44.3	0.104	0.014	**3.3E-13**
3p22	rs87938	A/G	0.45	G	CTNNB1	103.3	-0.043	0.012	1.7E-04
5q14	rs1366594	A/C	0.55	G	MEF2C	197.0	0.005	0.012	0.65
7p14	rs1524058	T/C	0.40	I	STARD3NL	81.7	-0.070	0.012	**1.1E-09**
7q21.3	rs4729260	C/G	0.68	I	FLJ42280	14.9	-0.081	0.013	**1.7E-10**
	rs7781370	T/C	0.34	I	FLJ42280	0.7	-0.074	0.012	**1.1E-09**
11p14.1	rs16921914	A/G	0.27	G	DCDC5	61.6	0.077	0.013	**2.3E-09**
11p15	rs7117858	A/G	0.80	G	SOX6	297.3	-0.042	0.014	0.004
16q24	rs10048146	A/G	0.81	G	FOXL1	95.4	-0.093	0.016	**1.7E-08**
17q12	rs9303521	T/G	0.46	G	CRHR1	56.5	-0.068	0.012	**1.4E-08**
Suggestive loci now associated with BMD at GWS level									
2p21	rs11898505	A/G	0.34	G	SPTBN1	1.1	0.067	0.012	**1.6E-08**
4q21.1	rs1471403	T/C	0.34	G	MEPE	7.3	0.068	0.012	**1.5E-08**
11p11.2	rs7932354	T/C	0.29	I	ARHGAP1	0.1	0.056	0.013	1.1E-05
17q21	rs228769	C/G	0.80	I	HDAC5	7.8	0.067	0.014	4.0E-06
Known loci associated with BMD at GWS level									
1p36	rs7524102	A/G	0.83	G	ZBTB40	79.9	0.094	0.015	**3.2E-10**
	rs6426749	C/G	0.17	I	ZBTB40	66.9	0.107	0.017	**7.6E-10**
6q25	rs2504063	A/G	0.40	G	ESR1	38.0	-0.078	0.012	**6.1E-11**
	rs2941740	A/G	0.57	I	C6orf97	67.3	0.070	0.012	**2.0E-09**
8q24	rs2062377	A/T	0.56	I	TNFRSF11B	43.0	0.094	0.012	**3.5E-16**
	rs11995824	C/G	0.45	I	TNFRSF11B	48.3	-0.093	0.012	**1.1E-15**
11q13.4	rs599083	T/G	0.69	G	LRP5	24.4	-0.067	0.012	**4.7E-08**
12q13	rs2016266	A/G	0.68	G	SP7	1.6	0.070	0.012	**1.3E-08**
13q14	rs9533090	T/C	0.50	I	AKAP11	54.0	-0.120	0.012	**5.4E-25**
18q21	rs884205	A/C	0.27	I	TNFRSF11A	1.4	-0.078	0.0136	**9.4E-09**

Type SNP : G=Genotyped (at least in 1 study) I=Imputed
Distance : to coding region
Bold: $P<5x10^{-8}$
OMA-GC : overall meta-analysis genomic control
NC : Not calculated: P>0.001
Q P-value: Q-statistic P-value

Lumbar spine BMD						Femoral neck BMD			Site Specificity
Heterogeneity			Effect estimate (in SD)			Heterogeneity			H o : β LS = β FN
OMA-GC P-value	Q P-value	I 2	Beta	SE	P-value	OMA-GC P-value	Q P-value	I 2	Q P-value
2.5E-12	0.26	21	0.100	0.014	**1.8E-12**	**1.2E-11**	0.75	0	0.82
3.1E-12	0.27	20	0.100	0.014	**1.7E-12**	**1.1E-11**	0.76	0	0.83
3.1E-04	0.24	23	-0.070	0.011	**8.1E-10**	**3.4E-09**	0.14	34.5	0.10
0.66	*NC*	*NC*	-0.085	0.011	**1.3E-13**	**1.1E-12**	0.62	0	3.2E-08
5.2E-09	0.18	30	-0.038	0.011	8.9E-04	1.4E-03	0.05	48	0.05
9.5E-10	0.14	35	-0.085	0.012	**9.4E-12**	**5.4E-11**	0.77	0	0.82
5.5E-09	0.12	37	-0.083	0.012	**4.7E-12**	**2.9E-11**	0.68	0	0.60
1.0E-08	0.52	0	0.038	0.013	0.003	0.005	*NC*	*NC*	0.03
0.005	*NC*	*NC*	0.088	0.014	**6.4E-10**	**2.7E-09**	0.70	0	1.5E-10
6.0E-08	0.21	28	-0.085	0.016	1.7E-07	4.9E-07	0.96	0	0.73
5.0E-08	0.05	49	-0.055	0.012	3.6E-06	8.3E-06	0.07	45	0.46
6.3E-08	0.39	6	0.027	0.012	0.02	0.03	*NC*	*NC*	0.02
5.7E-08	0.18	30	0.059	0.012	7.8E-07	2.0E-06	0.56	0	0.58
2.4E-05	0.61	0	0.073	0.012	**4.0E-09**	**1.5E-08**	0.68	0	0.32
1.0E-05	0.87	0	0.081	0.014	**1.7E-08**	5.8E-08	0.94	0	0.49
1.7E-09	0.27	19.5	0.079	0.015	**8.8E-08**	2.6E-07	0.64	0	0.48
3.8E-09	0.74	0	0.082	0.015	**4.8E-08**	1.5E-07	0.64	0	0.26
3.7E-10	0.03	52.7	-0.066	0.012	**3.0E-08**	9.6E-08	0.84	0	0.45
9.3E-09	0.01	59.0	0.073	0.012	**2.0E-10**	**9.1E-10**	0.02	57.2	0.84
5.7E-15	0.31	15.4	0.062	0.011	5.4E-08	1.7E-07	0.61	0	0.05
1.6E-14	0.24	22.3	-0.066	0.011	**7.1E-09**	**2.6E-08**	0.48	0	0.10
1.7E-07	0.50	0	-0.047	0.012	9.7E-05	0.0002	0.76	0	0.25
5.2E-08	0.93	0	0.046	0.012	1.9E-04	0.0003	0.81	0	0.16
4.6E-23	0.02	57.1	-0.041	0.011	3.9E-04	0.0006	0.39	5.7	1.1E-06
3.8E-08	0.90	0	-0.039	0.013	0.004	0.005	*NC*	*NC*	0.04

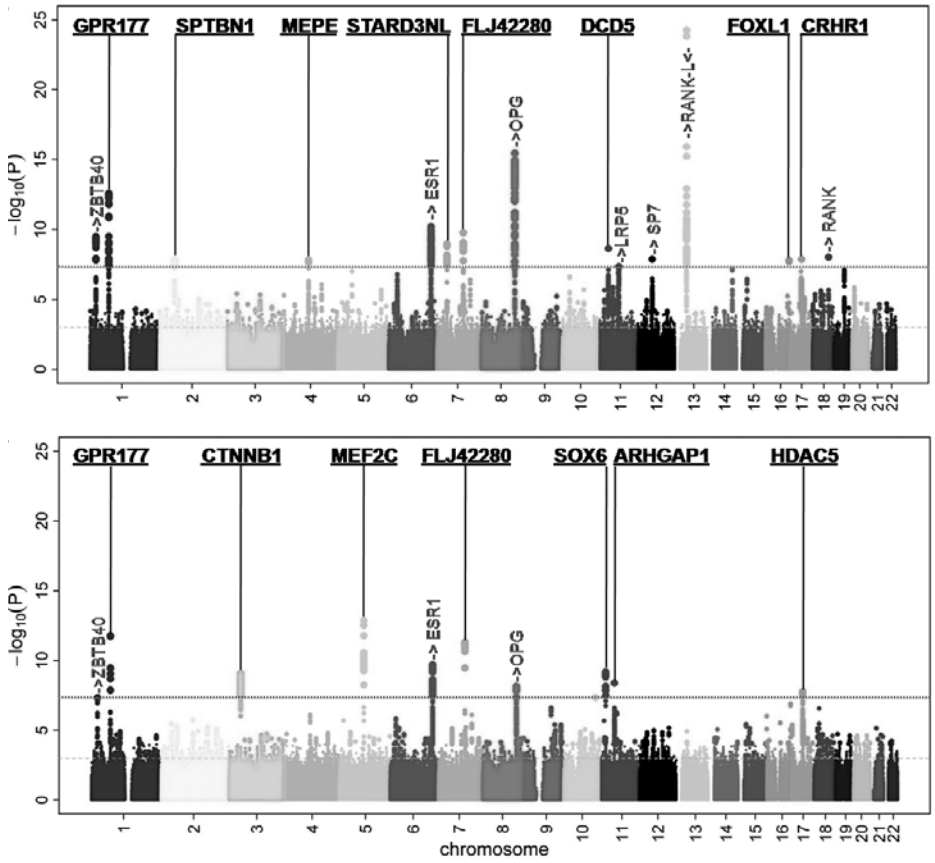

Figure 2. Manhattan plots displaying novel and previously reported (known) loci associated at genome-wide significant level (GWS) with Lumbar Spine BMD (top) and Femoral Neck BMD (bottom) for all 2,543,686 HapMap CEU-imputed SNPs analyzed using fixed-effects. The 13 new GWS loci are in bold and underlined type.

Tables 4B and 5B); five of these loci were associated with both skeletal sites. The effect sizes and significance of the top SNPs from the 20 regions containing markers associated with LS- and FN-BMD at GWS are presented in **Table 1**. Applying correction for overall- meta-analysis genomic control resulted in five of the 20 loci not to be GWS (with $7.1x10^{-7}<P<5.0x10^{-8}$). For most markers heterogeneity was not very large or statistically significant.

Despite the correlation between LS- and FN-BMD measurements, site-specific effects were observed. Seven of the 20 loci showed evidence for skeletal site-specificity ($P<0.05$), three of which displayed strong evidence for site-specificity ($P<1x10^{-6}$). This site-specificity is to be expected given the differences in heritability and that the genetic

correlation (or fraction of "shared" heritability) between the measurements is considerably less than 1 (Supplementary Table 6).

Genes in associated regions and their function

Of the 20 BMD loci identified in this genome scan at a GWS level, seven have been reported previously as GWS[13,24], whereas the remaining 13 have not. Of these 13 loci not previously associated with BMD at a GWS level, four were suggestively associated in previous reports ([12,13]), whereas nine are novel loci. In Supplementary Table 7 we present a summary of relevant gene annotations including related pathways, monogenic syndromes, knockout mouse models, and additional functional details of the genes most likely to be underlying the associated signals in these 13 loci.

Novel loci associated with BMD at GWS level

There are nine loci displaying novel associations with BMD for which we present Forest plots of effects (**Figure 3**) and regional association plots (Supplementary Figure 1) from the top SNPs.

1p31.3 locus [*GRP177*]

Two common SNPs (MAF=0.21) in complete pair wise LD (rs1430742 and rs2566755) were associated at a GWS level with both FN- and LS-BMD. Both top SNPs are located within an intronic region of the *G protein-coupled receptor 177* (*GPR177*, also named WNTLESS homologue) gene. *GPR177* is part of the highly evolutionary conserved *Wnt* signaling pathway,[25] involved in bone cell differentiation and development. The gene has been shown to be positive regulator of the *I-kappaB kinase/NF-kappaB* cascade, part of the RANK system. Cross-talk between the NF-*k*B and the mitogen-activated protein kinase (*MAPK*) pathway has been indicated by the identification of several overlapping genes expressed in both pathways, including several G protein-coupled receptors[26].

3p22 locus [*CTNNB1*]

The rs87939 SNP (MAF=0.45) located 103 Kb upstream of the *catenin (cadherin-associated protein), beta 1* (*CTNNB1*) gene on chromosome 3 was associated at a GWS level with FN-BMD. *CTNNB1* is integral to the *Wnt* signaling pathway, and as such, is an excellent candidate for BMD regulation, considering that *Wnt* signaling controls the process of bone resorption by (negative) regulation of *Opg* (*TNFRS11B*) expression in osteoblasts[27].

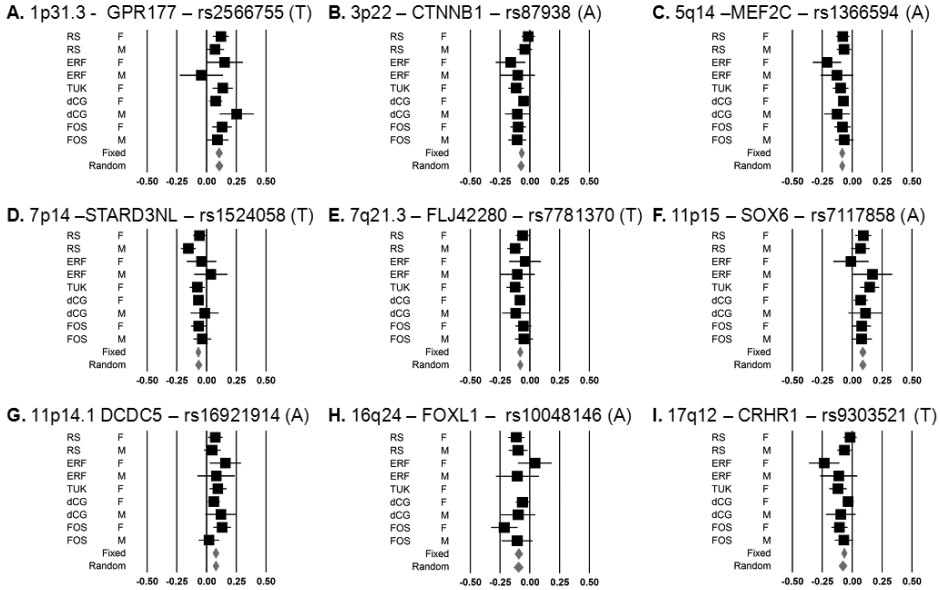

Figure 3. Forest Plots for the top SNPs for each of the 9 novel loci: **A.** *1p31.3*, **B**. *3p22*, **C.** *5q14*, **D.** *7p14*, **E.** *7q21.3*, **F.** *11p15*, ***G.*** *11p14.1*, **H.** *16q24*, **I.** *17q12*. Squares represent effect estimate and 95%CI for each study, and the diamonds are summary effect. Measurements units are in BMD standard deviations (SD).

5q14 **locus[*MEF2C*]**

The rs1366594 top SNP (MAF=0.45) displayed skeletal site-specificity being associated with FN-BMD only, together with other 60 GWS markers at this locus. The SNP is located 197 Kb upstream of the *MADS box transcription enhancer factor 2, polypeptide C* (*MEF2C*) gene with no other known annotation within the large LD block. *MEF2C* is a transcription factor highly expressed in muscle which allows transcriptional cross-talk between theCa2+/calmodulin-dependent kinase (*CaMK*) and mitogen-activated protein kinase (*MAPK)* signaling pathways by signal-dependent dissociation from histone deacetylases (HDACs). *MEF2C* interacts with *HDAC4* and *HDAC5* (see below 17q21 region), resulting in repression of the transcriptional activity of *MEF2C*. Potthoff et al. showed in mice that HDACs selectively degraded by the proteasome enables *MEF2C* to activate the slow myofiber gene program, resulting in enhanced endurance during physical exercise[28].

7p14-p13 **locus[*STARD3NL*]**

The rs1524058 SNP (MAF=0.40) located on the short arm of chromosome 7 is 81 Kb upstream of the *STARD3 n-terminal like* (*STARD3NL*) gene, a cholesterol endosomal transporter, was associated at a GWS level with LS-BMD but less strongly with FN-BMD. Very

recently, a study on Asian individuals[29] identified a SNP in this 7p14 region consistently associated with BMD measurements of the radius, tibia and heel (lowest $P=1.4x10^{-7}$). Yet, the rs1721400 SNP was not associated with either LS- or FN- BMD in our study ($P>0.05$). The rs1721400 marker (mapping close to *SFRP4*) is in very low LD with our top SNP rs1524058 in HapMap individuals of European descent ($r^2=0.015$, $D'=0.194$), while LD between markers is considerably higher in Chinese or Japanese individuals ($r^2=0.230$, $D'=0.931$). Thus, an underlying signal common to both Europeans and Asians may be still be captured by these SNPs considering the differences in LD across populations.

7q21.3 locus[*FLJ42280*]

Several SNPs are GWS in this region, with the two top SNPs being in moderate pair wise LD ($r^2=0.36$; $D'=0.84$) and associated at GWS with both LS- and FN-BMD. The SNPs are located within (rs4729260) and just upstream (rs7781370) of *FLJ42280*, a hypothetical protein of unknown function. There are several genes within the ~480 kb LD stretch region, of which the *split hand/foot malformation (ectrodactyly) type 1* (*SHFM1)* is the closest gene (87-185 kb away).

11p15 locus[*SOX6*]

The rs7117858 SNP (MAF=0.20) was associated at GWS level with FN-BMD only, displaying strong evidence for skeletal site-specificity. The SNP is located 297kb upstream from the *SRY (sex determining region Y)-box*(*SOX6*) gene, which is a transcription factor of the SOX gene family defined by a conserved high mobility group (*HMG*) DNA-binding domain. The gene is expressed in a wide variety of tissues, most abundantly in skeletal muscle. *Sox6* knock-out mice exhibit early lethality due to cardiac insufficiency and present with mild skeletal abnormalities affecting size and mineralization of endochondral elements[30]. Other SOX-family genes regulate *RUNX2*-mediated differentiation of mesenchymal cells during endochondral ossification (skeletogenesis)[31].

11p14.1 locus[*DCDC5*;*DCDC1*]

The rs16921914 SNP (MAF=0.27) is the only marker in the 11p14.1 region associated with LS-BMD at a GWS level. All other associated SNPs are in moderate LD with rs16921914 ($r^2<0.70$) and display less strong associations ($P>1x10^{-7}$). The SNP is located 62 kb downstream of the *doublecortin domain containing 1* (*DCDC1*) and 73 kbupstream of the *DCDC5*genes. Doublecortin domains are highly conserved elements which serve as protein-interaction platforms[32]. Mutations in members of this protein superfamily

are linked to several neurogenetic diseases and to our knowledge are not expressed in bone.

16q24.3 locus[*FOXC2*; FOXL1]

The rs10048146 SNP (MAF=0.19) was associated with LS-BMD (-0.09 and is located on the subtelomeric region of chromosome 16, about 95Kb downstream from a cluster of small (1 Kb) genes of the "forkhead" (or winged helix) gene family. The genes are mainly expressed in the gastrointestinal mucosa (*FOXL1*) or are involved in adipocyte metabolism and early stage chondrogenic differentiation (*FOXC2*). *FOXC2* stimulates osteoblast differentiation of mesenchymal cells through activation of canonical *Wnt-beta-catenin* signals[33] while *FOXC2* expression has been shown to occur via bone morphogenetic proteins[34]. Skeletal defects of the spine have been reported in *FOXC2* mouse knockout models[35] and recently, deletions and inactivating mutations affecting the FOX gene cluster have been identified as causing severe malformations of the VACTER type in humans, which include vertebral malformations.[36]

17q12-q22 [*CRHR1*]

The rs9303521 SNP (MAF=0.46) on chromosome 17 was associated with LS-BMD and is located 56 Kb from the *corticotrophin-releasing factor receptor* (*CRHR1*) gene. Among other genes in this LD region, MAP3K14 is another candidate potentially involved in bone-active pathways, particularly through the activation of NF-kappa-B.

SUGGESTIVE LOCI NOW ASSOCIATED WITH BMD AT GWS LEVEL

Four BMD loci which now reach for the first time a GWS level were "suggestively" associated with BMD in previous reports[12,13]. We present Forest plots of effects (**Figure 4**) and regional association plots (Supplementary Figure 2) from these SNPs.

2p21 locus [*SPTBN1*]

The rs11898505 (MAF=0.34) on chromosome 2 is intronic to the spectrin, beta, non-erythrocytic 1 (*SPTBN1*) gene which encodes a major cytoskeletal scaffolding protein, and was associated with LS-BMD. The same SNP was previously shown to be associated with BMD and fractures, even though it did not reach GWS level for BMD in the same study.[13] In mice, disruption of *beta-spectrin* isoforms (Elf) leads to the disruption of TGF-beta signaling by Smad proteins[37].

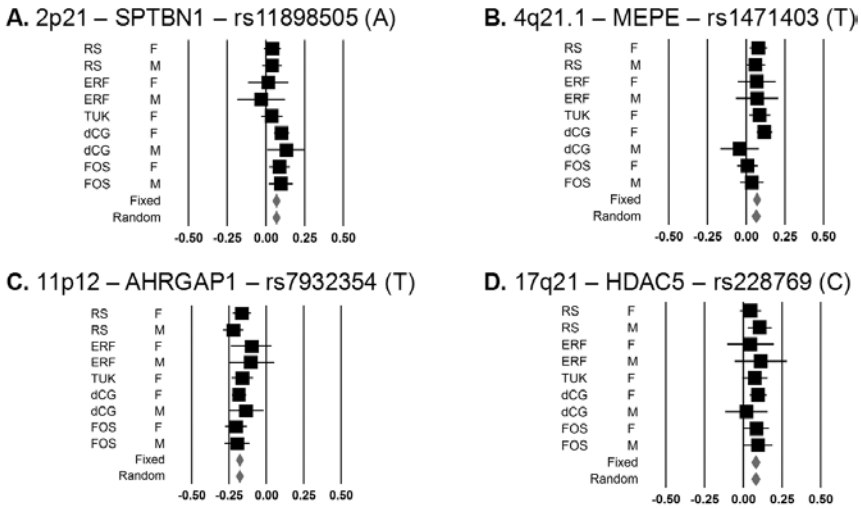

Figure 4. Forest Plots for the top SNPs for each of the 4 loci attaining GWS for the first time in this study: **A.** *2p21*, **B**. *4q21.1*, **C.** *11p11.2*, **D.** *17q21*. Squares represent effect estimate and 95%CI for each study, and diamonds are summary effect estimates. Measurements units are in BMD standard deviations (SD).

4q21.1 locus[*MEPE*]

The 4q21.1 region contains a cluster of structurally and phylogenetically related genes encoding matricellular phosphoglycoproteins with function in bone formation and growth[38]. The top associated rs1471403 SNP (MAF=0.34) is located 7 Kb 3′ to the matrix, extracellular, phosphoglycoprotein (*MEPE*) gene (also known as osteoblast/osteocyte factor 45), 42 Kb to the integrin-binding sialoprotein (*IBSP*) gene and 122 Kb 5′ to the secreted phosphoprotein 1 (*SPP1*) gene, also known as osteopontin. *IBSP* and *SPP1* are highly expressed in osteoblasts, osteoclasts and hypertrophic chondrocytes. *MEPE* is predominantly expressed by osteocytes in human bone, playing an inhibitory role in bone formation. All three genes display diverse skeletal phenotypes in mice knock out (KO) models. *MEPE* (Of45) KO show increased bone mass and inhibition of age-related bone loss[39], *IBSP* KO show high trabecular bone density with low bone turnover but respond to bone loss caused by disuse[40] and the *SPP1* KO have high trabecular bone mass and is resistant to bone loss[41]. Previously, a non-synonymous SNP in the IBSP gene (rs1054627, G195Q) in moderate LD with (r2=0.2, D′=0.8) was reported as suggestively associated with hip BMD.[12]

11p11.2 locus[*ARHGAP1*;*LRP4*]

At the 11p11.2 region a large LD block extends the region withholding several genes, including *C11orf49*, *LRP4*, *CKAP5*, *F2*, *ZN408* and *ARHGAP1* among others. Two fully cor-

related SNPs (r^2=1;MAF=0.29) including rs7932354, which lies in the promoter region of the *Rho GTPase activating protein 1* (*ARHGAP1*) gene; and rs2070852, located in intron 5 of the coagulation factor II (*F2*) gene were associated with FN-BMD at GWS level. Other correlated SNPs in the region (r^2 between 0.2 and 0.8) were previously suggestively associated with hip BMD and attributed to the *LRP4* gene[13]. *ARHGAP1*, a ubiquitous factor composing one of the GTPase activating proteins that represses RhoA, is another good candidate. RhoA is a small G-protein of the Rho family that regulates cell morphology via actin- cytoskeleton reorganization and which is thought to be a potential commitment switch in the differentiation of mesenchymal stem cells to osteoblasts[42]. In addition, data from *ARHGAP1* KO mouse models show a strong skeletal phenotype, including a 3-fold reduction in BMD, decreased cortical thickness and bone fragility in older animals[43].

***17q21* locus [*HDAC5;C17orf53*]**

The 17q21 region contains more than 30 genes in 1 Mb surrounding the top rs228769 SNP (MAF=0.20) which is located 8 Kb upstream of the*histone deacetylase 5* (*HDAC5*) and 26 kb upstream of the *C17orf53* genes. A non synonymous SNP in the *C17orf53* gene, rs227584 (T126P), moderately correlated to rs228769 (r^2=0.64) was found associated with hip BMD, albeit not at GWS level[12]. These SNPs likely represent the same signal. *HDAC5* is a class histone deacetylase II (homologous to yeast Hda1), ubiquitously expressed and responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4). Histone acetylation and deacetylation plays an important role in transcriptional regulation,cell cycle progression and developmental events, particularly for myocyte differentiation[44]. In undifferentiated myoblasts, *HDAC5* is present in the nucleus where it binds to the myocyte enhancer *MEF2C* (see above) to repress transcription and detain muscle maturation. In bone, recruitment of class II histone deacytylases like *HDAC5*, is needed for TGF-β mediated osteoblast differentiation[45], which occurs through inhibition of Runx2 function by Smad3[46].

Known loci associated with BMD at GWS level

We have replicated at GWS level 7 loci associated with BMD in previous GWA studies[12,13,24]. They include the 1p36 (*ZBTB40*), 6q25 (*ESR1*), 8q24 (*TNFRSF11B;*Opg), 11q13.4 (*LRP5*), 12q13 (*SP7*;Osterix), 13q14 (*TNFSF11*;RANKL), and 18q21 (*TNFRSF11A;*RANK) regions. Regional association plots for all seven regions are included in Supplementary Figure 3. In the 6q25 (*ESR1*) region at least two independent GWS signals are seen at the sides of a high recombination rate peak (Supplementary Figure 3B). This locus showed a complex pattern of association in a previous study indicating three independent signals at the locus[13]. In Supplementary Table 8 we report the associations observed in this study for

all loci reported previously as attaining GWS or "suggestive" association[12,13,24]. SNPs from the 6p21.32 (*MHC*), 14q32 (*MARK3*) and 17q21 (*SOST*) regions described previously to be GWS[13] were still significantly associated to BMD in our study, but not at a GWS level. It should be noted that in the previous study the 14q32 and the 17q21 regions were associated with total hip BMD, which differs from the femoral neck BMD phenotype used in the current study.

Gene expression (eQTL) associations

We tested the association of SNPs (or proxies) from the 13 newly GWS associated regions with cis-allelic expression of gene transcripts in primary human osteoblasts. All SNPs associated with gene expression at $P<0.05$ and located within the same LD block of the strongest associated variants ($D' \geq 0.8$) are presented in Supplemental Table 9. Associations were seen for transcripts of *GPR177*, *MEF2C* and *FOXC2*. Similarly, for variants in (or in LD with variants in) *MEPE* the most significant correlation with expression in osteoblasts was seen with the integrin bone sialoprotein (*IBSP)* gene, while *MEPE* seems not highly expressed in osteoblasts. Yet, the statistical evidence is not fully conclusive since only subtle overrepresentation of the associated loci was observed (10.5% vs 7% for non-associated control SNPs, $Chi^2=8.9$ and $P=0.003$). The small overrepresentation of the associated loci suggests several of the associated genes may be expressed in cell & tissues other than osteoblast lineages.

Combined effect of the 20 GWS BMD loci and fracture risk

We examined the combined effect of the top SNPs arising from the 20 associated BMD loci in 4,983 individuals from the prospective population-based Rotterdam study. Risk allele counts were derived from the top associated SNPs from the 15 LS-BMD and 10 FN-BMD loci, all of which followed a normal distribution of their frequency in the study population (**Figure 5**). The 15 LS-SNPs combined explained ~2.9% of the variance in LS-BMD and the 10 FN-SNPs combined explained ~1.9% of the variance in FN-BMD. A highly significant linear decrease in the mean LS-BMD and FN- BMD of individuals was seen with increasing numbers of "low BMD" risk alleles, i.e. those carrying 20 or more alleles that associated with low LS-BMD ("low BMD" alleles) (n=300) had 0.65 SD (~0.12 g/cm^2) lower BMD ($P=3x10^{-8}$) compared to those that carried 11 "low BMD" alleles or fewer (n=360). A similar (yet less pronounced) trend was seen for FN-BMD. The association between the compound allelic scores and the risk of fracture were assessed in 2727 radiographically screened individuals (302 vertebral fracture cases) and in 4865 individuals followed-up 8.2 years on average (672 non-vertebral fractures). The compound LS-BMD allelic score was not significantly associated with the risk of vertebral

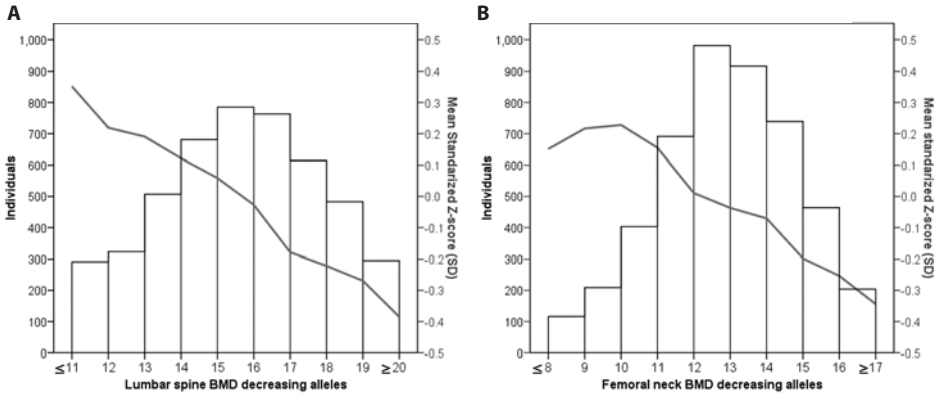

Figure 5. Histogram and line plot modeling in the Rotterdam Study the combined allelic effect across all genome-wide significant associated loci for **A**. Lumbar Spine BMD and **B**. Femoral neck BMD. Subjects were classified according to the number of BMD decreasing (risk) alleles at the lumbar spine BMD (20 SNPs) and the femoral neck (15 SNPs). The mean for each risk allele-count group was determined and the extremes of the distribution counts were pooled into the nearest risk allele-count group of size >100 individuals.

fracture in the Rotterdam Study dataset (OR=1.018, 95%CI[0.966,1.073]; P=0.50), while it was borderline significant for association with the risk of incident non-vertebral fracture (HR=1.031, 95%CI[0.997,1.066]; P=0.07). In contrast, the compound FN-BMD allelic score was consistently associated with the risk of vertebral (OR=1.057, 95%CI[1.010,1.107]; P=0.02) and non-vertebral (HR=1.033, 95%CI[1.004,1.063]; P=0.03) fracture. Adjustment for FN-BMD showed that at least 46% of the genetic effect on vertebral fracture could be explained by FN-BMD ($OR_{adjusted}$= 1.031, 95%CI[0.983,1.080]; P=0.21), and 54% of the genetic effect on incident non-vertebral fracture was through FN-BMD ($HR_{adjusted}$= 1.015, 95%CI [0.986,1.044]; P=0.32). Power limitations are a very plausible explanation to the absence of significant association between the LS-BMD compound allelic score and the risk of vertebral fracture.

DISCUSSION

The GEFOS consortium has been assembled to identify the genetic determinants of osteoporosis and fracture. This study represents the first step in a collaborative effort and expands the current knowledge on the underlying genetics of BMD, a clinical measurement used for the diagnosis of osteoporosis and the assessment of fracture risk. BMD measures of the lumbar spine and femoral neck were analyzed independently, because despite a relatively high phenotypic correlation, the genetic correlation is not perfect. This is illustrated by the site-specificity detected in some of the associations probably reflecting genuine biological mechanisms (i.e. differences in cortical vs trabecular bone

content), but also intrinsic measurement differences (i.e. artifacts influencing BMD values like osteophytes of the lumbar spine or aortic calcifications).

Performing meta-analysis of GWAS has limitations. False positive associations generated from multiple hypothesis testing and population stratification are inherent possibilities. Nevertheless, we applied well-established methods to minimize the impact of multiple testing by applying stringent GWS thresholds for determining significance. Also, our findings are constrained by the power of our current sample suited to identify effect sizes explaining ~0.2% of the variance of the trait. This means we are not powered to detect real effects of the same (small) magnitude arising within specific sex and/or age groups. Similarly, due to power limitations we cannot address potential gene-gene and gene-environment interactions, the effects of rare alleles that are not captured by the haplotype tagging approach employed in GWAS nor determine the effect on fracture risk. Despite being underpowered to assess heterogeneity of effects, some markers displayed significant heterogeneity and were not GWS when analyzed under the random effects model. Further evaluation of such markers in larger populations is needed to determine the source(s) of heterogeneity across datasets, like inaccuracies in the imputations, subtle differences in phenotype ascertainment, differences in linkage to the culprit, differences in environmental modifying factors, or genuinely different genetic effects across populations.[47] Achieving such sufficiently-powered setting is the target of the expanding GEFOS consortium. All our top associated markers displayed high quality imputation scores, high correlation after de-novo genotyping and/or at least one genotyped proxy in complete LD which was also associated at GWS level (Supplementary Table 10). Thus, we can exclude imputation inaccuracies as a source of heterogeneity and/or false-positive associations. In addition, we excluded individuals with non-European profiles strategy which confines our current findings to the context of populations of Northern European-descent. Similarly, test statistics corrected by the genomic inflation factors affecting each study, makes unlikely that population stratification or cryptic relatedness (like those observed in the ERF, TUK, and dCG populations) play an important role in our associations. In addition, we examined the effect of applying a second correction for overall meta-analysis genomic control in our results. Only one locus (*LRP5*) drifted away importantly from the GWS threshold after correction. This approach is likely to be over-conservative considering that the association of variants in LRP5 has been consistently replicated at GWS level in at least two previous efforts[10,24]In summary, we identified and/or confirmed at least 20 loci associated with lumbar spine and femoral neck BMD, highlighting the complex genetic architecture underlying the variation in BMD. Yet, these loci explain only a minor fraction of the variance in BMD, and hence, an even smaller fraction of the heritability for fracture risk. Nevertheless, these findings underscore molecules within novel and key-known biological pathways influencing BMD variation, particularly the *Wnt* and *NF-kappa-B*

signaling pathways (including about half of the identified loci GPR177, *CTNNB1,FOXC2, LRP5, SPTBN1,HDAC5,TNFSF11, TNFRSF11A* and *TNFRSF11B)*. None of the SNPs we have identified can be unequivocally designated as the underlying "true" variants driving the associations. Additional efforts to identify such variants are warranted to maximize the application of this genetic knowledge towards the prediction of risk in individuals and the translation into new pharmacological agents for the treatment of osteoporosis. Increasing further the sample size will aid the identification of additional loci associated not only with BMD, but more importantly will allow focusing on the risk of fracture, the ultimate consequence of osteoporosis.

OMIM[48,49] accession numbers

GPR177[611514]; *CTNNB1*[116806]; *MEF2C*[600662]; *STARD3NL*[611759]; *FLJ42280*[None],*SHFM1*[183600]; *SOX6*[607257]; *DCDC5*[612321],*DCDC1*[608062]; *FOXC2*[602402],*FOXL1*[603252]; *CRHR1*[122561]; *SPTBN1*[182790]; *MEPE*[6059 12],*IBSP*[147563],*SPP1*[166490]; *ARHGAP1*[602732],*LRP4*[604270],*F2*[176930]; *HDAC5*[605315],*C17orf53*[None]; *ZBTB40*[612106]; *ESR1*[133430], *C6orf97*[None]; *TNFRSF11B*[602343]; *LRP5*[603506]; *SP7*[606633]; *TNFSF11*[602642],AKAP11[604696]; *TNFRSF11A*[603499].

ACKNOWLEDGEMENTS

Above all, we thank all study participants for making this work possible. This research and the GEnetic Factors of Osteoporosis (GEFOS) consortium (http://www.gefos.org) have been funded by the European Commission (HEALTH-F2-2008-201865-GEFOS). **Rotterdam Study (RS):** This study was funded by the Netherlands Organization of Scientific Research NWO Investments (nr. 175.010.2005.011, 911-03-012), the Research Institute for Diseases in the Elderly (014-93-015; RIDE2), the Netherlands Genomics Initiative (NGI)/Netherlands Organization for Scientific Research (NWO) project nr. 050-060-810. We thank Pascal Arp, Mila Jhamai, Dr Michael Moorhouse, Marijn Verkerk, and Sander Bervoets for their help in creating the GWAS database. The Rotterdam Study is funded by Erasmus Medical Center and Erasmus University, Rotterdam, Netherlands Organization for the Health Research and Development (ZonMw), the Research Institute for Diseases in the Elderly (RIDE), the Ministry of Education, Culture and Science, the Ministry for Health, Welfare and Sports, the European Commission (DG XII), and the Municipality of Rotterdam. The authors are very grateful to the staff from the Rotterdam Study, particularly Lydia Buist and J. Hannie van den Boogert and also with the participating general practitioners and pharmacists. **Erasmus Rucphen Family (ERF):** The study was supported by grants from The Netherlands Organization for Scientific Research (NWO), Erasmus MC and the Centre for Medical Systems Biology (CMSB). We are grateful to

all general practitioners for their contributions, to Petra Veraart for her help in genealogy, Jeannette Vergeer for the supervision of the laboratory work and Peter Snijders for his help in data collection. **Twins UK (TUK):** The study was funded by the Wellcome Trust; the Arthritis Research Campaign; the Chronic Disease Research Foundation; the Canadian Institutes of Health Research (JBR); European Society for Clinical and Economic Aspects of Osteoporosis (JBR); the European Union FP-5 GenomEUtwin Project (QLG2-CT-2002-01254). The study also receives support from the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's & St Thomas' NHS Foundation Trust in partnership with King's College London. We thank the staff from the TwinsUK, the DNA Collections and Genotyping Facilities at the Wellcome Trust Sanger Institute for sample preparation; Quality Control of the Twins UK cohort for genotyping (in particular Amy Chaney, Radhi Ravindrarajah, Douglas Simpkin, Cliff Hinds, and Thomas Dibling); Paul Martin and Simon Potter of the DNA and Genotyping Informatics teams for data handling; Le Centre National de Génotypage, France, led by Mark Lathrop, for genotyping; Duke University, North Carolina, USA, led by David Goldstein, for genotyping; and the Finnish Institute of Molecular Medicine, Finnish Genome Center, University of Helsinki, led by Aarno Palotie **Icelandic deCODE Study (dCG):** We thank the staff of the deCODE core facilities and recruitment centre for their important contributions to this work. **Framingham Osteoporosis Study (FOS):** The study was funded by grants from the National Institute for Arthritis, Musculoskeletal and Skin Diseases and the National Institute on Aging (R01 AR/AG 41398; DPK and R01 AR 050066; DK). The Framingham Heart Study of the National Heart Lung and Blood Institute of the National Institutes of Health and Boston University School of Medicine were supported by the National Heart, Lung and Blood Institute's Framingham Heart Study (Contract No. N01-HC-25195) and its contract with Affymetrix, Inc for genotyping services (Contract No. N02-HL-6-4278). Analyses reflect intellectual input and resource development from the Framingham Heart Study investigators participating in the SNP Health Association Resource (SHARe) project. A portion of this research was conducted using the Linux Cluster for Genetic Analysis (LinGA-II) funded by the Robert Dawson Evans Endowment of the Department of Medicine at Boston University School of Medicine and Boston Medical Center. **eQTL HOb Study:**The study was supported by Genome Quebec, Genome Canada, and the Canadian Institutes of Health Research (CIHR). T.P. holds a Canada Research Chair. We thank Profs Olof Nilsson, Hans Mallmin and Östen Ljunggren at the Departments of Surgical and Medical Sciences, Uppsala University Hospital, Sweden for large-scale collection of primary bone samples.

NOTE: Supplementary information is available at the Nature Genetics website(www.nature.com/ng/index.html)

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Chapter 10

General discussion

In this thesis, studies are presented on the genetic and nutritional background of the age-related metabolic diseases obesity and osteoporosis, and the relation between the two conditions. In addition, determinants of survival in healthy subjects and in persons with type 2 diabetes are examined.

PART I: EPIDEMIOLOGICAL STUDIES

I a) Sex-specific genetic effects on body composition

Despite sex-differences in the occurrence of many diseases and polygenetic traits relatively few studies have investigated the sex-specificity of genetic effects. In the family-based Erasmus Rucphen Family (ERF) study, our shared heritability studies suggest that gene by sex interactions are present for most body composition traits such as fat mass, lean mass and fat distribution, but not for height and BMI. The genetic correlations between men and women were significantly different from 1 for fat percentage, lean mass, android fat, android-to-gynoid fat ratio and waist-to-hip ratio (WHR). These findings indicate that a given subset of genes contributes differently to the variance of these body composition parameters in men and women. Many claims of sex-related differences in genetic associations on diseases or traits in the past were found to be insufficiently documented or spurious [1]. However, a recent meta-analysis of genome-wide linkage scans provided evidence for sex- and site-specific regulation of bone mass although no genome-wide significant loci were found [2]. So far, few genome-wide association studies (GWAS) have formally addressed sex-differences in diseases or polygenic traits. In a recent meta-analysis from the GIANT consortium, a genetic locus near LYPLA1 associated with waist-to-hip ratio in women only [3]. Large-scale meta-analysis of GWAS with formal testing of gene by sex interaction in a sufficiently powered setting will allow the identification of sex-specific genetic effects when existing. This is an important area of future research for disease prediction, prevention and treatment.

I b.1) Relation between body composition and bone mineral density

Although it has been known for along time that heavier people have higher BMD and experience fewer fractures than lighter individuals, there is an ongoing debate about which part of body weight, namely fat mass or lean mass, is responsible for these beneficial effects on bone. Differences in study design and population characteristics may underlie these inconsistent findings. A complicating factor of all these analyses is that fat mass and lean mass are correlated in a complex manner with each other. When a person gains fat he or she will develop more muscle mass in response to the weight bearing demands of the extra fat mass. On the other hand, during physical inactiv-

ity muscle mass and bone mineral density may decrease while fat mass may increase. Cross-sectional relationships between lean and fat mass may be dependent on the level of adiposity. Therefore, it is difficult to accurately assess independent relations of these body composition parameters with bone. However, it is important to realize that the significance of BMI as a risk factor for fracture varies according to the level of BMI and the contribution to fracture risk is much more marked at low values of BMI than at values above the median [4]. As such, low BMI should be regarded as a stronger risk factor for fracture than obesity as a protective factor. This could be related to a greater protective effect of lean mass compared to fat mass on fractures, or to factors related to frailty (such as muscle weakness, greater risk of falls or nutritional deficiencies). In our study, we found that the positive association between android fat distribution and BMD is explained by higher BMI, but not by higher insulin and/or lower adiponectin levels in obesity states. After adjustment for BMI, android fat was inversely associated with BMD, suggesting that android fat deposition (as measured by circumference ratios and DXA-based android-to-gynoid fat ratio) is not beneficial and possibly even deleterious for bone health. Physical activity could be a potential intermediary of this inverse association but this needs further investigation together with the role of other potential mediators such as glucocorticoids, growth- and sex-steroid hormones, leptin and inflammatory adipokines. Additional prospective studies are needed to evaluate the relation of changes in body composition, including lean mass, fat mass and fat distribution, with changes in BMD and occurrence of fractures over time. Moreover, it will be of great interest to study if and how genetic loci emerging from GWAS on body composition traits (BMI, fat mass, lean mass and fat distribution) could influence bone traits like BMD, bone geometry and fractures and vice versa. Other areas of investigation on the complex cross-trait interactions between the metabolism of bone, fat, and lean tissue are needed, e.g., on the recently discovered intriguing role for the skeleton in energy metabolism in mice, related to osteocalcin [5].

I b.2) Will treatment of obesity induce osteoporosis?

Weight loss is associated with a decrease in bone mineral density in both men and women and irrespective of the level of adiposity [6, 7]. An advice to patients with osteoporosis not to loose weight might be in conflict with an advice for obesity related diseases like type 2 diabetes. The growing epidemic of obesity and related incidence of type 2 diabetes will necessitate weight loss measures for obese persons. It is predicted that many morbidly obese subjects with type 2 diabetes will undergo bariatric surgery within the near future, since this is the only approach that has consistently shown sustained weight loss and improvement or even disappearance of diabetes. Gut hormones are important in appetite regulation as a result of the signals from the periphery to the brain [8]. Changes

in the production of these hormones after bariatric surgery as well as changes in the production and/or sensitivity of leptin, insulin, adiponectin and other hormones and adipokines might impact bone mineral density [9, 10]. Prospective studies on the relation of changes in these hormones and adipokines with changes in BMD and body composition will give more mechanistic insight.

It will be very important for this group of patients to monitor their BMD and ensure optimal intake of calcium and vitamin D, since absorption of these nutrients can be severely compromised after bariatric surgery [11]. A diet high in protein, dairy, and calcium may attenuate bone loss during weight loss [12]. More studies are needed on the effect of the composition of the diet on BMD after bariatric surgery. Bone resorption inhibitors may be considered by physicians for treatment of decreased BMD with high bone turnover after bariatric surgery. However, it is essential to recognize osteomalacia in these patients as a cause for decreased BMD, as treatment with bisphosphonates in the presence of unrecognized osteomalacia is unwanted [13] and may lead to severe hypocalcaemia when administered intravenously [14]. It is also important to consider that oral bishosphonates may lead to increased local adverse events after gastric bypass procedures [15]. Follow-up studies after bariatric surgery should not only focus on BMI but on a wide range of physiological measurements, in particular on BMD and on fractures.

Increased physical exercise may counteract an effect of weight loss on BMD and bone turnover [16-18] but not all studies are consistent in this respect [19, 20]. Irrespective of body weight regulation, exercise programs may prevent falls in elderly persons by improving strength, flexibility, balance, and endurance [21]. Overall, promotion of active life styles may help reduce the incidence of obesity and osteoporosis.

I c) Effect of homocysteine-related B vitamins on BMD and fracture risk

An elevated homocysteine concentration is a novel and potentially modifiable risk factor for age-related osteoporotic fractures [22, 23]. The most common cause of homocysteine elevation is poor vitamin B status, most notably of cobalamin (vitamin B12) and folate (vitamin B11) but also of riboflavin (vitamin B2) and pyridoxine (vitamin B6). In the Rotterdam study we found that increased dietary intake of riboflavin and pyridoxine (but not of folate and cobalamin) was associated with higher BMD of the hip. Furthermore, a reduction was found in the risk of fracture in relation to dietary pyridoxine intake independent of BMD and, in a subset of subjects, also independent of serum homocysteine levels. Based on these findings it cannot be concluded if the association between pyridoxine and fracture risk is causal and this certainly needs further study. The efficacy of homocysteine reduction via supplementation with B vitamins has not been demonstrated in accurately designed clinical trials, despite the existing biological plausibility for an effect on fracture incidence. Currently, the effect of vitamin B supplementation

on fracture incidence is being assessed in elderly people in a double blind randomized placebo-controlled trial in collaboration between three academic centers in the Netherlands (Free University Amsterdam, Erasmus University Rotterdam and Wageningen University).

PART II: CANDIDATE GENE STUDIES ON *SIRT1*

Since the discovery of the *Sir2* gene in yeast as a regulator of gene silencing, ageing and survival, a wealth of information on this family of deacetylases, and especially on the mammalian homologue, *SIRT1*, has emerged in many high impact scientific journals. Sirtuins are NAD^+ dependent, which may form a link between nutritional status and ageing. They may mediate the life prolonging effect of caloric restriction, which is one of the most robust methods for extending lifespan and delaying age-related disease among various species [24, 25]. SIRT1 can be stimulated not only by caloric restriction but also by naturally occurring small molecules such as resveratrol, present for example in grapes and red wine. This observation has fuelled speculation that SIRT1 stimulators may beneficially influence metabolic and age-related diseases such as obesity, type 2 diabetes, cardiovascular disease and dementia, and possibly even prolong human survival.

II a) *SIRT1* and mortality and obesity in humans

Consistent with findings by others [26, 27], we found no association between *SIRT1* genetic variation and mortality in the overall population of elderly subjects from the Rotterdam study. However, in subjects with type 2 diabetes, *SIRT1* genetic variation influenced survival per se and in interaction with dietary niacin and smoking. These findings suggest that correction of niacin deficiency and treatment with *SIRT1* modulators may prolong the life span of patients with diabetes. Awaiting replication in independent populations and functional studies on the *SIRT1* variants, these findings may lead to prospective placebo controlled clinical trials with *SIRT1* modulators in subjects with type 2 diabetes.

The same genetic variants that were associated with mortality in type 2 diabetes were also associated with BMI and risk of obesity in subjects of the Rotterdam study. These findings were confirmed in the ERF Study. Carriers of the minor alleles of two *SIRT1* polymorphisms have 13-18 % decreased risk of obesity and they gain less weight over time. The availability of *SIRT1* stimulators like resveratrol makes these findings relevant in light of the growing epidemic of obesity. Currently, newer and more potent *SIRT1* stimulators are being tested in clinical studies. Diabetes and smoking are demanding metabolic situations with reduced *SIRT1* expression and/or activity and increased oxidative stress, where benefits of resveratrol may become evident. Whether resveratrol

and other newer compounds have therapeutic potential in such situations needs to be studied when more data on pharmacology and possible side effects in humans become available. Clinical trials will also be needed to study an effect on obesity and other age-related diseases. Regarding the utility of treating type 2 diabetics with such compounds, it should be considered that a positive effect of *SIRT1* stimulators through modulation of BMI and insulin secretion by the pancreas might be offset by a stimulatory effect on gluconeogenesis in the liver [28].

II b) *SIRT1* and bone

SIRT1 may also influence bone. We have shown that *SIRT1* is expressed in osteoblasts during differentiation and mineralization (J. P.T.M van Leeuwen et al, unpublished data). Activation of *SIRT1* during differentiation of mesenchymal stem cells decreases adipocyte formation and stimulates osteoblast formation via inhibition of peroxisome proliferator-activated receptor-gamma 2 (PPARγ2) [29, 30]. Age-related bone loss has been associated with high levels of marrow adipogenesis. A role of estrogen in this age-related bone loss might be mediated through the regulation of *SIRT1* expression within the bone marrow [31]. Moreover, *SIRT1* inhibits nuclear factor-κB (NF-κB), a 'master' protein that controls genes associated with inflammation and immunity, also mediating increased bone resorption. It was recently shown that this protein also inhibits new bone formation [32]. *SIRT1* could also influence bone indirectly by an effect on muscle mass [33, 34]. Human studies on the relation between *SIRT1* genetic variants and body composition and BMD and fractures are currently lacking and are being performed within our group.

II c) *SIRT1* and diet

Our findings that interactions between *SIRT1* genetic variants and diet influence obesity needs further follow up in larger studies to pin down the exact underlying dietary factors explaining these interactions. Vitamin E is abundant in foods such as nuts, seeds, vegetable oils and green leafy vegetables. High intake of vitamin E may thus represent a healthy diet or an otherwise healthy lifestyle. Although for many years the use of antioxidants has been advocated to protect the body from damaging free radicals, clinical trials have not provided evidence that routine use of for example vitamin E supplements prevents cardiovascular disease or mortality. Intriguing findings in some recent studies actually suggest that free radicals may under certain circumstances be beneficial to the body by a process called "hormesis". This represents an effect of adaptations where a toxic substance acts like a stimulant in small doses, but is an inhibitor in high doses. A recent paper found that vitamin C and E supplements inhibited exercise-induced beneficial effects on insulin sensitivity in healthy young men, by preventing the promotion

of endogenous antioxidant defense capacity [35]. It will be of interest to study a potential role of *SIRT1* in such exercise-induced anti-oxidant defense.

Further research on *SIRT1* gene-diet interactions is needed within large-scale studies. This will require meta-analysis of data from multiple studies within international consortia after standardization of different methods used to assess dietary intake. Gene-environment interactions may explain part of the so-called "dark matter" in unexplained heritability and it is of great importance to uncover such interactions in the growing epidemic of obesity. Knowledge on gene-diet interaction may modify dietary advices, which will be preferable to new and costly medication with potential side effects.

II d) Might *SIRT1* stimulators increase the risk of developing cancer?

The role of *SIRT1* in cancer is currently under debate due to recent discrepant findings. The concern that stimulating *SIRT1* may elevate cancer risk in mammals has arisen from the fact that *SIRT1* inhibits tumor suppressor P53 activity and also because increased *SIRT1* expression has been observed in certain types of cancer [36]. In fact, *SIRT1* inhibitors are currently under investigation as anti-tumor drugs [37, 38]. On the other hand, caloric restriction extends life span and decreases cancer risk. Also, it was recently demonstrated that increased expression of *SIRT1* reduces colon cancer formation in a mouse model [39]. In addition, resveratrol, exhibits chemo preventive activity against various cancers[40-42] despite its activating effect on *SIRT1*[43]. Furthermore, in a *SIRT1* mutant mouse model *SIRT1* was shown to play an important role in DNA damage repair, genomic integrity, and inhibition of tumorigenesis [44]. Another recent study showed that increased *SIRT1* levels mice protect mice from irradiation-induced cancer and also provided evidence that increasing *SIRT1* activity or quantity may actually reduce instead of promote tumorigenesis through increased genomic stability [39, 45]. However, because the effects of manipulation of *SIRT1* may be tissue and context dependent [46], any intervention in humans targeted at *SIRT1* should keep potential side effects in mind.

PART III: GENOME-WIDE ASSOCIATION STUDIES

There have been impressive advances recently in the understanding of the genetic background of common complex diseases. During the past 2 years, more than 200 GWA studies have reproducibly described over 300 common genetic variants associated with common diseases [47, 48]. Many successes in identifying genes for obesity and osteoporosis have occurred within the powered settings of international consortia. In our study within the international CHARGE consortium a new gene was uncovered, the neurexin 3 gene (*NRXN3*), influencing waist circumference. This gene has been previously implicated in

studies of addiction and reward behavior, providing further evidence that our genes may influence the desire for and consumption of food and, in turn, the susceptibility to obesity. Obesity and addiction may share common neurologic underlying mechanisms [49]. Other well-replicated obesity loci, including melanocortin 4 receptor gene (*MC4R*), have also been shown to be associated with centrally-mediated phenomena including binge eating behavior [50-52]. Studies in mice indicate that the fat mass and obesity associated (*FTO*) gene expression is particularly pronounced in regions of the brain known to regulate energy balance [53], and recent data suggest that variants in the *FTO* gene may regulate food intake and selection [54].

In our meta-analysis of five genome-wide association studies in 19,195 Caucasian subjects within the GEFOS consortium we identified 20 loci associated with femoral neck and/or lumbar spine BMD with genome-wide significance, of which 13 map to new regions. The novel SNPs map to genes in signaling pathways of potential relevance to bone metabolism.

In general, most top signals in GWAS are found within intronic or intergenic non-coding regions of the genome and often implicate genes with yet unknown function. Much work lies ahead in uncovering the causal variants involved, using techniques such as fine-mapping, re-sequencing and functional studies.

A recently criticized aspect of the findings from GWAS is that the combined effect of all discovered genetic variants explains only a small percentage of the heritability of the complex diseases or traits [47]. The explained trait variance is typically in the order of 3% as was also the case for the 20 loci found in our meta-analysis within the GEFOS consortium for bone mineral density. The currently discovered common variants explain together too little of the variance to make them a useful tool in disease prediction [47,55]. However, this is different for the potential to develop new therapeutic compounds and therapies. Even though effects of common variation in individual genes on BMD are quite small (such as for genes in the RANKL-RANK-OPG pathway), the functional impact of the related proteins on the physiological pathway can be substantial. Recently developed drugs that interfere with this pathway, (e.g., a monoclonal antibody against RANKL which mimics the endogenous effects of osteoprotegerin or OPG) can produce significant increases in BMD and decrease fracture risk. The same holds for drugs targeted at PPARγ (thiazolidinediones) for the treatment of type 2 diabetes. These examples show that there is not necessarily a relation between effect sizes of genetic variants and biological relevance. For patient care it will also be important to study whether genetic information can predict the effect of drugs or medication, in the so-called field of pharmaco-genomics but this is a so far largely unexplored area. The main impact from the GWAS studies at this moment is the potential for discovery of new biological pathways underlying common diseases and traits that will lead to better understanding

of health and disease. There is, however, still a long way to go before all this knowledge will be translated into clinical practice.

III a) Dark Matter in heritability

Part of the so-called "dark matter" or "missing genes" in heritability may be explained by structural variants such as copy number variations, inversions and rare variants with potentially larger effect sizes. Also, the sex chromosomes have not been systematically incorporated in GWA studies so far, mainly due to difficulties to impute SNPs from the sex chromosomes that are not on the arrays. Recent advances in techniques will allow investigation of all these variants on a large-scale basis in the near future. On the other hand, one should also realize that narrow-sense heritability estimates are population specific and largely ignore shared environmental and dominance effects as well as interactions and may be inflated, especially in twin studies. Part of the dark matter in heritability could thus be explained by overestimation of heritability for some traits so far. Also, it is possible that epigenetic changes are passed on to the next generation and thus influence heritability estimates without changes in the coding of DNA. Recent studies suggest that epigenetic mechanisms with histone modifications such as DNA methylation may underlie intrauterine programming [56]. It has been shown that some metabolic traits that result from low birth weight can be transmitted to subsequent generations, suggesting the possibility of epigenetic changes maintained during meiosis, as is observed for the agouti coat-color variant in mice [56]. A recent study on DNA methylation profiles in monozygotic and dizygotic twins suggests that also in humans molecular mechanisms of heritability may not be limited to DNA sequence differences [57]. It will be of great interest to examine the effects of intra-uterine programming using more global techniques for monitoring gene expression and chromatin structure. Since SIRT1 deacetylates histones, it is not unthinkable that epigenetic changes induced by SIRT1 during fetal malnutrition can be involved in an adverse metabolic response later in life.

III b) Interaction studies

Important aspects that have not been studied on a large-scale basis so far that may explain part of the dark matter in heritability are the gene-gene and gene-environment interactions. By definition these interactions underlie complex diseases and are therefore essential to incorporate in future research.

Gene-environment interactions:

Diet and physical activity are lifestyle and behavioral factors that play an important role in the etiology, prevention, and treatment of many chronic diseases, including obesity and osteoporosis. Other environmental factors influencing both obesity and osteoporo-

sis are habits like tobacco smoking and the use of alcohol. An improved understanding of how gene-environment interactions affect disease risk may lead to better prevention and treatment options. Limitations to current gene-environment interaction studies are 1) the need for very large studies in order to have sufficient statistical power to detect them, 2) difficulty in harmonizing environmental trait assessment between studies, and 3) general difficulties in measuring accurately environmental effects, since exposures may vary in time and instruments used to quantify these variables (e.g. questionnaires) are subject to several sources of bias.

For more accurate assessment of dietary intake and physical activity, new techniques are being developed or existing technologies are being adapted. They include the use of devices such as sensors, scanning and imaging techniques, wireless technologies and bio-informatics tools. Examples of more accurate dietary assessment include the use of digital cameras to identify foods and estimate portion sizes. This can be combined with audio recording and bar code scanners and wireless technology for real-time data transmission. Also, personal digital assistant (PDA) technologies are being refined to assess dietary and supplement intake. Assessment of physical activity is complex because several important elements have to be taken into account like the type, intensity, frequency and duration of activity. New devices to estimate physical activity can integrate sensors to detect motion and posture with physiologic markers such as heart rate, respiration, and temperature. This will also generate information on time a person spends sleeping, which will be important given the effect of sleep on metabolic diseases. These measurements should be repeated over time in order to detect changes in lifestyle factors. Adoption of these new techniques with more accurate data on dietary intake and physical activity into large-scale epidemiological studies will allow better identification and understanding of the determinants of health and disease and provide better assessments to study gene-environment interactions. It will also be of interest for the determination of heritability of outcomes measured by these techniques (e.g., physical activity, sleep patterns, food preferences) and to search for genes underlying these traits using GWAS.

Gene-Gene interactions:

Another challenge that lies ahead of us is the investigation of gene-gene interactions. To study this with about 2.5 million polymorphisms currently used in GWAS will require large computational capacities and well-powered studies within international consortia. Recent studies imply that systems-based approaches might be a valuable tool to address complex interactions in both animals and humans [58-60].

III c) Systems biology

Systems biology is a biology-based inter-disciplinary study field that focuses on the systematic study of complex interactions in biological systems. It can integrate data that come from studies of DNA variation, global mRNA expression array analysis (providing information on transcript levels from gene expression) and high-throughput technologies for screening of proteins and metabolites (proteomic and metabolomic data) such as mass spectrometry. Subsequently, these data can be used in combination with clinical phenotypes [58-60]. Loci that control transcript levels, named expression or e-QTL's can be mapped and used to prioritize positional candidates [61]. System-based approaches aim to study beyond simple correlations of levels to determine how components interact. These interactions are often described in terms of networks that consist of parts (collaborating proteins) and their connections. The network concept has proven very useful in studies of metabolic traits [59]. We also recently adopted system-based approach in prioritizing candidate genes from GWAS on BMD and bone geometry (Y Hsu et al., manuscript under review).

FUTURE DIRECTIONS

There have been impressive advances recently in elucidating the genetic background of many complex diseases, including obesity and osteoporosis but much, if not most, is yet to be discovered. Important aspects of future research are integrated studies on interactions such as gene by sex, gene by environment (diet, physical activity, tobacco smoking, use of drugs and alcohol), and gene-by-gene interactions. Adopting new strategies to more accurately assess dietary intake and physical activity in population-based studies will greatly enhance our understanding of how gene-environment interactions affect disease risk. This may ultimately lead to better prevention and treatment options.

More genetic variants underlying complex diseases and traits will be discovered by expanding international consortia but the discovered genetic effects will consequently be even smaller. As discussed, genes with small effect sizes may still have significant biological relevance. Most top signals from GWAS are found within intronic and sometimes intergenic regions and often implicate genes with yet unknown functions. Follow-up studies are needed to find the causal genetic variants involved using techniques such as fine-mapping, resequencing and functional studies. Future studies will target other types of genetic information, e.g., on structural variants such as copy number variations, inversions and rare variants with potentially larger effect sizes. The sequencing of 1000 genomes within the '1000 Genomes' project (www.1000genomes.org) will identify more common, rare and structural genetic variants.

It will be important to investigate how functional genetic variants operate in a tissue-specific manner. Studies on DNA methylation will give more insight into the contribution of epigenetics on complex diseases. An important challenge for future research in obesity lies in uncovering the mechanisms leading to a set point in body weight regulation, which determines a person's predestined weight range. This set point is likely to be determined by genes and by environmental conditions, especially by conditions evolving during pregnancy.

Despite all technological innovations, hypothesis-based studies with candidate genes will remain important as is shown by our findings on the *SIRT1* gene. Associations of *SIRT1*with obesity were not detected by GWAS in large-scale studies because of very stringent criteria for multiple testing needed in these studies. Well-powered association studies with *SIRT1* are needed for other age-related diseases such as diabetes, hyperlipidaemia, osteoporosis, dementia and cancer. Deep sequencing of the gene may lead to the discovery of rare variants with larger effects. It will be of great interest to study the other 6 human *SIRT* genes and their interactions. This could be extended to pathway analyses using interacting genes such as those in the NAD^+ salvage pathway (*CD38 and Visfatin*), FOXO genes, *PGC-1α*, and one of the master regulators of metabolism, i.e., AMP-activated protein kinase (*AMPK*). One of the potential translations of genetic research into clinical practice lies in identifying and testing modulators of genes discovered in relation to the phenotype of interest. This can be done by analyzing gene-environment interaction (e.g. *SIRT1* with diet and physical exercise). Natural or chemical modulators of AMPK and SIRT1 may also aid in the prevention of age-related loss of muscle mass (sarcopenia), which may have a large effect on morbidity and mortality in the elderly. Very recently it was demonstrated that also in rhesus monkeys moderate adult onset caloric restriction lowers the incidence of ageing-related deaths and delays the onset of age-associated pathologies such as diabetes, cancer, cardiovascular disease, brain atrophy and loss of muscle mass [62]. Given the obvious parallels between rhesus monkeys and humans, this suggests that beneficial effects of caloric restriction may also occur in humans. These findings should give a boost to studies on the ability of potential caloric restriction mimetics such as SIRT1 stimulators to improve health and survival in humans.

CONCLUDING REMARK

The medical problems associated with ageing, obesity, osteoporosis and type 2 diabetes are far from being resolved and still require extensive investigations at different multi-disciplinary levels. Nevertheless, the findings presented in this thesis provide improved insight into how underlying genetic and nutritional factors influence these morbid conditions. Hopefully, such insight may be translated into future interventions capable of providing humans in general, and patients with obesity, osteoporosis and diabetes in particular, a longer and healthier life.

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Chapter 11

Summary and conclusions

SUMMARY AND CONCLUSIONS

Obesity and osteoporosis are common and complex diseases resulting from the interplay between genetic factors and environment with increasing prevalence worldwide. The incidence of both conditions increases with age but obesity appears to protect from osteoporosis. Despite the high heritability of body mass index (BMI) and bone mineral density (BMD), knowledge on the genetic background of obesity and osteoporosis is still very limited and even less is known about interactions between genes and environmental factors like diet, tobacco smoking and physical activity.

In this thesis, epidemiological, genetic and nutritional studies are presented on the metabolic diseases obesity and osteoporosis, and on survival. In the epidemiological studies, the relationship is investigated between body fat distribution and bone mineral density, and a relation is described between the intake of dietary B vitamins and bone mineral density and fractures. The genetic studies in this thesis use two main approaches to disentangle the genetic background of these conditions, namely 1) a family-based design for the estimation of trait heritability, and 2) the association design with both the candidate gene and genome-wide association (GWA) approach. In the heritability study, evidence for the existence of sex-specific genetic effects on body composition is presented. In addition, using the candidate gene approach we show how variants in the *SIRT1* gene influence BMI, the risk of obesity and longitudinal BMI-changes. Within the context of large-scale collaborative genome-wide association studies we identified *NRXN3* as a novel locus influencing waist circumference and identified 20 loci influencing BMD variation. Furthermore, results are described of investigations on gene-diet interactions on body mass index and survival at the *SIRT1* locus.

Chapter 1 presents the introduction and outline of the thesis.

The main concepts introduced in the next chapter emphasize the well-known sexual dimorphism in human body composition with adult males having greater body height, bone mineral density (BMD), and total lean mass and a lower fat mass than females. Moreover, there are differences in fat distribution with males showing more upper body (trunk) or so called "android" distribution of adipose tissue, and females more peripheral or "gynoid" distribution with larger hips and thighs. Android or so-called "apple-shaped" obesity is associated with a greater risk of disease, e.g., type 2 diabetes and cardiovascular disease than the gynoid or "pear-shaped" type of obesity. **Chapter 2** shows the results of the study on heritability and on sex-specific genetic and environmental effects in body composition, assessed by dual energy x-ray absorptiometry (DXA) and anthropometry in 2,506 subjects from the Erasmus Rucphen Family (ERF) study. After accounting for age, sex and inbreeding, heritability estimates ranged from 0.39 for fat mass index (FMI) to 0.84 for height. Sex-specific genetic effects were found for several body composition traits including fat percentage, lean mass, android fat and parameters of fat distribution

but not for BMI and height. It was concluded that sex-specific genetic effects underlie sexual dimorphism in various body composition traits, including fat distribution. The findings are relevant for studies on the relationship of body composition with common diseases like cardiovascular disease and type 2 diabetes, and for genetic association studies.

Despite a clear positive association between body weight or BMI and BMD, the role of fat distribution on BMD is far from clear. **Chapter 3** presents the results of the study on relationships between fat distribution and BMD in 2,631 participants from the ERF study. Also, the role of BMI and serum insulin and adiponectin levels as possible confounders or intermediaries in these relations is described. BMD was measured at the hip and lumbar spine and BMC at the total body by dual-energy x-ray absorptiometry (DXA). Fat distribution was assessed by the waist-to-hip ratio, waist-to-thigh ratio and DXA-based trunk-to-leg fat ratio and android-to-gynoid fat ratio measurements. Using linear regression (adjusting for age, height, smoking and use of alcohol) most relationships between fat distribution and BMD and BMC were positive. After adjustment for BMI, most correlations were negative. No consistent influence of age, gender or menopausal status was found. Insulin and adiponectin levels did not explain either positive or negative associations. It was concluded that positive associations between android fat distribution and BMD and BMC are explained by higher BMI but not by higher insulin and/or lower adiponectin levels found in obesity. Inverse associations after adjustment for BMI suggest that android fat deposition is not beneficial and possibly even deleterious for BMD.

Recently, elevated homocysteine concentrations were identified as a novel and potentially modifiable risk factor for age-related osteoporotic fractures. In **Chapter 4** the association is described between intake of the homocysteine-related B vitamins, including riboflavin (vitamine B2), pyridoxine (vitamine B6), folate (vitamine B11) and cobalamin (vitamine B12) and femoral neck BMD (FN-BMD) and the risk of fracture in 5,304 elderly individuals from the Rotterdam study. A small but statistically significant positive association was observed between dietary pyridoxine ($ß$=0.09, p=1x10^{-8}) and riboflavin intake ($ß$=0.06, p=0.002) and baseline FN-BMD. In addition, after controlling for gender, age and BMI, pyridoxine intake was inversely correlated to the risk of non-vertebral fracture during a mean follow-up period of 7.4 years. This relation was independent of BMD and, in a subgroup, also independent of homocysteine levels. In conclusion, we found that increased dietary riboflavin and pyridoxine intake are associated with higher FN-BMD and that higher dietary pyridoxine intake is associated with a reduced risk of fracture. Clinical trials are needed to show if lowering of homocysteine levels in the elderly using vitamin B supplements will reduce the incidence of fractures.

SIRT1 is an intriguing new candidate gene for metabolic diseases and survival. **Chapter 5** presents the results of the analysis on the association between *SIRT1* genetic varia-

tion and BMI, the risk of obesity (BMI > 30kg/m^2), and with longitudinal BMI-changes. In 6,251 elderly men and women from the Rotterdam study, three Single Nucleotide Polymorphisms (SNPs) in the *SIRT1* gene were genotyped. Cross-sectional data in 2,347 participants from the ERF study were used for replication. Minor alleles of two common *SIRT1* SNPs were significantly associated with lower BMI in the Rotterdam study. These associations were replicated in the ERF study. Combined analysis in both study populations showed a 13-18% decreased risk in carriers of these alleles for being obese (p<0.02). In the Rotterdam study, the two variants were also associated with lower increase in BMI after 6.4 years of follow-up. In conclusion, carriers of two common genetic variants in *SIRT1* have a lower BMI and a 13-18% decreased risk of obesity. The availability of SIRT1 stimulators, like the polyphenol resveratrol found in grapes and red wine, makes these findings relevant in light of the growing epidemic of obesity.

In **Chapter 6** the results are shown of the study on the association of *SIRT1* genetic variation with mortality in elderly subjects and in persons with increased oxidative stress (type 2 diabetes and smokers) in relation with dietary niacin intake (vitamin B3). In 4,573 participants from the Rotterdam Study with dietary information, including 413 subjects with prevalent and 378 with incident type 2 diabetes, 3 *SIRT1* tagging SNPs were genotyped and all-cause mortality was studied (average follow-up12 years). No association was found between *SIRT1* variation and mortality in the total population and smokers. In subjects with prevalent type 2 diabetes, homozygous carriers of the most common *SIRT1* haplotype 1 had 1.5 times (95%CI 1.1-2.1) increased mortality risk compared to non-carriers. This risk further increased when these subjects smoked or when they had low niacin intake. In the lowest tertile of niacin intake, mortality risk was increased 2.3 (95%CI 1.1-4.9) and 5.7 (95%CI 2.5-13.1) times for heterozygous and homozygous carriers of haplotype 1, respectively. Subjects with incident diabetes showed similar findings but only when they smoked. In conclusion, *SIRT1* genetic variation influences survival in subjects with type 2 diabetes per se and in interaction with dietary niacin and smoking. Correction of niacin deficiency and SIRT1 modulators may prolong life of patients with diabetes.

In **Chapter 7** results are reported of a study of gene-diet interaction on BMI at the *SIRT1* locus. In 4,575 participants from the Rotterdam study an interaction was studied with nutrients that may modify SIRT1 expression or activity, i.e., dietary intake of energy, fat, calcium, milk, antioxidant vitamins (betacarotene, vitamin C and E) and niacin as precursor for NAD^+. No difference was found in energy and fat intake by *SIRT1* genotypes, suggesting that the effect of *SIRT1* genetic variants on BMI is not mediated by altered caloric intake (or a change in appetite). Significant associations were found between two *SIRT1* SNPs and haplotype 1 with BMI in the lowest tertiles of intake of energy, fat and vitamins and in the highest tertile of calcium and milk intake. Significant interaction between *SIRT1* variants and intake of vitamin E and calcium was found. In ERF, where

only information on milk intake was available, similar associations between the two SNPs and BMI were found in the highest tertiles of milk intake. It was concluded that dietary intake of anti-oxidants (especially vitamin E) and of calcium and milk modify the associations of *SIRT1* variants with BMI. These data support evidence that gene-diet interactions influence complex traits like BMI. Replication of our findings and further in-depth study of specific dietary patterns that can modify SIRT1 can pave the way for prospective studies on dietary modification of SIRT1 to influence obesity.

Chapter 8 shows the results of a large-scale genome-wide association study (GWAS) for (abdominal) obesity susceptibility genes in 31,373 Caucasian individuals from eight population-based studies from the CHARGE consortium. In this study, the role of *FTO* and *MC4R* in obesity was confirmed. In addition, a novel association of the neurexin 3 gene (*NRXN3*) with waist circumference is reported, which was subsequently replicated in additional 38,641 participants of the GIANT consortium. The SNP in *NRXN3* was also significantly associated with BMI and the risk of obesity. The *NRXN3* gene has been previously implicated in addiction and reward behavior, providing further evidence that our genes may influence the desire for and consumption of food and, in turn, the susceptibility to obesity.

In **Chapter 9** results from a meta-analysis of five genome-wide association studies within the GEFOS consortium are presented. The study aimed to find new loci associated with femoral neck and lumbar spine BMD in 19,195 Caucasian subjects. Twenty loci reaching genome-wide significance (GWS $p<5x10^{-8}$) were identified, of which 13 are new and map to genes in signaling pathways of potential relevance to bone metabolism, including *GPR177, SPTBN1, CTNNB1, MEPE, MEF2C, STARD3NL, FLJ42280, LRP4/ARHGAP1/F2, DCDC5, SOX6, FOXC2, HDAC5 and CRHR1*. The meta-analysis also confirmed the role of 7 known BMD loci, mapping to *ZBTB40, ESR1, TNFRS11B/OPG, LRP5, SP7/osterix, TNSF11/RANKL and TNFRS11A/RANK*. These findings highlight the complex genetic architecture underlying osteoporosis and identify novel genetic variants that are associated with BMD. Additional studies are warranted to identify the underlying causal variants.

In Chapter 10 the thesis is concluded with a general discussion where the findings are placed into perspective and suggestions for future research are given.

Chapter 12

Samenvatting en conclusies

SAMENVATTING EN CONCLUSIES

Obesitas (ook wel vetzucht genaamd) en osteoporose (ook wel botontkalking genaamd) zijn veelvoorkomende complexe aandoeningen met een toenemende prevalentie. Zij worden veroorzaakt door een wisselwerking tussen genen en omgeving. De incidentie van beide aandoeningen neemt toe met de leeftijd maar obesitas lijkt te beschermen tegen osteoporose. Ondanks een hoge mate van erfelijkheid van de body mass index (BMI) en van de botminerale dichtheid (BMD) is de kennis over de genetische achtergrond van obesitas en osteoporose is nog steeds erg beperkt. Nog minder is bekend over interacties tussen genen en omgevingsfactoren, zoals voeding, roken en lichamelijke activiteit.

In dit proefschrift worden de resultaten beschreven van onderzoek op het gebied van de epidemiologie, genetica (erfelijkheid) en voeding naar de metabole zieken obesitas en osteoporose en naar overleving. In de epidemiologische studies wordt de relatie tussen verdeling van lichaamsvet en de BMD beschreven en wordt een verband getoond tussen de inname van B vitaminen met de voeding en de BMD en fracturen. De genetische studies in dit proefschrift maken gebruik van twee methoden om de genetische achtergrond van beide aandoeningen te ontrafelen namelijk 1) een op familieonderzoek gebaseerde methode om de erfelijkheid van lichaamssamenstelling te berekenen en 2) associatieonderzoek met zowel de kandidaatgenmethode als genoomwijde associatie (GWA). In de erfelijkheidstudie worden aanwijzingen gepresenteerd voor het bestaan van geslachtspecifieke genetische effecten op de lichaamssamenstelling. Vervolgens wordt met de kandidaatgenmethode aangetoond hoe genetische variaties in het *SIRT1* gen van invloed zijn op de BMI, het risico op obesitas en longitudinale veranderingen in BMI. Tenslotte wordt beschreven dat binnen grootschalige samenwerkingsverbanden met GWA het *NRXN3* gen werd ontdekt als een nieuw locus voor de middelomvang en dat 20 loci met de BMD zijn geassocieerd. Tevens wordt beschreven hoe interacties tussen variaties in het *SIRT1* gen en voeding de BMI en overleving beïnvloeden.

Hoofdstuk 1 geeft een algemene inleiding en de indeling van het proefschrift.

In het volgende hoofdstuk wordt uitgegaan van de bekende verschillen in lichaamssamenstelling tussen mannen en vrouwen. Mannen zijn langer, hebben een grotere BMD en spiermassa (vetvrije massa) en een lager vetpercentage dan vrouwen. Bovendien hebben mannen een andere vetverdeling met meer vet op het bovenlichaam oftewel een zogenaamde “androide” of “appelvormige” vetverdeling, terwijl vrouwen relatief meer vet hebben op de heupen en dijen, een zogenaamde “gynoide” of “peervormige” vetverdeling. Het appelvormige type van overgewicht vormt een groter risico op het krijgen van ziekten zoals bijvoorbeeld type 2 diabetes mellitus en hart- en vaatziekten dan het peervormige type.

Hoofdstuk 2 toont de resultaten van het onderzoek naar de erfelijkheid van lichaamssamenstelling en naar het bestaan van geslachtspecifieke genetische en omgevingsfactoren hierop. De lichaamssamenstelling werd gemeten met behulp van dual energy x-ray absorptiometrie (DXA) and anthropometrie bij 2506 deelnemers van de Erasmus Rucphen Familie (ERF) studie. Rekening houdend met leeftijd, geslacht en mate van verwantschap berekenden wij dat lichaamssamenstelling voor 39% (vetmassa gecorrigeerd voor lengte) tot 84% (lengte) door genetische factoren wordt bepaald. Wij vonden dat er geslachtspecifieke genetische factoren zijn voor verschillende componenten van lichaamssamenstelling, zoals het vetpercentage, de vetvrije massa, de hoeveelheid buikvet en de vetverdeling maar niet voor de body mass index of voor lengte. Concluderend heeft onze studie aangetoond dat er geslachtspecifieke genetische effecten ten grondslag liggen aan verschillen in lichaamssamenstelling en vetverdeling tussen mannen en vrouwen. Deze bevindingen zijn van belang om verbanden tussen lichaamssamenstelling en ziekten zoals suikerziekte en hart- en vaatziekten te onderzoeken en voor genetische associatie studies.

Ondanks overtuigende aanwijzingen dat een hogere BMI gepaard gaat met een hogere BMD is de relatie tussen vetverdeling en BMD is niet precies bekend. **Hoofdstuk 3** beschrijft de resultaten van onderzoek naar deze relatie bij 2631 deelnemers van het ERF studie en van de invloed hierop van de BMI en van serumspiegels van insuline en adiponectine. Met behulp van dual-energy x-ray absorptiometrie (DXA) werd de BMD gemeten ter plaatse van de heup en de lendenwervels alsmede de hoeveelheid botmineraal van het hele lichaam (BMC). De vetverdeling werd bepaald middels de verhouding tussen de middel- en heupomtrek en tussen de middel- en dijbeenomtrek alsmede met de verhouding tussen romp- en beenvet en tussen buik- en dijbeenvet gemeten met DXA. In een lineaire regressie (met correctie voor leeftijd, lengte, roken en alcoholgebruik) werden voornamelijk positieve verbanden gevonden tussen appelvormige vetverdeling en BMD en BMC. Na correctie voor de BMI waren de verbanden voornamelijk negatief. Er werd geen duidelijke invloed vastgesteld van leeftijd, menopauze of van insuline en adiponectine spiegels. Concluderend worden positieve verbanden tussen appelvormige vetverdeling en de BMD en BMC verklaard door een hogere BMI en niet door hogere insuline en/of lagere adiponectine spiegels bij obesitas. Negatieve verbanden na correctie voor BMI suggereren dat een appelvormige vetverdeling zeker niet gunstig en mogelijk zelfs ongustig is voor de BMD.

Recent is vastgesteld dat verhoogde serum spiegels van homocysteine een nieuwe en mogelijk behandelbare risicofactor vormen voor botbreuken door osteoporose. In **Hoofdstuk 4** wordt de relatie beschreven tussen de inname met de voeding van met homocysteine geassocieerde B vitaminen riboflavine (vitamine B2), pyridoxine (vitamine B6), foliumzuur (vitamine B11) en cobalamine (vitamine B12) en de BMD van de heup en het risico op botbreuken bij 5304 deelnemers van de Rotterdam studie. Er

werd een klein maar statistisch significant positief verband gevonden tussen inname van vitamine B6 (*ß*=0,09, $p=1x10^{-8}$) en vitamine B2 (*ß*=0,06, *p*=0,002) en de heup BMD. Bovendien werd na correctie voor geslacht, leeftijd en BMI een omgekeerd verband gevonden tussen inname van vitamine B6 en het risico op (niet-wervel) botbreuken na een gemiddelde observatieduur van 7,4 jaar. Deze relatie was onafhankelijk van de BMD en, in een subgroep, eveneens onafhankelijk van homocysteine spiegels. Concluderend vonden wij dat een hogere inname met de voeding van vitamine B2 en vitamine B6 gepaard gaat met een hogere BMD van de heup en dat een hogere inname van vitamine B6 gepaard gaat met een kleinere kans op botbreuken. Klinische studies zullen moeten aantonen of verlaging van de homocysteine spiegels met behulp van B vitaminen zal leiden tot minder botbeuken bij ouderen.

SIRT1 is intrigerend nieuw kandidaatgen voor metabole ziekten en overleving. **Hoofdstuk 5** toont de resultaten van het onderzoek naar de associatie tussen variaties in het *SIRT1* gen en BMI en het risico op obesitas (BMI > 30kg/m^2) en BMI veranderingen in de tijd. Bij 6251 ouderen in de Rotterdam studie werden drie zogeheten Single Nucleotide Polymorphismen (SNPs) in the *SIRT1* gen gegenotypeerd. Gegevens van 2347 deelnemers aan de ERF studie werden gebruikt om de bevindingen te bevestigen. Dragers van de minst voorkomende allelen van twee SNPs in *SIRT1* hadden een significant lagere BMI in de Rotterdam studie en in ERF. Een gecombineerde analyse in beide populaties toonde een afname van 13-18% op het risico van obesitas bij dragers van deze allelen (*p*<0,02). In de Rotterdam studie waren deze twee genetische varianten ook geassocieerd met een geringere toename van de BMI bij een vervolgonderzoek na gemiddeld 6.4 jaar. Er werd geconcludeerd dat dragers van twee veelvoorkomende varianten in het *SIRT1* gen een lagere BMI hebben en minder kans op obesitas. Het bestaan van SIRT1 stimulatoren, zoals resveratrol in druiven en rode wijn, maakt deze bevindingen van belang in het licht van de groeiende obesitas epidemie.

In **Hoofdstuk 6** worden de bevindingen getoond van onderzoek naar de associatie tussen variatie in het *SIRT1* gen en mortaliteit bij ouderen en bij personen met verhoogde oxidatieve stress (type 2 diabetes mellitus en rokers) in relatie tot inname van niacine (vitamin B3) met de voeding. Bij 4573 deelnemers van de Rotterdam studie met dieetgegevens, inclusief 413 personen met type 2 diabetes mellitus bij aanvang en 378 personen die diabetes ontwikkelden tijdens follow-up, werden drie SNPs in the *SIRT1* gen gegenotypeerd . Een relatie met mortaliteit, ongeacht de oorzaak, werd bestudeerd met een gemiddelde follow-up duur van 12 jaar. Er werd geen verband gevonden tussen variaties in het *SIRT1* gen en mortaliteit in de gehele populatie en bij rokers. Bij personen met type 2 diabetes bij aanvang hadden homozygote dragers van het meest voorkomende *SIRT1* haplotype 1 een 1,5 maal (95%CI 1,1-2,1) grotere kans op overlijden dan niet-dragers. Dit risico nam toe wanneer deze personen tevens rookten en wanneer ze weinig inname hadden van vitamine B3. In het laagste tertiel van vitamine B3 inname

was de kans op overlijden 2,3 maal (95%CI 1,1-4,9) and 5.7 (95%CI 2,5-13,1) verhoogd voor, respectievelijk, heterozygote en homozygote dragers van haplotype 1. Personen die diabetes tijdens follow-up ontwikkelden vertoonden vergelijkbare verschillen maar alleen wanneer zij rookten. Er werd geconcludeerd dat bij mensen met type 2 diabetes genetische variaties in *SIRT1* de overleving beïnvloeden dat er een interactie bestaat met vitamine B3 inname en roken. Herstel van een mogelijk vitamine B3 tekort en toepassing van middelen die SIRT1 beinvloeden zouden de levensverwachting van mensen met type 2 diabetes kunnen verlengen.

Hoofdstuk 7 beschrijft de bevindingen van het onderzoek naar een mogelijke interactie tussen variaties in het *SIRT1* gen en voeding op de BMI. Bij 4575 deelnemers van de Rotterdam studie werd een interactie bestudeerd met nutriënten die de expressie of activiteit van SIRT1 zouden kunnen beïnvloeden zoals de inname van energie, vet, calcium, melk, anti-oxidante vitamines (betacaroteen, vitamine C en E) alsmede van vitamine B3 als bouwsteen van NAD^+. Er werd geen verschil gevonden in inname van energie of vet tussen de verschillende *SIRT1* genotypen, hetgeen suggereert dat het effect van *SIRT1* genetische varianten op de BMI niet het gevolg is van een verandering in energieinname of eetlust. Significante associaties werden gevonden tussen twee SNPs and haplotype 1 in *SIRT1* met de BMI in het laagste tertiel van inname van o.a. energie, vet en van de vitaminen en in het hoogste tertiel van inname van calcium en melk. Er werd een statistisch significante interactie gevonden tussen *SIRT1* varianten en de inname van vitamine E en calcium. In ERF, waar alleen gegevens over melkinname beschikbaar waren, werden vergelijkbare associaties gevonden voor de twee SNPs met BMI bij personen in de hoogste tertielen van melkinname. Geconcludeerd werd dat inname in de voeding van anti-oxidante vitaminen (met name van vitamine E) en van calcium en melk de relatie tussen *SIRT1* genetische variaties en BMI beïnvloedt. Deze bevindingen ondersteunen aanwijzingen dat interacties tussen genen en voeding complexe ziekten of kenmerken zoals BMI kunnen beïnvloeden. Bevestiging van deze bevindingen en verdere analyse naar specifieke voedingspatronen met invloed op SIRT1 kunnen leiden tot prospectieve studies met als doel om via SIRT1 obesitas te beïnvloeden.

Hoofdstuk 8 toont de bevindingen van een grootschalige genoomwijde associatie studie (GWAS) naar gevoeligheidsgenen voor (abdominale) obesitas in 31.373 caucasische deelnemers van 8 bevolkingsonderzoeken binnen het CHARGE consortium. In deze studie werd de rol van *FTO* and *MC4R* bij obesitas bevestigd en er werd bovendien een nieuw locus gevonden dat geassocieerd was met de middelomvang in het zogenaamde neurexine 3 gen (*NRXN3*). Deze associatie werd bevestigd bij 38.641 deelnemers van het GIANT consortium. De SNP in *NRXN3* was ook significant geassocieerd met BMI and het risico op obesitas. Dit *NRXN3* gen werd eerder in verband gebracht met verslaving en met beloningsgedrag, hetgeen verdere steun geeft aan de gedachte dat onze genen

van invloed zijn op verlangen naar en inname van voedsel en derhalve op gevoeligheid voor obesitas.

In **Hoofdstuk 9** worden de resultaten gepresenteerd van een meta-analyse van vijf GWA studies in 19.195 Caucasische deelnemers binnen het GEFOS consortium met als doel om nieuwe loci te vinden die geassocieerd zijn met botminerale dichtheid van de heup en/of lumbale wervels. Er werden 20 loci geïdentificeerd met een genomewijde significantie ($p<5x10^{-8}$), waarvan 13 in nieuwe regio's, ter plaatse van genen in signaalpaden die mogelijk van belang zijn voor botmetabolisme waaronder *GPR177, SPTBN1, CTNNB1, MEPE, MEF2C, STARD3NL, FLJ42280, LRP4/ARHGAP1/F2, DCDC5, SOX6, FOXC2, HDAC5* and *CRHR1*. Tevens werd in deze meta-analyse de rol bevestigd van 7 bekende BMD loci ter plaatse van *ZBTB40, ESR1, TNFRS11B/OPG, LRP5, SP7/Osterix, TNSF11/RANKL* and *TNFRS11A/RANK*. Concluderend benadrukken deze bevindingen de complexe genetische architectuur van osteoporose en identificeren nieuwe genetische variaties geassocieerd met BMD. Toekomstige studies zijn nodig om precies vast te stellen welke causale varianten ten grondslag liggen aan de gevonden associaties.

In **Hoofdstuk 10** wordt besloten met een algemene discussie waarbij de bevindingen in perspectief worden geplaatst en suggesties worden gedaan voor toekomstig onderzoek.

Chapter 13

Dankwoord

Curriculum Vitae

Publications

CURRICULUM VITAE

Carola Zillikens was born on May 6th 1957 in Arnhem. After graduating from high school in 1975 (Gymnasium Beta, Stedelijk Gymnasium, Arnhem) she entered Medical School at the Medical Faculty, University of Groningen. During her medical training she did rotations in General Surgery at the University Hospital in Freiburg, Germany (1976), in General Surgery in Medan, Indonesia (1979) and in Internal Medicine at the University of Denver, Colorado, USA (1982). From 1976-1981 she worked as student assistant at the operating rooms and at the ICU of the Thoracic Surgery Department Academic Hospital Groningen.

From 1982-1987 she did her specialty training in Internal Medicine at the Department of Internal Medicine II, Erasmus Medical Center Rotterdam, former University Hospital Rotterdam, Dijkzigt (heads of the Department Prof. Dr. M. Frenkel and Prof. J.H.P. Wilson) after which she worked there as a junior internist for one year. From 1988 till 1989 she was employed as internist at the Ikazia Hospital Rotterdam and returned to the Department of Internal Medicine III at the Erasmus Medical Center, Lipid Clinic, in 1990 (heads: Prof. dr. J.C. Birkenhäger, Prof dr. H.A.P. Pols and currently Prof. dr. E.J. Kuipers). Since 1998 she works as an internist at the Division of Endocrinology at the Erasmus MC, where she is a staff member with special interest in calcium and bone metabolism (heads: Prof. dr. SWJ Lamberts and Prof. dr. A.J. van der Lelij). In October 2005 she was registered as endocrinologist.

From 2002-2005 she has been involved as a senior investigator in the organization and data collection of the Erasmus Rucphen Family (ERF) study (PI's: Prof. dr. ir. C.M. van Duijn and Prof. dr. B.A. Oostra, Erasmus MC). She also participates in the locomotor and genetics group of the Rotterdam study (PI's Prof. dr. H.A.P. Pols and Prof. dr. A.G. Uitterlinden). Data from the ERF study and the Rotterdam study were used for her PhD project that she commenced in 2005.

Since 1988 she is married to Lex Eggermont, professor of Surgical Oncology at the Erasmus University MC. They have three children, Rogier (1988) Celeste (1992) and Eline (1992).

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