A study into genes encoding longevity in humans

Kuningas, M.

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Maris Kuningas

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Kuningas, Maris
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Contents

CHAPTER 1

General Introduction

Introduction

The lifespan of an organism is determined by a complex network of environmental-, genetic- and stochastic factors. Each of these components contributes to the wide variability in lifespan between and within species. In recent years, a central question has been to what extent the variability in human life span is related to genetic differences and whether there are common genetic determinants that influence lifespan. To date, we know that 20-30 % of the overall variation in human lifespan is accounted for by genetic factors, which become increasingly important for survival at oldest ages (Herskind et al., 1996; Mitchell et al., 2001; vB et al., 2006). In addition, a number of candidate genes have arisen for the study of longevity in humans, of which the majority has emerged from studies with model organisms.

The most commonly studied model organisms are the budding yeast *Saccharomyces cerevisiae*, the nematode worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and the house mouse *Mus musculus*. These organisms have many advantages for ageing studies, most notably their relatively short life spans, well-characterized biology and completely sequenced genomes, which have allowed rapid progress in the discovery of pathways underlying longevity. The first pathway that was identified was the evolutionarily conserved insulin/IGF1-like signal (IIS) transduction pathway. In *C. elegans*, mutations in the *daf-2* and *age-1* genes, which are related to the mammalian insulin receptor and to the catalytic subunit of phosphatidylinositol-3-kinase (PI-3-kinase), respectively, both lead to increased lifespan (Friedman and Johnson, 1988; Kenyon et al., 1993; Larsen et al., 1995; Morris et al., 1996). In following studies, similar effects were observed for other genes in the IIS pathway and in other pathways involved in metabolic- and physiologic processes, that regulate stress resistance, fertility and genomic maintenance (Christensen et al., 2006; Vijg and Suh, 2005). These findings have provided evidence that individual genes can have major effects on lifespan, but it is largely unknown whether the same genes and processes are also important for the observed variation in human life span. Human genes homologous to the longevity genes identified in model organisms represent relevant candidate genes for the study of longevity in humans.

In addition to the longevity genes identified in model organisms, other candidate genes can de deduced from the biology of human ageing and lifespan. A consistent feature of environmental and genetic factors that influence lifespan is their influence on stress resistance. Most types of stressors are perceived first by the nervous system and the responses of the whole body to such stressors are coordinated by the brain. In humans, the ability to cope with stress and maintain a good mental performance are essential for a long life. Therefore, genes involved in both central nervous system- and peripheral stress responses may play important roles in lifespan determination (Mattson et al., 2002). Yet another set of candidate genes can be derived from human premature ageing syndromes, such as Hutchinson-Gilford syndrome, Werner syndrome, Bloom syndrome, Cockayne's syndrome and Xeroderma pigmentosum. It is unknown whether subtle variations in these genes influence ageing in the population at large. Taken together, different approaches have yielded a number of candidate longevity genes that await testing in humans. Understanding the role of specific genetic factors in the variation of lifespan among humans is central to the understanding of human ageing and lifespan, including exceptional longevity.

The candidate longevity genes

Daf-16 - forkhead transcription factors

The main downstream target of the IIS pathway is the transcription factor dauer formation -16 (*daf-16*), which regulates the expression of numerous downstream genes that mediate stress resistance, innate immunity and metabolic processes (McElwee et al., 2003; Murphy et al., 2003). In *C. elegans*, increased activity of Daf-16 has been associated with increased lifespan (Kenyon et al., 1993; Kimura et al., 1997). In contrast, mutations in the *daf-16* gene have been shown to suppress the life-extending effects of decreased insulin signaling (Lin et al., 1997). These data indicate that *daf-16* is negatively regulated by insulin signaling and is the major downstream effector of the IIS pathway. In mammals, the *daf-16* gene homologues are forkhead/winged-helix transcription factors (FOXOs) of which to date, four different family members have been identified: *FOXO1a*, *FOXO3a*, *FOXO4* and *FOXO6* (Anderson et al., 1998; Biggs et al., 2001; Jacobs et al., 2003). From these, cellular functions have been described best for FOXO1a and FOXO3a. Both of these genes are involved in a variety of cellular processes, including metabolism, cell differentiation, cell cycle arrest and DNA repair. In addition, FOXO1a has been specifically implicated in mediating the effects of insulin on hepatic glucose production (Altomonte et al., 2003; Barthel et al., 2005; Nakae et al., 2002) while FOXO3a has been specifically implicated in female fertility through suppression of follicular activation (Castrillon et al., 2003).

Daf-12 - liver X receptors and vitamin D receptor

Dauer formation-12 (*Daf-12*) gene belongs to the nuclear hormone receptor (NHR) superfamily, a large and diverse family of transcription factors (Laudet, 1997), and it has been placed at the convergence of several signal transduction pathways, including the IIS pathway. Similar to other Daf proteins in *C. elegans*, Daf-12 regulates dauer diapause, developmental timing and metabolism in response to environmental signals (Rottiers and Antebi, 2006). Mutations in the daf-12 gene can result in dauer defective or dauer constitutive worms, which are short- and long-lived, respectively. In addition, it has been shown that the long-lived phenotype of germline-ablated worms depends, besides on daf-16, also on daf-12 (Hsin and Kenyon, 1999). In humans, the closest *daf-12* homologues are the liver X receptors (*LXRA* and *LXRB*) and the vitamin D receptor (*VDR*) (Mooijaart et al., 2005). These genes belong to NHR super-family, but have distinct functions. The LXRs are mainly involved in lipid metabolism and -transport (Peet et al., 1998; Tontonoz and Mangelsdorf, 2003), whereas the VDR is involved in diverse functions that include bone metabolism, cellular proliferation and differentiation, immunomodulation and neuroprotection (Lin and White, 2004).

Sir2 - sirtuins

The Sirtuins represent an evolutionarily conserved family of Silent Information Regulator 2 (Sir2) NAD-dependent protein deacetylases that interact with and influence the activity of vari-

ous transcription factors and co-regulators (Bordone and Guarente, 2005). Increased expression of the *Sir2* gene, either due to an extra copy of the gene or to caloric restriction, has been shown to prolong lifespan in various model organisms (Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001; Wood et al., 2004). In mammals, there are seven *Sir2* homologues (*SIRT1-7*), of which *SIRT1* is the most closely related to *Sir2* (Frye, 2000). Studies with mouse models have shown that in response to environmental signals, SIRT1 regulates glucose and fat metabolism, stress resistance and cell survival (Bordone and Guarente, 2005; Cohen et al., 2004; Picard et al., 2004; Yang et al., 2006). Some of the target genes through which SIRT1 exerts these effects include the *FOXOs*, *p53* and *PPAR-gamma* (Leibiger and Berggren, 2006).

Mineralocorticoid- and glucocorticoid receptor

The mineralocorticoid receptor (*MR*) and glucocorticoid receptor (*GR*) genes, which belong to the NHR family, and are activated by cortisol to regulate metabolism, inflammation, and immunity (Meijer et al., 2006). Cortisol is the primary active stress hormone that mediates counter-responses to stress, aimed to re-establish homeostasis and coordinate behavioral adaptations (de Kloet et al., 2005). By targeting many genes through the MR and GR, cortisol functions in a binary fashion, and serves as a master switch in the control of neuronal and network responses that underlie behavioral adaptation. Various studies have shown that stress-responsiveness is highly variable among human subjects, and an inadequate stress-response increases vulnerability for disease. Changes in the stress hormone system have been shown to play a role in cognitive impairment (Lupien et al., 2005) and in the development of depression (Belanoff et al., 2001; Holsboer, 2000). The ability to cope with stress and maintain a good mental performance are essential for a long life, which place the MR and GR genes on the list of candidate genes important for human longevity.

WRN

The *WRN* gene encodes a nuclear protein with both helicase and exonuclease activities (Liu et al., 1999; Morozov et al., 1997; Mushegian et al., 1997). The WRN protein is capable of a multitude of functions and is involved in DNA replication, repair, recombination, transcription and/or a combination of these events. Loss-of function mutations in the *WRN* gene lead to Werner syndrome (WS), which is a segmental progeroid disorder with an autosomal recessive pattern of inheritance. Patients with WS exhibit a number of symptoms that resemble premature ageing. Characteristic clinical features of the syndrome include diabetes, osteoporosis, vascular diseases and a high incidence of malignant neoplasms (Martin, 1978; Salk, 1982). Since mutations in the *WRN* gene lead to accelerated ageing, it has been reasoned that common polymorphism in the *WRN* gene could contribute to the differences in the prevalence of disease and lifespan in the general population.

Candidate-gene-based association studies

Candidate-gene-based association studies assess correlations between genetic variants in the candidate gene and differences in the trait of interest on a population scale. In analyzing the role of a candidate gene in humans, the association between DNA variants in, around and nearby the candidate gene and the trait of interest are analyzed. The DNA variants investigated most often are single nucleotide polymorphisms (SNPs). Until recently, there were relatively few SNPs available for study, but advances in the past two decades have identified millions of such polymorphisms. The availability of a large number of SNPs in public databases has facilitates the selection of genetic variants for association studies. There are two approaches that can be used for the identification of DNA variants related to the phenotype of interest: the direct- and the indirect association approach (Figure 1) (Carlson et al., 2004; Cordell and Clayton, 2005).

Direct association studies make use of polymorphisms which are themselves putative causal variants (Carlson et al., 2004; Cordell and Clayton, 2005). This type of study is the easiest to analyze and the most powerful, but its difficulty is the identification of candidate polymorphisms. A mutation in a codon, which leads to an aminoacid change, is a candidate causal variant. However, it is likely that many causal variants responsible for heritability of common complex disorders will be non-coding. For example, such variants may cause variation in gene regulation and expression, or differential splicing. The exponential increase in annotation of common variants has generated a catalog of variants of which we know nothing about the function for the vast majority. Thus, for the direct association approach prior knowledge of functionality of the SNPs has to be first gained through functional studies.

The second, indirect approach, does not rely on the functionality of the polymorphisms but on linkage disequilibrium (LD) between a disease susceptibility allele and either a single marker allele or a multilocus haplotype (Carlson et al., 2004; Cordell and Clayton, 2005; Newton-Cheh and Hirschhorn, 2005). Several polymorphisms are commonly selected from a candidate gene and the polymorphisms under study serve as surrogates for the causal locus. Much recent meth-

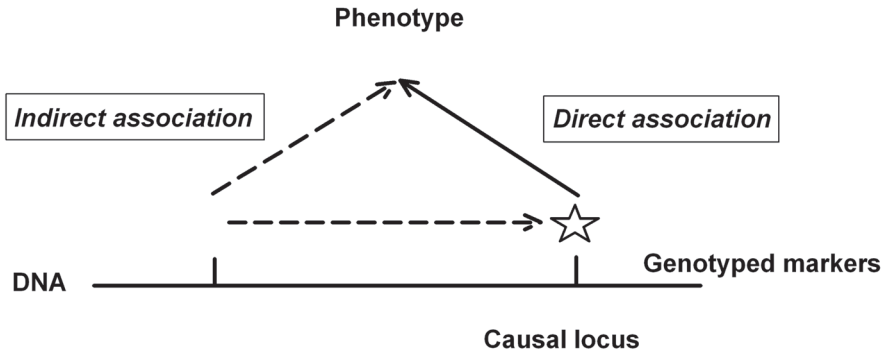

Figure 1. The direct- and indirect association approach

odological work has been conducted to optimize this indirect approach, including the investigation of haplotype-block structure and techniques for selecting haplotype-tagging SNPs. The systematization of the indirect approach is the aim of the HapMap Project (The International HapMap Consortium, 2005). In this thesis, both the direct- and indirect association strategies were used.

The Leiden 85-plus Study

All studies presented in this thesis were performed within the Leiden 85-plus Study. The Leiden 85-plus Study is a prospective population-based study, in which all 85 year old or older inhabitants of the city Leiden, The Netherlands, were invited to take part. The study population consists of two cohorts, cohort '87 and '97. The Medical Ethical Committee of the Leiden University Medical Centre approved the study.

Cohort '87

On December 1, 1986, the community of Leiden in the Netherlands had 105 000 inhabitants, of whom 1258 (1.2 %) were 85 years and older. Among these oldest old, a population-based prospective follow-up study was initiated to assess the association of HLA antigens with human lifespan (Izaks et al., 1997; Lagaay et al., 1992). During an assessment, which lasted from December 1986 to March 1989, 221 participants died before they could be visited. From the remaining 1037 people, 977 (94 %) agreed to participate in study (Weverling-Rijnsburger et al., 1997). All participants were interviewed at their place of residence by an internist experienced in geriatric medicine. After oral informed consent was obtained, a Mini-Mental State Examination (MMSE) and General Health Questionnaire (GHQ-12) were administered to detect cognitive impairment. The Dutch version of the Geriatric Mental State Schedule (GMS) was used to diagnose psychiatric disorders according to Diagnostic and Statistical Manual of Mental Disorders III (DSM-III) criteria. A complete medical history was taken with special emphasis on cardiovascular disease, diabetes mellitus, and other chronic disorders together with information about the living situation and demography. When it was not possible to get reliable information from the participant, a family member or a caretaker was asked to provide the information. In addition, diastolic- and systolic blood pressure, and glucose levels were measured. Blood samples were taken at their homes, according to predefined protocols under non-fasting conditions. After isolation of the leucocytes for HLA typing, which was the primary goal of the study, the remaining serum was available for other laboratory measurements. For DNA extraction, sufficient cell material was available for 682 participants.

Cohort '97

Between September 1997 and September 1999, 705 inhabitants of the city Leiden, in The Netherlands, reached the age of 85 years, and in the month after their 85th birthday, they were asked to participate in the Leiden 85-plus Study. Of the 705 eligible subjects 14 died before they

could be enrolled and 92 refused to participate, resulting in a cohort of 599 subjects (85 %) who were enrolled (der Wiel et al., 2002). All the study participants were visited at their place of residence, where face-to-face interviews were conducted, cognitive testing was performed, and a venous blood sample was drawn. Of the 599 participants, a venous blood sample was available for 563 participants. During the main interview with participants, global cognitive function was assessed with the Mini-Mental State Examination (MMSE), attention with the Stroop Test (Klein et al., 1997), processing speed with the Letter Digit Coding Test (LDT) (Houx et al., 2002), and memory with the 12-Word Learning Test, which assesses immediate recall (WLTI) and delayed recall (WLTD)(Brand and Jolles, 1985). The prevalence of depressive symptoms was assessed with the 15-item Geriatric Depression Scale (GDS-15) (De Craen et al., 2003). All participants were visited annually for re-measurement of cognitive functioning and depressive symptoms during a mean follow-up period of 4.2 years. For all subjects socio-demographic characteristics such as gender, marital state, and type of housing were available from the municipal registry. Informed consent was obtained from all participants, or in case of severe cognitive impairment, from their guardian.

Follow-up of mortality

All participants of the cohort '87 and '97 were followed for mortality until August 1 2005. Primary causes of death were obtained from the Dutch Central Bureau of Statistics and categorized according to the 10th International Classification of Diseases (ICD-10). At the date of censoring, August 1 2005, 681 (99 %) participants of the cohort '87 and 320 (57 %) participants of the cohort '97 had died. The most frequent primary cause of death in the participants of the Leiden 85-plus Study was death due to cardiovascular diseases (Table 1), as is the case in the general population.

Table 1. The causes of death in the Leiden 85-plus Study

	The Leiden 85-plus Study		
	Cohort '87 (n=682)	Cohort '97 (n=563)	Combined (n=1245)
All-cause mortality*	681 (99 %)	320 (57 %)	1001 (80 %)
CVD	277 (40 %)	129 (40 %)	406 (41 %)
Cancer	101 (15 %)	61 (19 %)	162 (16 %)
Infectious disease	65 (10 %)	20 (6 %)	85 (8 %)
Other	237 (35 %)	109(34 %)	346 (35 %)

*A cause of death could not be obtained for one participant from both cohort '87 and cohort '97; CVD – cardiovascular disease

Outline of the thesis

The general objective of the thesis was to test the impact of the most prominent longevity candidate genes on the prevalence of age-related diseases and lifespan in a population-based prospective study of the oldest old. The thesis consists of ten chapters, of which in **chapter 1** a general introduction to the thesis is given. In the following chapters, the effect of genetic variance in the evolutionarily conserved genes *FOXO1* and *FOXO3a* (**chapter 2**), *LXR* (**chapter 3**), *VDR* (**chapter 4**) and *SIRT1* (**chapter 5**) on the prevalence of age-related diseases and lifespan was examined. In **chapter 6**, the influence of cortisol levels and of polymorphisms in the mineralocorticoid receptor (*MR*) and glucocorticoid receptor (*GR*) genes on mental performance and on the prevalence of depressive symptoms in old age was assessed. The influence of polymorphisms in the *GR* gene and in the *WRN* gene on the prevalence of age-related diseases and lifespan were studied in **chapter 7** and in **chapter 8**, respectively. In **chapter 9**, the results are summarized and discussed, and the main conclusions are drawn. The last chapter of the thesis, **chapter 10**, contains a summary in Dutch of the thesis.

The research presented in this thesis was carried out within the framework of an "Innovative Oriented Research" (IOP) project entitled "Genetic determinants of longevity and disease in old age", subsidized by the Dutch Ministry of Economic Affairs (grant number IGE010114). This project brought together medical doctors, evolutionary biologist, geneticists and bioinformaticians with the aim to identify mechanisms that determine longevity and disease in old age. In addition there was tight collaboration with industrial partners to maximize the opportunity to generate knowledge with the potential to exploite commercially.

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CHAPTER 2

Haplotypes in the Human *FOXO1a* and *FOXO3a* Genes; Impact on Disease and Mortality at Old Age

Maris Kuningas, Reedik Mägi, Rudi G.J. Westendorp, P. Eline Slagboom, Maido Remm and Diana van Heemst

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Abstract

Recently, the *Daf-16* gene has been shown to regulate the lifespan of nematodes and flies. In mammals, Daf-16 homologues are forkhead (FOXO) transcription factors, of which specific functions have been identified for FOXO1a and FOXO3a. Despite that, their influence on human age-related trajectories and lifespan is unknown. Here, we analyzed the effect of genetic variance in *FOXO1a* and *FOXO3a* on metabolic profile, age-related diseases, fertility, fecundity and mortality. This study was carried out in the prospective population-based Leiden 85-plus Study, which includes 1245 participants, aged 85 years or more. The mean follow-up time was 4.4 years. Haplotype analyses of *FOXO1a* revealed that carriers of haplotype 3 'TCA' have higher HbA1c levels ($p=0.025$) and a 1.14-fold higher all-cause mortality risk ($p=0.021$). This increase in mortality was attributable to death from diabetes, for which a 2.43-fold increase was observed ($p=0.025$). The analyses with *FOXO3a* haplotypes revealed no differences in metabolic profile, fertility or fecundity. However, increased risks of stroke were observed for *FOXO3a* block-A haplotype 2 'GAGC' ($p=0.007$) and haplotype 4 'AAAT' ($p=0.014$) carriers. In addition, the haplotype 2 'GAGC' carriers had a 1.13-fold increased risk for all-cause mortality ($p=0.036$) and 1.19-fold increased risk for cardiovascular mortality ($p=0.052$). In conclusion, this study shows that genetic variation in evolutionarily conserved *FOXO1a* and *FOXO3a* genes, influences lifespan in our study population.

Introduction

Insulin signaling has emerged as a conserved mechanism that influences the lifespan of several organisms (Guarente and Kenyon, 2000; Tatar et al., 2003). In *Caenorhabditis elegans* down-regulation of the insulin/IGF-1 signalling (IIS) pathway activates Daf-16, and leads to increased lifespan (Ogg et al., 1997; Tissenbaum and Ruvkun, 1998). Among the genes regulated by Daf-16 are those implicated in glucose and lipid metabolism, fertility, stress response and defense mechanisms (Murphy et al., 2003). In mammals, the main downstream targets of the IIS pathway are the forkhead box group O (FOXO) transcription factors, which are Daf-16 homologues (Lin et al., 1997). However, it remains to be elucidated whether FOXO proteins in mammals have a similar role as Daf-16 in *C. elegans*.

In mammals, the FOXO family consists of *FOXO1a*, *FOXO3a*, *FOXO4* and *FOXO6*. These genes are expressed in all tissues albeit at varying degrees, suggesting that their physiological roles might be different (Anderson et al., 1998; Biggs et al., 2001; Furuyama et al., 2000). Distinct functions have been identified for FOXO1a and FOXO3a. Compared to other family members, FOXO1a seems to be the most important and functionally the most indispensable, as only the *FOXO1a* knock-out mice were not viable (Furuyama et al., 2004; Hosaka et al., 2004). It has been shown that FOXO1a predominantly mediates the effects of insulin on metabolism, including its effects on hepatic glucose production (Barthel et al., 2005). Mice over-expressing *FOXO1a* in liver and pancreatic β-cells have fasting hyperglycaemia and hepatic insulin resistance leading to the development of diabetes in an age-dependent manner (Altomonte et al., 2003; Nakae et al., 2002; Zhang et al., 2006). On the other hand, FOXO3a has been implicated in the suppression of follicular activation and thus in female fertility (Castrillon et al., 2003; Hosaka et al., 2004). These female FOXO3a knock-out mice also displayed signs of premature ageing. Reduced lifespan in reproductively active females has been noted for a variety of species over the years (Partridge et al., 2005). Hence, the phenotypes described above provide strong clues to the basic functions of FOXO1a and FOXO3a. Despite that, the role of FOXO proteins in humans has hardly been assessed. Recently, genetic variants in FOXO1a were associated with increased glucose levels and with a trend for early onset type-2 diabetes in a case-control study consisting of middle-aged participants (Karim et al., 2006). The influence of FOXO1a and FOXO3a on human lifespan has not been assessed yet.

In this study, we analyzed the effect of genetic variance in *FOXO1a* and *FOXO3a* on metabolic profile and mortality. In addition, associations with the prevalence of age-related diseases, fertility and fecundity were assessed. We used a haplotype-based approach, and the study was carried out in participants aged 85 years and older of the prospective population based Leiden 85-plus Study.

Participants and methods

Participants

The Leiden 85-plus Study is a prospective population based study, in which inhabitants of

Leiden, The Netherlands, aged 85 years or above, were invited to take part. There were no selection criteria related to health or demographic characteristics. The study population consists of two cohorts, cohort '87 and '97. Cohort '87 includes 977 participants aged 85 years and older, enrolled between 1987 and 1989 (Weverling-Rijnsburger et al., 1997). Cohort '97 consists of 599 subjects, all members of the 1912-1914 birth cohort, who were enrolled in the month of their 85th birthday between 1997 and 1999 (der Wiel et al., 2002). DNA was available for 682 participants from cohort '87 and for 563 people from cohort '97. All the participants of the Leiden 85-plus Study were followed for mortality until August 1, 2005. Primary causes of death were obtained from the Dutch Central Bureau of Statistics and categorized according to the 10th International Classification of Diseases (ICD-10). The Medical Ethical Committee of the Leiden University Medical Center approved the study and informed consent was obtained from all the participants. We also genotyped 370 blood donors from Leiden and surrounding areas (Heijmans et al., 1999), in order to compare allele and haplotype frequencies between the elderly and the young.

Metabolic profile and BMI at baseline in cohort '97

HbA1c (hemoglobin A1c), triglycerides, C-reactive protein (CRP) and high-density lipoprotein (HDL)-cholesterol concentrations in serum were determined using fully automated analyzers (Hitachi 747 and 911; Hitachi, Ltd, Tokyo, Japan). Low-density lipoprotein (LDL)-cholesterol was estimated with the Friedewald equation (Friedewald et al., 1972). Body weight (kg) and height (cm) were measured in all participants and body mass index (BMI, kg/m^2) was calculated.

Diabetes and cardiovascular pathologies at baseline in cohort '97

Participants were classified as having diabetes when they met at least one of the following criteria: 1) history of diabetes obtained from the general practitioner or the subject's treating physician; 2) use of sulfonylureas, biguanides, or insulin, based on information obtained from the subject's pharmacist; or 3) nonfasting glucose of 11.1 mmol/l. The prevalence of and the number of cardiovascular pathologies were obtained from the participants' general practitioners or nursing home physicians. In addition, electrocardiograms were recorded on a Siemens Siccard 440 and transmitted by telephone to the ECG Core Lab in Glasgow for automated Minnesota coding (Macfarlane and Latif, 1996). Cardiovascular pathologies were classified as follows: myocardial infarction, myocardial ischemia, intermittent claudication, arterial surgery and stroke (van Exel et al., 2002).

Fertility and fecundity in the combined cohort

Birth dates of all the participants and their children, and the date(s) of marriage(s) were obtained from the registry of births, deaths, and marriages of the municipality of Leiden and from the Central Bureau of Genealogy, The Netherlands. These participants were of childbearing age at a time of minimal fertility control for lack of modern contraceptive methods. Fertility and fecundity were assessed only in married female participants who were younger than 40 at the time of their marriage (n=701). Women older than 40 years at the time of their marriage were

excluded from further analyses owing to the rapid decline of fertility and fecundity that can be expected from that age onwards. Fertility was defined as by having children or not. Fecundity was defined as the calculated time interval between the date of the first marriage and the birth date of the firstborn child. To minimize the selection of pregnancies conceived before marriage, women whose children were born before marriage or within the first 36 weeks (250 days) of marriage were excluded from analyses (van Dunne et al., 2006).

SNP selection and genotyping

The single nucleotide polymorphisms (SNPs) from *FOXO1a* (GeneID 2308) and *FOXO3a* (GeneID 2309) were selected using the CEPH population (Utah residents with northern and western European ancestry) data from the International HapMap Project release no. 15 (The International HapMap Consortium, 2005). All polymorphisms were genotyped with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), using the Sequenom MassARRAYtm methodology (Sequenom Inc, San Diego, CA, USA). Amplification reactions and parameters were based on the manufacturer's instructions.

Statistical analysis

The program Haploview (Barrett et al., 2005) was used to estimate the allele frequencies of the polymorphisms, test for Hardy-Weinberg equilibrium and estimate pair-wise linkage disequilibrium (LD) between the SNPs. Haplotypes and haplotype frequencies were calculated using SNPHAP software (*http://www-gene.cimr.cam.ac.uk/clayton/software*). Differences in allele and haplotype frequencies between the elderly and the young control group were tested using Fisher's exact test. The posterior probabilities of pairs of haplotypes per subject, as estimated by the SNPHAP, were used as weights in all the analyses. The haplotype analyses approach used in this study assumes an additive effect of the haplotypes, and details of this approach have been described elsewhere (Wallenstein et al., 1998). Haplotypes with a frequency < 5 % were combined and included in the following analyses, without reporting the results. Continuous variables were normally distributed except for HbA1c, triglycerides and CRP levels, which were therefore ln-transformed. Associations between haplotypes and metabolic profiles were analyzed using a general linear model. Differences in the prevalence of cardiovascular pathologies, fertility and fecundity between haplotypes were tested using binary logistic regression. All-cause and cause-specific mortality risks with 95 % confidence intervals (CI) were calculated with Cox proportional hazard model, using left censoring to correct according to age for the delayed entry into the risk set. All analyses were sex adjusted, and clustered by the individual identification number to obtain robust standard errors. The common allele homozygote haplotype was used as the reference group. All the analyses were performed using STATA statistical software, version 9.0 (StataCorp LP, TX, USA).

Results

Using HapMap data, we first examined the extent of LD in *FOXO1a* and *FOXO3a*. The polymorphisms of both genes were in LD (Figure 1a), which enabled us to select haplotype tagging SNPs (htSNPs) that would tag all haplotypes with frequencies > 1 %. From the *FOXO1a* gene three htSNPs that define one haplotype block, and from the *FOXO3a* gene nine htSNPs that define two haplotype blocks were chosen. In order to mark non-haploblock regions, one SNP from *FOXO1a* and two SNPs from *FOXO3a* were selected (Figure 1a).

The 1245 participants of the Leiden 85-plus Study and 370 young blood bank donors were genotyped for these polymorphisms. The genotype frequencies of the SNPs were in Hardy-Weinberg equilibrium and similar between the two elderly cohorts and the young control group (Table 1). As expected, all the htSNPs were in strong LD and in *FOXO1a* gene they defined six haplotypes, of which four had frequencies > 5 % and described 99.6 % of the haplotype diversity (Figure 1b). In *FOXO3a*, the nine htSNPs defined two haplotype blocks. All five haplotypes in block-A and three haplotypes in block-B had frequencies > 5 % and described respectively 100 % and 94.6 % of haplotype diversity (Figure 1b). The overall and individual haplotype frequencies were not different between the elderly and young control group for neither of the genes (data not shown).

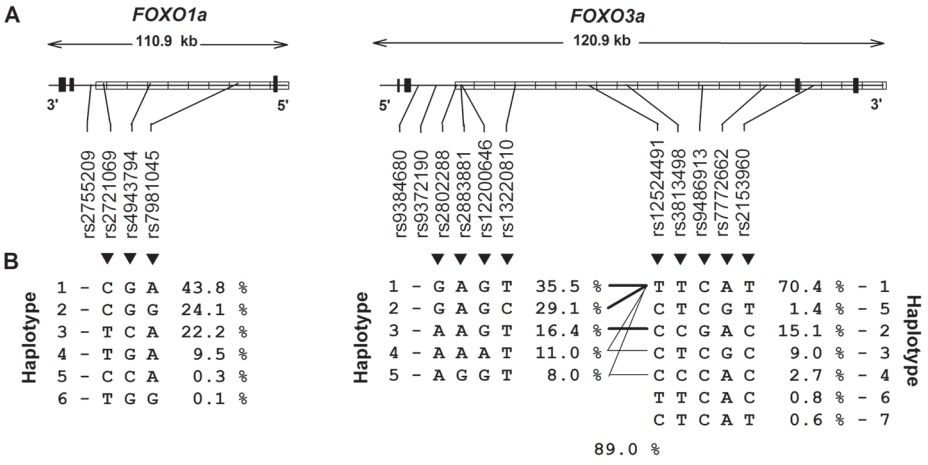

Figure 1. *FOXO1a* and *FOXO3a* gene structure, LD and haplotypes. (a) Genomic structure of *FOXO1a* and *FOXO3a* genes, where exons are represented by black boxes, and introns and intragenic regions by lines. The long striped horizontal box indicates the extent of LD based on the Hapmap data. Long vertical lines show the relative position of the SNPs analyzed in this study; (b) Haplotypes and their frequencies. For *FOXO3a* the lines between the block-A and block-B show the most common crossings from one block to the next, with thicker lines showing more common crossings than thinner lines. Beneath the crossing lines is shown the multilocus D', which is a measure of the LD between the blocks.

Table 1. Demographic characteristics of the study participants, and minor allele frequencies of the FOXO1a and FOXO3a polymorphisms

	Leiden 85-plus Study		
	Cohort '87	Cohort '97	Young control
Number	682	563	370
Female (n, %)	491 (72 %)	375 (67 %)	220 (60 %)
Age (median, IQR)	89 (88-92)	85 (-)	32 (27-36)
FOXO1a[1]			
rs2755209 (A/C)	0.385	0.398	0.376
rs2721069 (C/T)	0.321	0.310	0.292
rs4943794 (G/C)	0.229	0.218	0.181
rs7981045 (A/G)	0.229	0.263	0.260
FOXO3a[1]			
rs9384680 (T/G)	0.029	0.039	0.036
rs9372190 (T/G)	0.072	0.087	0.074
rs2802288 (G/A)	0.365	0.341	0.354
rs2883881 (A/G)	0.073	0.087	0.069
rs12200646 (G/A)	0.113	0.109	0.128
rs13220810 (T/C)	0.291	0.290	0.271
rs12524491 (T/C)	0.300	0.270	0.290
rs3813498 (T/C)	0.193	0.163	0.171
rs9486913 (C/G)	0.166	0.136	0.142
rs7772662 (A/G)	0.104	0.103	0.116
rs2153960 (T/C)	0.293	0.262	0.281

IQR- interquartile range; [1]minor allele frequencies. htSNPs are indicated in bold

The data on metabolic profile, BMI, prevalence of diabetes and cardiovascular diseases were available for 563 participants of the cohort '97. Haplotype analyses of the *FOXO1a* gene revealed that carriers of haplotype 3 'TCA' have 0.25 mmol/l higher HbA1c levels (95 % CI: 0.02-0.48, *p=0.025*) compared with the levels in the carriers of the most common haplotype 1 'CGA' (Table 2). In addition, haplotype 3 'TCA' carriers had a trend for higher CRP levels and lower BMI (Table 2). Their risks for diabetes and myocardial infarction were also increased, although the association with diabetes was non-significant (OR 1.29, 95 % CI: 0.86-1.92, *p=0.360*) and the association with myocardial infarction just failed to reach significance (OR 1.41, 95 % CI: 1.00-2.00, *p=0.051*) (Supplementary Table 1). No differences in metabolic profile, diabetes or cardiovascular diseases were observed for any other *FOXO1a* haplotype.

FOXO1a haplotypes were also analyzed for association with histories of fertility and fecundity in married women of the combined cohort (n=701). These analyses revealed no associations with *FOXO1a* haplotypes (Supplementary Table 2).

Table 2. Metabolic profile and BMI dependent on *FOXO1a* haplotypes in cohort '97 (n=563)

	FOXO1a						
	Haplotype 1	Haplotype 2		Haplotype 3		Haplotype 4	
	Mean (95% CI)	Dif (95% CI)[1]	p-value[1]	Dif (95% CI)[1]	p-value[1]	Dif (95% CI)[1]	p-value[1]
HbA1c (mmol/l)[2]	5.80 (5.61-5.98)	-0.11 (-0.25-0.03)	0.185	+0.25 (0.02-0.48)	0.025*	-0.05 (-0.24-0.14)	0.700
Triglycerides (mmol/l)[2]	1.57 (1.44-1.69)	+0.07 (-0.04-0.17)	0.593	+0.09 (-0.03-0.21)	0.152	+0.04 -0.12-0.19)	0.816
CRP (mg/l)[2]	6.10 (2.99-9.21)	+0.88 (-1.22-2.97)	0.803	+1.90 (-1.05-4.86)	0.070	1.56 (-2.34-5.46)	0.346
HDL (mmol/l)	1.42 (1.35-1.48)	-0.01 (-0.06-0.04)	0.651	-0.04 (-0.10-0.01)	0.128	-0.06 (-0.13-0.01)	0.053
LDL (mmol/l)	3.71 (3.56-3.86)	+0.12 (-0.01-0.25)	0.068	0.00 (-0.13-0.14)	0.971	+0.12 (-0.08-0.31)	0.245
BMI (kg/m^2)	28.3 (27.5-29.1)	-0.48 (1.10-0.15)	0.132	-0.57(-1.24-0.10)	0.094	-0.38 (-1.42-0.67)	0.478

HbA1c - hemoglobin A1c; CRP - C-reactive protein; HDL - high-density lipoprotein cholesterol; LDL - low-density lipoprotein cholesterol; BMI - body mass index; [1]Difference compared to the most common haplotype 1; [2]Estimates presented for non-transformed and p-values for ln-transformed data; *p-value < 0.05

To study the role of *FOXO1a* further, we assessed the association between *FOXO1a* haplotypes and mortality in 1245 participants of the combined cohort. During the mean follow-up time of 4.4 years, 1001 (80 %) of the participants had died. Of these deaths 406 (41 %) were due to cardiovascular causes, 162 (16 %) were due to cancer and 431 (43 %) owing to other causes. Causes of death could not be obtained for two participants. Mortality analyses dependent on *FOXO1a* haplotypes revealed that carriers of haplotype 3 'TCA' had 1.14-fold increased all-cause mortality risks (95 % CI: 1.02-1.28, *p=0.021*) compared to the reference haplotype (Figure 2). This increase was not attributable to cardiovascular or cancer mortality, but to death from other causes (HR 1.28, 95 % CI: 1.09-1.51, *p=0.002*). This category also included death due to diabetes (n=14), for which an association with haplotype 3 'TCA' was observed (HR 2.43, 95 % CI: 1.12-5.27, *p=0.025*). For the other *FOXO1a* haplotypes, no associations with all-cause or cause-specific mortalities were found (Figure 2).

The analyses with *FOXO3a* haplotypes revealed no differences in the various parameters of the metabolic profile (Supplementary Table 3), fertility and fecundity (Supplementary Table 4). In contrast, increased risks of stroke for haplotype 2 'GAGC' (OR 1.92, 95 % CI: 1.19-3.08, *p=0.007*), and for haplotype 4 'AAAT' (OR 2.17, 95 % CI: 1.17-4.03, *p=0.014*) in *FOXO3a* block-A were observed (Table 3). In addition, the haplotype 2 'GAGC' carriers had 1.13-fold increased all-cause mortality (95 % CI; 1.01-1.26, *p=0.036*) and 1.19-fold increased cardiovascular mortality risks (HR 1.19, 95 % CI: 1.00-1.42, *p=0.052*) (Figure 3). There were no differences in cancer risk or in the risk from other causes of mortality in the *FOXO3a* haplotypes (Figure 3).

All the above-mentioned analyses were also performed with the individual SNPs, which were selected to cover the non-haploblock regions of the *FOXO1a* and *FOXO3a* genes. None of these polymorphisms were associated with any of the phenotypes analyzed (data not shown).

Table 3. Risks of diabetes and cardiovascular disease (CVD) dependent on *FOXO3a* haplotypes in cohort '97 (n=563)

	FOXO3a							
	Block-A					Block-B		
	Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4	Haplotype 5	Haplotype 1	Haplotype 2	Haplotype 3
	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)
Diabetes (n=92)	1 (Ref)	1.07 (0.73-1.57)	0.93 (0.59-1.48)	1.03 (0.60-1.74)	1.18 (0.69-2.02)	1 (Ref)	0.85 (0.52-1.38)	0.83 (0.47-1.47)
CVD total (n=365)	1 (Ref)	1.15 (0.86-1.54)	1.09 (0.75-1.57)	1.04 (0.69-1.57)	1.38 (0.86-2.23)	1 (Ref)	1.02 (0.71-1.47)	0.88 (0.57-1.35)
Myocardial infarction (n=137)	1 (Ref)	0.74 (0.52-1.06)	1.01 (0.67-1.52)	1.18 (0.74-1.86)	1.51 (0.94-2.40)	1 (Ref)	1.09 (0.73-1.62)	1.56 (0.98-2.49)
Myocardial ischemia (n=286)	1 (Ref)	1.09 (0.82-1.45)	1.09 (0.76-1.55)	1.03 (0.69-1.55)	1.30 (0.84-2.02)	1 (Ref)	1.03 (0.73-1.46)	0.96 (0.63-1.47)
Intermittent claudication (n=36)	1 (Ref)	0.98 (0.56-1.73)	1.42 (0.82-2.47)	1.20 (0.50-2.85)	0.92 (0.32-2.67)	1 (Ref)	1.64 (0.92-2.90)	1.12 (0.41-3.01)
Arterial surgery (n=37)	1 (Ref)	0.75 (0.41-1.36)	0.71 (0.37-1.36)	0.42 (0.16-1.11)	0.52 (0.20-1.37)	1 (Ref)	0.77 (0.38-1.55)	0.28 (0.07-1.14)
Stroke (n=57)	1 (Ref)	1.92 (1.19-3.08)*	1.14 (0.63-2.08)	2.17 (1.17-4.03)*	1.50 (0.72-3.12)	1 (Ref)	0.94 (0.53-1.65)	1.74 (0.95-3.18)

The most common haplotype 1 was used as a reference group (Ref); *p-value < 0.05

Figure 2. *FOXO1a*, all-cause and cause-specific mortality. Mortality risks were calculated in the combined cohort (n=1245). Data are presented as hazard ratios (HR) with 95 % confidence intervals (CI). The most common haplotype 1 was used as a reference group (Ref); *p-value < 0.05 (see text)

Figure 3. *FOXO3a*, all-cause and cause-specific mortality. Mortality risks were calculated in the combined cohort (n=1245). Data are presented as hazard ratios (HR) with 95 % confidence intervals (CI). The most common haplotype 1 was used as a reference group (Ref); *p-value < 0.05 (see text)

Discussion

In this study, we report for the first time associations between haplotypes in the evolutionarily conserved *FOXO1a* and *FOXO3a* genes, and mortality in humans. For *FOXO1a*, haplotype 3 'TCA' was associated with higher HbA1c levels, with a trend for higher prevalence of diabetes and myocardial infarction, and increased mortality. Moreover, haplotype analyses of the *FOXO3a* gene revealed increased risks of stroke and mortality for haplotype 2 'GAGC' carriers.

FOXO transcription factors have emerged as candidate genes that are involved in lifespan regulation of various organisms. On the basis of the results from mouse models, it has been reasoned that FOXO1a influences mortality mainly by modifying the risks of diabetes (Barthel et al., 2005). In this study, we observed an association between FOXO1a haplotype 3 'TCA' and HbA1c, which is the main risk factor for diabetes. In these haplotype carriers, the risks of diabetes and mortality were also increased. The observation that BMI was lower, suggests that the susceptibility to diabetes in these elderly participants was not influenced by body composition. In principle, all diabetes at old age is due to type-2 diabetes and is driven by insulin resistance and secondary exhaustion of the β-cell function. This implies that the FOXO1a transcription factors, which are normally downregulated by insulin signaling, are activated leading to increased transcription of FOXO1a target genes.

The role of FOXO1a in the development of diabetes has been previously assessed in a case-control study consisting of middle-aged participants (Karim et al., 2006). In that study, haplotypes in *FOXO1a* were associated with increased glucose levels, and with a trend for diabetes. In this study, we observed similar results, even though only one polymorphism was the same (rs2721069) between the studies. This difference in the analyzed polymorphisms might explain the stronger associations observed in this study. However, on the basis of the evidence from both the studies, we conclude that, in humans, FOXO1a may influence glucose metabolism and contribute to the predisposition to diabetes, leading to increased mortality. In contrast, we found no evidence for the FOXO1a involvement in female fertility and fecundity, which were respectively defined as the ability to have children, and the probability to conceive within a specific period of time (van Dunne et al., 2006).

The other FOXO transcription factor, FOXO3a, has been implicated in a variety of biological processes, including metabolism, fertility, stress response and ageing. In this study, we found no associations between *FOXO3a* haplotypes, human fertility and fecundity. In mouse models, the lack of FOXO3a resulted in an age-dependent decline of fertility in homozygous knockout mice, whereas heterozygous mice were indistinguishable from the wild-types (Hosaka et al., 2004). This suggests that mutations or severe disruptions of human *FOXO3a* might lead to phenotypes similar to those observed in mice. Similar to the results with fertility and fecundity, we found no association with metabolic profile and *FOXO3a* haplotypes. Despite that, carriers of haplotype 2 'GAGC' in *FOXO3a* block-A, had increased risks of stroke, and increased mortality, which was partly attributable to increased cardiovascular mortality. The mechanisms through which FOXO3a influences the occurrence of stroke are unknown, but the involvement of FOXO3a in the mediation of oxidative stress responses (Lehtinen et al., 2006) might be a possibility.

Several studies have implicated FOXO1a and FOXO3a in the development of tumors (Galili et al., 1993; Hillion et al., 1997). In addition, FOXO proteins have been shown to induce cell-cycle arrest, DNA repair and apoptosis, thereby making them attractive candidates for tumor

suppression. The results of this study did not reveal any significant differences in the estimates of cancer mortality risk for the different *FOXO1a* and *FOXO3a* haplotypes. For *FOXO1a*, we expected opposite results, as predisposition to diabetes and protection against cancer have been associated with FOXO gain of function (Hu et al., 2004; Yang et al., 2005).

The regulation of an organism's lifespan is complex and depends not only on multiple genetic, epigenetic and environmental factors, but also on the interaction between them. In this study, we used a candidate gene approach, which relies on predicting the identity of the correct gene or genes, on the basis of biological hypothesis, or the location of the candidate within a previously determined region of linkage. This approach, however, will identify only a fraction of the genetic factors that contribute to the complex phenotype. A complementary approach would be a whole genome association study that surveys most of the genome for causal genetic variants. Such an approach could reveal valuable additional information on the genetic bases of human lifespan regulation.

The first strength of this study is the haplotype-tagging SNP approach, which most probably captured the common genetic variations present in both the genes. The *FOXO1a* haplotype 3 'TCA' consists of intronic SNPs. This suggests that these SNPs might be in LD with a nearby functional polymorphism that drives the observed associations. Since the LD in the *FOXO1a* gene extends beyond 5' UTR then the functional SNP hypothesized probably is located in the regulatory region. Therefore, in addition to replication, further studies are needed to pinpoint the location of the functional variant and to prove its influence on the FOXO1a function. Other strengths of the study were the possibility of estimating several intermediate phenotypes in one cohort, and the prospective analyses. The high prevalence of age-associated diseases and mortality in this cohort excludes the possibility that this cohort consists of healthy survivors only. A limitation of the study concerns the reproductive data, which were acquired from registries; therefore all conception times and fecundity rates were calculated. In addition, taking into account the number of tests performed, adjustment for multiple testing would eliminate all the significant p-values observed.

In conclusion, the present study shows that human homologues of genes identified as influencing the life spans of model organisms, have the same impact in humans. In this study, we observed biologically plausible influences of *FOXO1a* and *FOXO3a* haplotypes on age-related trajectories and mortality.

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Supplementary materials

Supplementary Table 1. Risks of diabetes and cardiovascular disease (CVD) dependent on *FOXO1a* haplotypes in cohort '97 (n=563)

	FOXO1a			
	Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4
	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)
Diabetes (n=92)	1 (Ref)	0.68 (0.44-1.03)	1.29 (0.86-1.92)	1.01 (0.60-1.70)
CVD total (n=365)	1 (Ref)	0.85 (0.63-1.16)	1.08 (0.78-1.50)	0.90 (0.60-1.36)
Myocardial infarction (n=137)	1 (Ref)	0.88 (0.63-1.23)	1.41 (1.00-2.00)*	1.08 (0.69-1.70)
Myocardial ischemia (n=286)	1 (Ref)	0.90 (0.68-1.20)	1.09 (0.80-1.48)	1.12 (0.75-1.66)
Intermittent claudication (n=36)	1 (Ref)	1.03 (0.65-1.63)	0.94 (0.52-1.71)	0.91 (0.36-2.28)
Arterial surgery (n=37)	1 (Ref)	0.95 (0.60-1.52)	0.59 (0.28-1.22)	1.61 (0.75-3.46)
Stroke (n=57)	1 (Ref)	0.93 (0.60-1.44)	0.94 (0.58-1.51)	0.99 (0.52-1.88)

The most common haplotype 1 was used as a reference group (Ref); *p-value < 0.05

Supplementary Table 2. Fertility and fecundity dependent on *FOXO1a* haplotypes in females of the combined cohort (n=701)

	FOXO1a			
	Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4
	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)
Fertility (n=701)	1 (Ref)	1.00 (0.66-1.51)	1.06 (0.70-1.62)	0.87 (0.51-1.49)
Fecundity (n=610)[1]				
≤3 months	1 (Ref)	1.29 (0.89-1.87)	1.03 (0.70-1.51)	1.38 (0.80-2.39)
≤6 months	1 (Ref)	0.94 (0.66-1.32)	1.01 (0.71-1.45)	1.35 (0.83-2.20)
≤12 months	1 (Ref)	1.18 (0.84-1.66)	1.07 (0.75-1.51)	1.28 (0.80-2.06)

[1] Conception interval, calculated as the time interval between the date of marriage and the birth date of the firstborn child.
The most common haplotype 1 was used as a reference group (Ref)

Supplementary Table 3. Metabolic profile and BMI dependent on *FOXO3a* haplotypes in cohort '97 (n=563)

	FOXO3a							
	Block-A					Block-B		
	Haplotype1	Haplotype 2	Haplotype 3	Haplotype 4	Haplotype 5	Haplotype 1	Haplotype 2	Haplotype 3
	Mean (95% CI)	Dif. (95% CI)*	Dif. (95% CI)*	Dif. (95% CI)*	Dif. (95% CI)*	Mean (95% CI)	Dif. (95% CI)*	Dif. (95% CI)*
HbA1c (mmol/l)	5.81 (5.62-5.99)	+0.01 (-0.13-0.16)	0.00 (-0.23-0.23)	-0.05 (-0.25-0.15)	+0.17 (-0.09-0.44)	5.88 (5.73-6.02)	-0.09 (-0.26-0.07)	-0.09 (-0.33-0.14)
Triglycerides (mmol/l)	1.68 (1.52-1.84)	-0.02 (-0.14-0.10)	+0.02 (-0.12-0.16)	-0.06 (-0.20-0.08)	-0.07 (-0.24-0.10)	1.67 (1.56-1.78)	+0.02 (-0.12-0.16)	-0.03 (-0.19-0.12)
CRP (mg/l)	7.88 (4.13-11.6)	-0.72 (-3.68-2.23)	-1.52 (-4.36-1.32)	+2.58 (-2.17-7.33)	+0.16 (-4.31-4.63)	7.44 (5.41-9.46)	-1.39 (-3.59-0.81)	+4.42 (-2.24-11.1)
HDL (mmol/l)	1.36 (1.29-1.44)	+0.01 (-0.05-0.06)	+0.02 (-0.06-0.09)	0.00 (-0.08-0.07)	+0.04 (-0.05-0.12)	1.37 (1.32-1.42)	+0.01 (-0.06-0.09)	+0.01 (-0.07-0.09)
LDL (mmol/l)	3.90 (3.72-4.09)	-0.11 (-0.24-0.03)	+0.02 (-0.15-0.20)	-0.11 (-0.31-0.08)	-0.12 (-0.33-0.09)	3.80 (3.68-3.92)	+0.04 (-0.14-0.21)	-0.02 (-0.24-0.20)

HbA1c - hemoglobin A1c; CRP - C-reactive protein; HDL - high-density lipoprotein cholesterol; LDL - low-density lipoprotein cholesterol; BMI - body mass index
*Differences (Dif) compared to the most common haplotype 1

Supplementary Table 4. Fertility and fecundity dependent on *FOXO3a* haplotypes in females of the combined cohort (n=701)

	FOXO3a							
	Block-A					Block-B		
	Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4	Haplotype 5	Haplotype 1	Haplotype 2	Haplotype 3
	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)	OR (95 % CI)
Fertility (n=701)	1 (Ref)	1.25 (0.83-1.90)	0.88 (0.57-1.36)	1.18 (0.72-1.94)	1.09 (0.64-1.84)	1 (Ref)	0.89 (0.57-1.37)	1.00 (0.59-1.70)
Fecundity (n=610)[1]								
≤3 months	1 (Ref)	0.95 (0.66-1.38)	0.77 (0.49-1.20)	1.06 (0.66-1.70)	1.13 (0.67-1.93)	1 (Ref)	0.82 (0.54-1.27)	1.03 (0.63-1.69)
≤6 months	1 (Ref)	0.96 (0.69-1.32)	0.88 (0.58-1.33)	1.33 (0.86-2.06)	1.13 (0.70-1.82)	1 (Ref)	0.95 (0.63-1.42)	1.30 (0.82-2.05)
≤12 months	1 (Ref)	1.06 (0.76-1.46)	0.99 (0.65-1.50)	1.19 (0.77-1.84)	1.27 (0.79-2.04)	1 (Ref)	1.03 (0.69-1.54)	1.11 (0.70-1.75)

[1] Conception interval, calculated as the time interval between the date of marriage and the birth date of the firstborn child. The most common haplotype 1 was used as a reference group (Ref)

CHAPTER 3

The Liver X Receptor Alpha Associates with Human Lifespan

Simon P. Mooijaart, Maris Kuningas, Rudi G. J. Westendorp, Jeanine J. Houwing-Duistermaat, P. Eline Slagboom, Patrick C. N. Rensen and Diana van Heemst

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Abstract

In the nematode *Caenorhabditis elegans*, nuclear hormone receptor DAF-12 regulates the decision to go into a resistant dauer diapause, in which the worm exhibits a decreased rate of aging. Using sequence similarity searches, we previously identified the liver X receptor alpha (LXRA) as one of the human nuclear hormone receptors the protein sequence of which is most similar to *C. elegans* DAF-12. Here, we studied whether variation in the gene encoding LXRA associates with human life span. In the Leiden 85-plus Study, a population-based prospective follow-up study, we genotyped four polymorphisms spanning the gene coding for *LXRA* (NR1H3) and tagged four common haplotypes. Among 563 participants, haplotype 2 associated with reduced mortality during the 7-year follow-up (hazard ratio 0.78; $p=0.015$), predominantly caused by reduced mortality from infectious disease (hazard ratio 0.31; $p=0.023$). Haplotype 2 also associated with higher levels of plasma apolipoprotein E, a target gene of the LXRA ($p=0.018$), and higher levels of triglycerides ($p=0.041$). Genetic variation in the gene coding for the LXRA (NR1H3) associates with human lifespan.

Introduction

Human lifespan is under genetic control (Schoenmaker et al., 2006; vB et al., 2006), but only few specific genes modulating lifespan have been identified. In the nematode worm *Caenorhabditis* elegans, DAF-12 is a nuclear hormone receptor (NHR) that in response to environmental cues regulates the entry into dauer diapause (Antebi et al., 1998). Under adverse environmental conditions, unliganded DAF-12 coordinately turns down essential traits—such as metabolism, feeding, and reproduction— making the worm more stress resistant and extending larval survival up to 5-fold (Klass and Hirsh, 1976), which suggests that during the diapause the worm ages at a lower rate. Genetic mutations in *daf-12* can be either dauer defective or dauer constitutive (Antebi et al., 2000) and, in parallel, can decrease or increase adult life span of *C. elegans* (Fisher and Lithgow, 2006). Using sequence similarity searches, we previously identified the liver X receptor alpha (LXRA) as one of the human NHRs the protein sequence of which is most similar to *C. elegans* DAF-12 (Mooijaart et al., 2005). However, nothing is known about the association of genetic variants in the gene coding for the LXRA (NR1H3) with human lifespan.

In humans, the *LXRA* is expressed in the liver, kidney, macrophages, astrocytes, and other tissues (Peet et al., 1998). Oxysterols are breakdown products of cholesterol and serve as ligands for the LXRA (Janowski et al., 1999). Binding of ligands leads to the transcription of target genes that coordinately regulate various processes that together result in increased catabolism and excretion of cholesterol from the body (Lu et al., 2001; Repa et al., 2000). In humans, cholesterol is a major determinant of mortality in old age, especially from infectious disease (Weverling-Rijnsburger et al., 1997).The LXRA is also involved in innate immunity, as activation of human macrophages that produce cytokines is dependent on LXRA (Joseph et al., 2003). In humans, cytokine production is a highly heritable characteristic (de Craen et al., 2005) and associates with diseases and mortality up to the highest age category (van den Biggelaar et al., 2004a). These observations make the *LXRA* a candidate to affect human life span.

To test the hypothesis that the *LXRA* is involved in modulating human lifespan, we made use of genetic variation in the gene coding for the LXRA (NR1H3). Out of the data that recently came available from the HapMap Project, we selected four evenly spaced haplotype-tagging single nucleotide polymorphisms (SNPs) spanning the NR1H3 gene. In the Leiden 85-plus Study, a prospective population-based follow-up study of 563 elderly persons aged 85 years or older and onwards, we studied the association of the common haplotypes with survival during a mean follow-up period of almost 5 years. To further explore a potential role of the LXRA in biological mechanisms associated with modulation of human lifespan, we associated the genetic variation to mortality-related phenotypic markers.

Participants and methods

Participants

The Leiden 85-Plus Study is a prospective population-based study, in which inhabitants of Leiden, The Netherlands, aged 85 years, were invited to take part. There were no selection

criteria related to health or demographic characteristics. The study population consists of 599 individuals (all members of the 1912–1914 birth cohort) who were enrolled in the month of their 85th birthday between 1997 and 1999 (der Wiel et al., 2002). DNA was available for 563 people. The Medical Ethical Committee of the Leiden University Medical Center approved the study, and written informed consent was obtained from all participants.

Causes of death

All participants in the Leiden 85-plus Study were followed for mortality until August 1, 2005. Primary causes of death were obtained from death certificates registered at the Dutch Central Bureau of Statistics and categorized according to the 10th International Classification of Diseases (ICD). Specific causes of death were categorized into cardiovascular disease (ICD codes I00–I99), infectious disease (ICD codes A00–B99 or J11–J18), cancer (ICD codes C00–D48), or other causes (all other ICD codes).

Plasma measurements

At baseline, participants were visited twice at their place of residence within 1 month after their 85th birthday. All blood samples were collected early in the morning, but fasting was not required.

Plasma levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and C-reactive protein (CRP) were analyzed on fully automated computerized analyzers (Hitachi 747 and 911; Hitachi, Ltd, Tokyo, Japan). The level of low-density lipoprotein (LDL) cholesterol was estimated by the Friedewald equation (LDL cholesterol [mmol/L] ¼ total cholesterol – HDL cholesterol – [triglycerides/2.2]), whereby participants with a triglyceride concentration higher than 443 mg/dL (5 mmol/L) were excluded (n=5).

Apolipoprotein E (ApoE) levels were determined in 2005 in one batch of plasma samples that were collected at age 85 years at study baseline and stored frozen. Plasma ApoE levels were determined using a human ApoE-specific sandwich enzyme-linked immunosorbent assay (ELISA) essentially as described (van Vlijmen et al., 1994). The detailed procedure is described in (Mooijaart et al., 2006).

Cytokine production capacity of the innate immune system

The cytokine production capacity of the innate immune system was assessed by stimulating *ex vivo* whole-blood samples with lipopolysaccharide (LPS) as described elsewhere (van der Linden et al., 1998). In short, all venous blood samples were drawn in the morning before 11 AM to exclude circadian variation, diluted 2-fold with RPMI-1640, and stimulated with *Escherichia coli*–derived LPS (10 ng/mL; Difco Laboratories, Detroit, MI). After 4 hours and after 24 hours of incubation at 378 C^0 and 5 % CO_2, supernatants were collected and stored at -808 C^0 to measure tumor necrosis factor-alpha (TNF-a), interleukin-1 beta (IL-1b), IL-6, IL-10, IL-12, IL-1 receptor antagonist (IL-1Ra), and interferon-gamma (IFN-c), respectively. Standard ELISA techniques were performed according to the manufacturer's guidelines (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). Because of a possible distortion

by frailty (van den Biggelaar et al., 2004b), we restricted these analyses to those participants who survived for at least 2 years (n=463).

SNP selection

Four SNPs in the NR1H3 gene were selected using the HapMap database (*http://www.hapmap.org*; version June 2005). Only validated SNPs were selected, and calculations on linkage disequilibrium (LD) and frequencies were performed using data from the European Centre d'Etude du Polymorphisme Humain (CEPH) population. As boundaries, the first SNP upstream of the ATG start site (LXRA5 untranslated region [UTR], rs11039149) and the first SNP downstream of the stop codon (LXRA3UTR, rs1449627) were selected. The expected D' between these two SNPs was 1, indicating that the entire region is in strong LD. We additionally selected one SNP in exon 3 (LXRAex3, rs227923) and one in intron 6 (LXRAint6, rs712011), resulting in a set of four evenly spaced SNPs, separated by 5 kb.

Genotyping

The polymorphisms were genotyped using either an Assay-by-Design or an Assay-on-Demand (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands), consisting of PCR primers and TaqMan Major Groove Binding (MGB) probes. For LXRA5UTR an Assay-by-Design was used with forward primer GAGCATCTGCAGGGTTCTCA, reverse primer GCCAGTGAAGTGCTGTAATGGAA, one probe CCCCTGTAGCCCACC labeled with VIC, and one probe CCCTGTGGCCCACC labeled with FAM. For the LXRAex3 SNP an Assay-on-Demand was used with identification number C_15967384_10. For LXRAint6 an Assay-on-Demand was used with identification number C_1301060_20. For LXRA3UTR, an Assay-by-Design was used with forward primer CCTCACGTGCATGTGTAGCAT, reverse primer AGGTCTTTCAGGTTGTGCCTTTT, one probe CCTTGGTTTTTCC labeled with VIC, and one probe CCTTGGGTTTTCC labeled with FAM. Amplification reactions were performed at standard conditions except for the following modifications. A qPCR core kit was used (Eurogentec, Maastricht, The Netherlands) with half of the amount of primers and probes. Real-time PCR was performed on an ABI 7900 HT (Applied Biosystems), and genotypes were called using the Sequence Detection System 2.1 (Applied Biosystems). A random 10 % of all genotypes were performed in duplicate, and genotyping errors were < 2 % for all assays.

Statistical analysis

The program Haploview (Barrett et al., 2005) was used to estimate allele frequencies, test the consistency of genotype frequencies at each SNP locus with Hardy–Weinberg equilibrium, and estimate and plot pairwise LD between the SNPs examined. LD was estimated for all two-way comparisons of individual SNPs using two common measures: the r2 (the square of the standardized correlation coefficient) and the Lewontin D' (D'=D/Dmax if D > 0 or D'=D/Dmin if D < 0). Haplotypes and haplotype frequencies were estimated using the SNPHAP software (*http://www-gene.cimr.cam.ac.uk/clayton/software*). The posterior probabilities of pairs of haplotypes per subject as estimated by SNPHAP, were used as weights in the following analyses.

Continuous variables were normally distributed, except for plasma ApoE levels, triglycerides, CRP levels, and cytokines, which therefore were ln-transformed. All analyses were sex adjusted, using homozygosity for the most common haplotype as the referent group. Associations between haplotypes and metabolic profile were analyzed using linear regression. Mortality risks and 95 % confidence intervals (CI) were calculated with the Cox proportional hazard model. These analyses included all the estimated haplotypes in the model weighted for probability, except the reference haplotype. Clustered robust standard errors were calculated using individuals as clustering variable. These models assume an additive effect of the haplotypes. Haplotypes with low frequencies (< 5 %) fully participated in these analyses, but results on these haplotypes are not reported as their accuracy is low due to small numbers. The analyses were performed using STATA statistical software, version 9.0 (STATA Corp., College Station, TX).

Results

The baseline characteristics of the study populations are listed in Table 1. All participants were aged 85 years, and 67 % were female.

The position of the selected SNPs relative to the gene structure is shown in Figure 1a. The SNPs were in strong LD (D' > 0.97) and constituted one haplotype block (Figure 1b) with seven haplotypes, of which the predicted frequencies are listed in Table 2. For the present analyses

Table 1. Baseline characteristics of the study population

Characteristic	Value
Total number	563
Female (n, %)	375 (67 %)
Age (mean, SD)*	85.0 (-)
Lipid and lipoprotein plasma level	
Total cholesterol, mean mmol/L (SD)	5.71 (1.13)
LDL cholesterol, mean mmol/L (SD)	3.68 (0.97)
HDL cholesterol, mean mmol/L (SD)	1.31 (0.40)
Triglycerides, median mmol/L (IQR)	1.34 (1.01–1.95)
CRP, median mg/L (IQR)	4.00 (1.00–8.00)
LPS-stimulated cytokines	
IL-1b, median ng/mL (IQR)	3.50 (2.10–6.50)
IL-1RA, median ng/mL (IQR)	30.8 (28.3–46.0)
IL-6, median ng/mL (IQR)	60.7 (43.2–82.9)
IL-10, median pg/mL (IQR)	762 (487–1089)
IL-12, median ng/mL (IQR)	6.70 (4.30–10.20)
IFN-c, median ng/mL (IQR)	139 (43.0–448)
TNF-a, median ng/mL (IQR)	10.3 (7.40–13.3)

*All participants were enrolled within the month of their 85th birthday; SD=standard deviation; LDL=low-density lipoprotein; HDL=high-density lipoprotein; IQR=interquartile range; CRP=C-reactive protein; LPS=lipopolysaccharide; IL=interleukin; IFN=interferon; TNF=tumor necrosis factor; RA=receptor antagonist

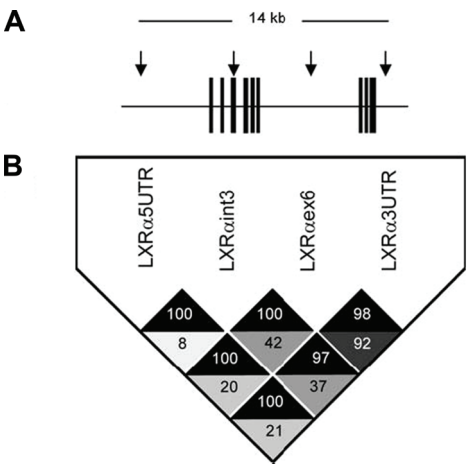

Figure 1. Haplotype structure of the NR1H3 gene in the Leiden 85-plus Study population. (A) Relative position of the selected single nucleotide polymorphisms (SNPs) in the NR1H3 gene. Vertical bars: exons 1–9. Arrows: positions of SNPs. (B) Visual representation of linkage disequilibrium within the gene. Based on standard confidence interval criteria, all four SNPs constitute one haplotype block. Top triangles (pointing upwards, all black) indicate strong linkage disequilibrium of all four SNPs. Pairwise linkage disequilibrium is indicated by the numbers in the top triangles. Pairwise R^2 values are indicated by the numbers in the bottom triangles (pointing downwards), in which light gray triangles indicate low R^2, and dark gray triangles indicate high R^2.

Table 2. Haplotype structures and frequencies

	SNP allele				
Haplotype	LXRa5UTR	LXRaex3	LXRaint6	LXRa3UTR	Frequency
1	A	C	T	T	0.367
2	G	C	T	T	0.273
3	A	T	C	G	0.176
4	A	C	C	G	0.164
5	A	C	T	G	0.016
6	A	T	C	T	0.004
7	A	C	C	T	0.003

Minor alleles are depicted in bold. Minor allele frequencies of the four polymorphisms were: LXRa5UTR, 0.27 (G); LXRaex3, 0.18 (T); LXRaint6, 0.35 (C); LXRa3UTR, 0.36 (G). All genotype distributions were in Hardy–Weinberg equilibrium, SNP=single nucleotide polymorphism; LXR=liver X receptor; UTR=untranslated region; ex = exon; int=intron

we report the results of the four most common haplotypes (frequency > 5 %) that cumulatively account for > 97% of the haplotypes.

During a mean follow-up time of 4.9 years, 320 participants (57 %) had died. We compared the mortality risk associated with the various haplotypes, using the most common haplotype 1 as the reference category (Figure 2). The mortality risk was lower for haplotype 2 compared to haplotype 1 (hazard ratio [HR] = 0.78; 95 % CI, 0.64–0.95; *p=0.015*), whereas other haplotypes were not significantly associated with a higher or lower mortality risk. When assessing specific causes of death, the lower mortality risk that was associated with haplotype 2 was mainly caused by a lower mortality risk from infectious disease (HR=0.31, 95 % CI, 0.12–0.85; *p=0.023*) and from mortality in the category "other causes" (HR= 0.71, 95 % CI, 0.50–1.00; *p=0.052*).

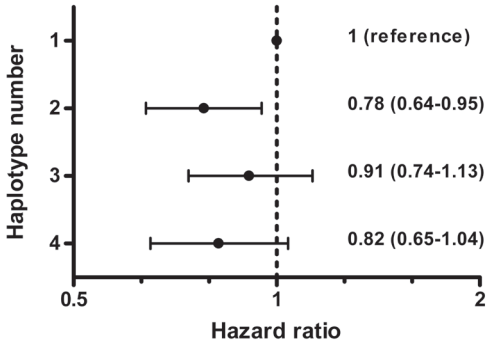

Figure 2. Dots represent hazard ratios calculated using a Cox proportional model adjusting for gender; bars represent 95 % confidence intervals. Mean follow-up time was 4.9 years, in which time a total number of 320 participants (57 %) had died.

The relationship between the four common haplotypes and variables in lipid metabolism is shown in Table 3. Haplotype 2 associated with significantly higher plasma ApoE levels (+0.48 mg/dL, *p=0.018*) and triglyceride levels (+0.098 mmol/dL, *p=0.041*) compared to haplotype 1. Haplotype 4 also associated with higher plasma ApoE levels compared to haplotype 1, although the association was borderline statistically significant (+0.45 mg/dL, *p=0.057*), possibly due to the lower haplotype frequency.

To explore the association of NR1H3 haplotypes with innate immune function, we assessed cytokine production capacity by *ex vivo* whole-blood LPS-stimulated cytokine levels (Table 4). We found no association of any haplotype with cytokine production capacity. Finally, to investigate the possibility that the LXRA regulates inflammation through alternative mechanisms, we associated the haplotypes with circulating CRP level, a plasma marker of systemic inflammation. We found no association for any haplotype with circulating levels of CRP (Table 4).

Discussion

In *C. elegans*, the NHR DAF-12 has been shown to be one of the key components that modulate lifespan in response to environmental cues. Based on protein sequence comparisons, we recently identified the LXRA as one of the human NHRs most similar to *C. elegans* DAF-12 (Mooijaart et al., 2005). Here we report that genetic variation in the gene coding for the LXRA (NR1H3) associates with human life span.

We found that a common haplotype of the NR1H3 gene associated with lifespan extension, predominantly attributable to decreased death from infectious disease. The LXRA is involved in various processes that contribute to infectious disease. The LXRA regulates specific processes that increase resistance to pathogens. For instance, LXRA regulates the expression of APa, a scavenging receptor that inhibits macrophage apoptosis and promotes the killing of the bacteria (Joseph et al., 2004). Although LXR agonists reduce inflammatory gene expression in models

Table 3. Association of NR1H3 haplotypes with parameters of lipid metabolism

	Haplotype 1	Haplotype 2		Haplotype 3		Haplotype 4	
Plasma Component	Mean (95 % CI)	Difference (95 % CI)[1]	p-value[1]	Difference (95 % CI)[1]	p-value[1]	Difference (95 % CI)[1]	p-value[1]
Apolipoprotein							
ApoE (mg/dL)[2]	4.95 (4.52–5.42)	0.48 (0.08-0.91)	0.018	0.30 (-0.13-0.79)	0.188	0.45 (-0.01-0.96)	0.057
Lipids							
Total cholesterol, mmol/L	5.93 (5.74–6.13)	0.06 (-0.10-0.21)	0.473	-0.021 (-0.18-0.14)	0.802	-0.10 (-0.29-0.09)	0.294
LDL cholesterol, mmol/L	3.84 (3.67–4.02)	0.02 (-0.12- 0.15)	0.806	-0.072 (-0.21-0.07)	0.311	-0.10 (-0.27-0.06)	0.226
HDL cholesterol, mmol/L	1.39 (1.32–1.46)	-0.01 (-0.07-0.20)	0.813	-0.004 (-0.07-0.06)	0.890	-0.01 (-0.08-0.06)	0.813
Triglycerides, mmol/L[2]	1.38 (1.28–1.49)	0.10 (0.00-0.20)	0.041	0.07 (-0.04-0.19)	0.195	0.02 (-0.08-0.14)	0.662

[1] Compared to Haplotype 1; [2] Geometric mean; Freq= frequency; ApoE=apolipoprotein E; LDL=low-density lipoprotein; HDL=high-density lipoprotein. Data represent sex-adjusted means and 95% confidence intervals (CI). All participants were aged 85 years

Table 4. Association of NR1H3 haplotypes with whole-blood lipopolysaccharide (LPS)-stimulated cytokine levels at baseline

	Haplotype 1	Haplotype 2		Haplotype 3		Haplotype 4	
	Mean (95 % CI)	Difference (95 % CI)[1]	p-value[1]	Difference (95 % CI)[1]	p-value[1]	Difference (95 % CI)[1]	p-value[1]
Innate immunity[2]							
IL-1b, ng/mL	3.30 (2.80-3.80)	0.10 (-0.30-0.60)	0.680	-0.20 (-0.60-0.30)	0.440	0.40 (-0.10-1.00)	0.141
IL1-RA, ng/mL	35.4 (32.4-38.6)	-1.20 (-3.70-1.40)	0.340	0.10 (-2.80-3.20)	0.972	-0.20 (-3.30-3.30)	0.859
IL-6, ng/mL	57.4 (51.8-63.4)	-0.90 (-5.20-3.70)	0.682	-1.40 (-6.20-3.90)	0.592	0.60 (-4.30-6.10)	0.794
IL-10, pg/mL	709 (623-807)	5.00 (-64.0-82.0)	0.888	-6.00 (-85.0-83.0)	0.892	-19.0 (-101.0-75.0)	0.680
IL-12, ng/mL	6.00 (5.20-6.90)	0.20 (-0.40-1.00)	0.494	-0.60 (-1.30-0.01)	0.054	0.50 (-0.30-1.30)	0.249
IFN-c, ng/mL	151 (108-209)	10.0 (-27.0-59.0)	0.617	-35.0 (-66.0-8.0)	0.100	15.0 (-32.0-82.0)	0.575
TNF-a, ng/mL	9.80 (9.00-10.7)	0.40 (-1.10-0.30)	0.240	-0.80 (-1.60-0.10)	0.035	0.30 (-0.50-1.30)	0.432
Chronic inflammation[2]							
CRP, mg/L	2.00 (1.50-2.70)	0.20 (-0.30-0.80)	0.440	0.40 (-0.20-1.10)	0.224	0.20 (-0.40-0.90)	0.599

[1] Compared to Haplotype 1; [2] Geometrical means; Freq=frequency; IL=interleukin; RA=receptor antagonist; IFN=interferon; TNF=tumor necrosis factor; CRP=C-reactive protein. Data represent sex-adjusted means and 95% confidence intervals (CI). All participants were aged 85 years

of dermatitis and atherosclerosis (Joseph et al., 2003), LXRA$^{-/-}$ knockout mice are more susceptible to infection with *Listeria monocytogenes* (Joseph et al., 2004).

In the search for an intermediate phenotype, we associated genetic variation in the NR1H3 gene with mortality-related markers in lipid metabolism and immunity. We selected these markers because these phenotypes are known to associate with mortality and a functional relationship with the LXRA protein was plausible. Cholesterol metabolism is related to various causes of death (Weverling-Rijnsburger et al., 1997), and the LXRA is involved in various components of lipid metabolism, such as reverse cholesterol transport, cholesterol excretion, and fatty acid synthesis (Lu et al., 2001). We observed an association of haplotype 2 of the NR1H3 gene with increased plasma ApoE and triglyceride levels. ApoE is a component of triglyceride-rich lipoproteins, such as very low- density lipoprotein (VLDL), which may explain why haplotype 2 associates with plasma levels of both ApoE and triglycerides. Furthermore, LXRA agonists have been suggested for therapeutic use against cardiovascular disease, but a serious side effect of the use of LXR agonists as therapeutic agents is the concomitant increase in liver and serum triglycerides (Grefhorst et al., 2002). These effects are caused by a strong induction of lipogenic genes in the liver and an increased VLDL production (Grefhorst et al., 2002). In line with these animal data, we found that haplotype 2 also associated with higher ApoE levels. Thus, the associations with increased levels of triglycerides and ApoE presented here suggest that haplotype 2 associates with increased LXRA activity in the liver. It is interesting that triglyceride-rich lipoproteins (of which ApoE is a component) act as agents of the innate immune system (Barcia and Harris, 2005), for example, by binding and neutralizing bacterial components. ApoE redirects lipopolysaccharides (bacterial components) in the liver from Kupffer cells to hepatocytes and protects against endotoxemia in rats (Rensen et al., 1997). Recently, it was discovered that ApoE is also involved in lipid antigen presentation (van den et al., 2005) and that high plasma ApoE levels associate with increased systemic inflammation (Mooijaart et al., 2006).

LPS-stimulated cytokine production levels are highly heritable (de Craen et al., 2005), and cytokine production profiles associate with patterns of old age mortality (van den Biggelaar et al., 2004a). However, genetic variation in the genes coding for the cytokines has so far been insufficient to explain the heritable component (Haukim et al., 2002). In the present study, variation in the NR1H3 gene did not associate with *ex vivo* LPS-stimulated whole-blood cytokine levels or with circulating CRP levels. Others have demonstrated an association of the LXRA with inflammation in macrophage and monocyte cell cultures (Joseph et al., 2003; Landis et al., 2002). However, inflammatory cytokines and other serum mediators were not different between LXRA and LXRB knockout mice and wild types (Joseph et al., 2004). We interpret that NR1H3 may not be a major determinant of cytokine production capacity in blood upon stimulation by LPS. This interpretation does not, however, exclude the possibility that in other cell types NR1H3 haplotypes may influence the local production of cytokines.

We did not observe a beneficial effect of haplotype 2 on death from cardiovascular causes. In mouse models, LXR agonists reduce the formation of atherosclerotic lesions (Joseph et al., 2002) whereas macrophage-specific LXRA knockout aggravates atherosclerosis development (Tangirala et al., 2002). To date, the function of the LXRA in lipid and cholesterol metabolism has been studied in mouse models and in human cultured cell lines, mostly macrophages. However, caution must be taken in extrapolating these results based on cultured cells and mouse models of atherosclerosis to humans (Repa and Mangelsdorf, 2002). Whereas macrophage LXR

has been shown to be antiatherogenic (Levin et al., 2005; Tangirala et al., 2002), this beneficial effect on cardiovascular disease may be balanced by proatherogenic effect of liver LXR activation.

Genetic variation in both *C. elegans* daf-12 and the human NR1H3 gene associates with differences in life span, suggesting that the two genes may, at least to some extent, be functionally conserved. Other evolutionarily conserved pathways have previously been implicated in life-span regulation. For example, it was first discovered that the *C. elegans* daf-2 mutant was long-lived (Kenyon et al., 1993). Later it was discovered that the daf-2 gene shows homology to the mammalian genes encoding the insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF-1R) (Kimura et al., 1997), which are conserved throughout evolution. Extended life span was then also demonstrated in IR mutants in *Drosophila melanogaster* (Tatar et al., 2001) and in IR and IGF-1R mutants in mice (Bluher et al., 2003; Holzenberger et al., 2003). Recently we showed that reduced insulin signaling in humans also associates with longevity (van Heemst et al., 2005). These observations suggest that the approach of studying evolutionarily conserved pathways is fruitful in identifying genes that regulate human life span.

Very recently, several articles report on the biological function of daf-12 in *C. elegans*. Two hormones were identified that function as DAF-12 ligands (Motola et al., 2006), and the biosynthetic pathway of production of these ligands was described in more detail (Rottiers et al., 2006). Furthermore, cholestenoic acid was found to rescue the worm from dauer diapause in a DAF-12–dependent manner (Held et al., 2006). These studies provide important new hints to investigate the functional conservation of life-span regulation throughout evolution and the biological function of the human LXRs or other NHRs.

A limitation of our study is that it does not include analyses of the gene encoding the LXRB (NR1H2). The LXRA and LXRB are highly similar proteins, as their amino acid sequences are very alike and the proteins have similar functions in lipid metabolism and inflammation. It could therefore be hypothesized that a loss of function of one of the genes will be compensated for by the function of the other receptor and will therefore not have dramatic effects. The LXRA and LXRB are encoded by different genes and have different expression patterns. Whereas the LXRA is expressed only in a limited number of tissues, the LXRB is expressed ubiquitously. Indeed, in activated macrophages the inhibiting effect of LXR ligands on cytokine expression is completely abrogated in double knockout macrophages (nr1h3$^{-/-}$ nr1h2$^{-/-}$). However, there was also a partial reduction of this effect in nr1h3$^{-/-}$ macrophages (Joseph et al., 2003). Furthermore, whereas LXRA$^{-/-}$ knockout increases susceptibility to bacterial infection, additional knockout of LXRB does not increase this susceptibility (Jonsson et al., 2003). These observations suggest that genetic variation in LXRA may have functional significance independent of LXRB. A second limitation is that it is unclear which genetic variant within the haplotype is responsible for the observed functional variation. The SNP that tags haplotype 2 is located in the 5` UTR of the gene. It is, however, unknown whether this SNP itself has functional significance—for instance, by affecting promoter function or interaction domains. Alternatively, the 5` UTR SNP may be in LD with an SNP elsewhere in the genome, for instance in the coding region of the gene, which leads to an alteration with functional significance.

The strong point of our study is that we selected genetic variants tagging all common haplotypes of the NR1H3 gene and associated them with a range of variables in inflammation and lipid metabolism. Furthermore, the prospective nature of the mortality analyses and the rela-

tively large number of deaths yield a powerful tool to study mortality risk.

As this is the first report on the effect of common genetic variants in the human NR1H3 gene on human life span, the results of our observational study need further replication. Furthermore, more research is warranted to confirm which specific genetic variation on the haplotypes actually changes the function of the gene or protein.

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CHAPTER 4

VDR Gene Variants Associate with Cognitive Function and Depressive Symptoms in Old Age

Maris Kuningas, Simon P. Mooijaart, Jelle Jolles, P. Eline Slagboom, Rudi G. J. Westendorp and Diana van Heemst

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Abstract

Vitamin D has been recently implicated in brain function. Our objective was to test whether genetic variance in the vitamin D receptor (*VDR*) gene is associated with cognitive functioning and depressive symptoms in old age. The study was carried out in the prospective population-based Leiden 85-plus Study. All 563 participants of the study were genotyped for *Cdx-2*, *FokI*, *BsmI*, *ApaI* and *TaqI* polymorphisms in the *VDR* gene. Our data revealed an overall worse performance on tests measuring cognitive functioning for carriers of *BsmI* ($p=0.013$) and *TaqI* ($p=0.004$) polymorphisms, and of haplotype 2 (BAt) ($p=0.004$). In contrast, carriers of *ApaI* variant-allele and of haplotype 1 (baT) had better cognitive functioning together with less depressive symptoms. These associations could not be explained by differences in calcium levels, and by selective survival, since no associations between the *VDR* gene variants and calcium levels and mortality were observed. In conclusion, our results show that genetic variance in the *VDR* gene influence the susceptibility to age-related changes in cognitive functioning and in depressive symptoms.

Introduction

Ample data provide evidence that vitamin D is involved in brain function. The reported biological processes influenced by vitamin D in the brain include neuroprotection, immunomodulation and detoxification (Brown et al., 2003; Garcion et al., 2002). The neuroprotective effects of vitamin D appear to be exerted via the regulation of calcium homeostasis (Brewer et al., 2001; Brewer et al., 2006; de Viragh et al., 1989), and synthesis of neurotrophins, such as nerve growth factor and neurotrophin 3 (Naveilhan et al., 1996; Neveu et al., 1994a; Neveu et al., 1994b; Saporito et al., 1994; Wang et al., 2000). These biological effects suggest that vitamin D could influence cognitive functioning and the prevalence of depressive symptoms.

The functions of vitamin D are mediated by vitamin D receptor (*VDR*), which belongs to the nuclear hormone receptor (NHR) super-family, and which is ubiquitously expressed in the organism (Kamei et al., 1995; Langub et al., 2001). Defects in the vitamin D signaling system have been associated with multiple sclerosis, and various behavioral- and mood disorders in animals and humans (Cantorna et al., 1996; Garcion et al., 2002; Lansdowne and Provost, 1998; Munger et al., 2004). It has been shown that animals exposed to prenatal vitamin D deficiency have alterations in brain morphology (Eyles et al., 2003), locomotion (Burne et al., 2004; Kesby et al., 2006), and learning and memory (Becker et al., 2005). In addition, mice lacking a functional *VDR* gene appear to suffer from anxiety-like behavior (Kalueff et al., 2004; Kalueff et al., 2006). In humans, vitamin D deficiency has been associated with the presence of an active mood disorder and with worse cognitive functioning (Przybelski and Binkley, 2007; Wilkins et al., 2006). In contrast, little is known about whether and how disturbed function of the *VDR* gene influences these endpoints. The *VDR* gene contains several polymorphisms of which five; *Cdx-2*, *FokI*, *BsmI*, *ApaI* and *TaqI*, have been most often investigated, and associated with a number of phenotypes, such as bone mineral density, and risks for fractures and cancer (Uitterlinden et al., 2004b). In addition, haplotype alleles have been identified that influence the risk of osteoporotic fractures and the expression of the *VDR* gene (Fang et al., 2005; Grundberg et al., 2007). The risk haplotypes that have recently emerged, baT and BAt, are composed of the *BsmI*, *ApaI* and *TaqI* polymorphisms, located in the 3‘ untranslated region (UTR).

The aim of this study was to assess the influence of these five polymorphisms in the VDR gene and the risk haplotypes on cognitive functioning and depressive symptoms in old age. Furthermore, the association with calcium levels, and the incidence of fractures and mortality were assessed for the VDR polymorphisms and the haplotypes. The study was carried out in the Leiden 85-plus Study, a population-based prospective study of the oldest old.

Participants and methods

Participants

The Leiden 85-plus Study is a prospective population based study in which all 85-year-old inhabitants of the city Leiden, in The Netherlands, were invited to take part. There were no selection criteria related to health or demographic characteristics. The population under study

consists of 599 subjects, all Caucasians and members of the 1912-1914 birth cohort, enrolled in the month of their 85th birthday between 1997 and 1999 (Bootsma-van der Wiel et al., 2002). For the present study, DNA was available for 563 participants. All participants of the Leiden 85-plus Study were followed for mortality until August 1, 2005. Primary causes of death were obtained from the Dutch Central Bureau of Statistics and categorized according to the 10th International Classification of Diseases (ICD-10). The Medical Ethical Committee of the Leiden University Medical Centre approved the study and informed consent was obtained from all participants or in case of severe cognitive impairment, from their guardian.

Calcium levels at baseline

Calcium and albumin concentrations were determined in serum using fully automated analyzers (Hitachi 747 and 911; Hitachi, Ltd, Tokyo, Japan). Total calcium levels were adjusted for albumin using the following formula: corrected calcium = uncorrected calcium - [(40 – albumin) × 0.02] (Palmer et al., 1988).

Cognitive function and depressive symptoms

Overall cognitive function was measured with the Mini-Mental State Examination (MMSE) (Folstein et al., 1975). From the specific domains of cognitive functioning, attention was assessed with the Stroop Test (Klein et al., 1997), processing speed with the Letter Digit Coding Test (LDT) (Houx et al., 2002) and memory with the 12-Word Learning Test, which assesses immediate recall (WLTI) and delayed recall (WLTD)(Brand and Jolles, 1985). The prevalence of depressive symptoms was assessed with the 15-item Geriatric Depression Scale (GDS-15) (De Craen et al., 2003). The tests assessing specific domains of cognitive functioning could not be administered to 92 participants because of severe cognitive impairment (MMSE score < 19 points). All participants were visited annually for re-measurement of cognitive functioning and depressive symptoms during a mean follow-up period of 4.2 years. During the study, parallel versions of the tests were used and details of testing are described elsewhere (Houx et al., 2002). In addition to the specific tests, a composite cognitive score was calculated by converting the scores of the individual tests (Stroop Test, LDT, WLTI and WLTD) into a z-score ((individual level – mean level)/SD), and computing the average. A higher composite cognitive score reflects better performance on the tests measuring cognitive functioning.

The incidence of fractures

The incidence of fractures was assessed yearly during the five-year follow-up period. The number of fractures was obtained by self-reporting using a standardized yes/no format. When the participant was severely cognitively impaired, a guardian was asked for the information. In addition, the general practitioner, or the nursing home physician in case of institutionalization, was interviewed concerning fracture related contacts with the participant. The composite of self-reported and physician reported fractures were used. Fractures included hip, wrist and other fractures.

Possible confounders

Socio-demographic characteristics, such as sex and level of education were considered as possible confounders. Education was divided into two levels: a lower education level, including individuals without schooling or with only primary school education (less than 6 years of schooling), and a higher education level (6 years or more of schooling).

Genotyping

The *Cdx-2* G/A (rs11568820) and *BsmI* C/T (rs1544410) single nucleotide polymorphisms (SNPs) were genotyped using an Assay-by-Design (Applied Biosystems, Foster City, CA, USA), consisting of PCR primers and TaqMan MGB probes, on an ABI 7900 HT real-time PCR (Applied Biosystems, Foster City, CA, USA). Amplification reactions were made at standard conditions except for the following modifications. A qPCR core kit was used (Eurogentec, Liege, Belgium) and one-third of the amount of assay mix. *FokI* G/A (rs10735810), *ApaI* A/C (rs7975232) and *TaqI* A/G (rs731236) polymorphisms were genotyped using MassArray platform according to the protocols of the manufacturer (Sequenom Inc., San Diego, CA, USA).

Statistical analysis

The program Haploview (Barrett et al., 2005) was used to estimate allele frequencies, test for Hardy-Weinberg equilibrium and to estimate pair-wise linkage disequilibrium (LD) between the SNPs. Haplotypes and haplotype frequencies were calculated using the program SNPHAP (*http://www-gene.cimr.cam.ac.uk/clayton/software*). In order to take into account the uncertainty in haplotype probabilities, the multiple imputation approach was used (Rubin, 1987), and with SNPHAP ten datasets were generated by randomly assigning a haplotype to each subject according to its haplotype probabilities. All statistical analyses were performed with the ten datasets. The haplotype specific estimates were calculated by averaging the ten dataset-specific estimates, and the standard errors were estimated using the estimated variance within and across the datasets. The associations between baseline calcium levels and *VDR* polymorphisms and haplotypes were tested using sex adjusted linear regression. Associations between cognitive functioning, depressive symptoms and *VDR* polymorphisms and haplotypes were analyzed using a sex and education adjusted linear mixed model, estimating the overall mean difference in cognitive functioning or depressive symptoms during follow-up. Cox proportional hazard model, measuring time-to event was used to estimate the risk of incident fractures, and mortality during the follow-up period, in relation to the polymorphisms or haplotypes. The reference group contained zero-copies of a risk allele or haplotype. All analyses were performed with SPSS, version 12.0 (SPSS Inc., Chicago, IL, USA) statistical software.

Results

Demographic characteristics and baseline measures of cognitive functioning and depressive symptoms of the 563 participants of the Leiden 85-plus Study are presented in Table 1. All

study participants were genotyped for *Cdx-2*, *FokI*, *BsmI*, *ApaI* and *TaqI* polymorphisms in the VDR gene. The genotype frequencies of the SNPs (Table 1) were in agreement with Hardy-Weinberg equilibrium and similar to those reported in other Caucasian populations (Uitterlinden et al., 2004a). The *BsmI*, *ApaI* and *TaqI* polymorphisms were in strong linkage disequilibrium (LD) (D'> 0.99) (Figure 1), and defined five haplotypes, of which the first three had frequencies > 5 %. These haplotypes have previously been described as baT, BAt and bAT, respectively. The haplotype frequencies were similar to those reported in other Caucasian populations (Fang et al., 2005; Grundberg et al., 2007).

Global cognitive functioning, attention, processing speed, memory and the prevalence of depressive symptoms were assessed at baseline, age 85 years, and re-examined annually during a mean follow-up period of 4.2 years. During follow-up, a significant decline in cognitive functioning, and an increase in depressive symptoms were observed in all participants (all $p<0.001$) (Vinkers et al., 2005). These changes were not attributable to the *Cdx-2* or *FokI* polymorphisms, since during follow-up no differences in cognitive functioning and depressive symptoms were observed for carriers of these polymorphisms (data not shown). On the other hand, carriers of the *BsmI* and *TaqI* polymorphisms performed worse on all tests measuring cognitive functioning (Table 2). This worse performance was reflected by a lower composite cognitive score (*BsmI* $p_{trend}=0.013$; *TaqI* $p_{trend}=0.004$), but not by a lower MMSE, which measures global cognitive functioning (*BsmI* $p_{trend}=0.999$; *TaqI* $p_{trend}=0.899$). From specific domains of cognitive functioning, attention, immediate- and delayed memory were affected most, whereas for the prevalence

Table 1. Characteristics of study participants

Number	563
Age[1]	85 (-)
Female (%)	375 (67 %)
Low level of education (%)	362 (65 %)
Calcium (mmol/l)[1]	2.23 (2.16-2.29)
MMSE (points)[1]	26 (22-28)
MMSE >18 points (%)	471 (84 %)
Specific domains of cognitive functioning[1]	
Stroop Test (seconds)	74 (60-97)
LDT (digits)	16 (12-21)
WLTI (pictures)	25 (20-28)
WLTD (pictures)	9 (7-11)
GDS-15 (points)[1]	2 (1-3)
Polymorphisms[2]	
Cdx-2 (G/A)	0.19
FokI (G/A)	0.34
BsmI (C/T)	0.43
ApaI (A/C)	0.46
TaqI (A/G)	0.42

[1] Data are presented as medians with interquartile ranges; [2] Data are presented as minor allele frequencies; MMSE - Mini-Mental State Examination; LDT - Letter Digit Coding Test; WLTI - Word Learning Test Immediate Recall; WLTD - Word Learning Test Delayed Recall; GDS-15 - 15-item Geriatric Depression Scale

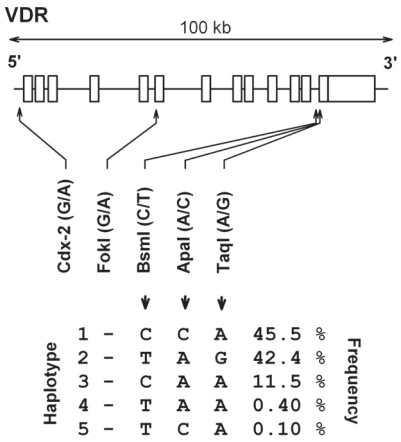

Figure 1. The VDR gene structure and haplotypes. The VDR gene spans a genomic region of 100 kb and contains 14 exons (indicated with boxes). The approximate positions of the five polymorphisms analyzed in this study are indicated with arrows. The BsmI, ApaI and TaqI polymorphisms are in strong linkage disequilibrium (LD) and define five haplotypes. The first three haplotypes, haplotype 1, haplotype 2 and haplotype 3 have previously been described as baT, BAt and bAT, respectively.

Table 2. Cognitive functioning and depressive symptoms during follow-up dependent on the *VDR* polymorphisms

	VDR genotypes			
	wt/wt	wt/var	var/var	
	Mean (SE)	Difference (SE)	Difference (SE)	p_{trend}
BsmI (C/T)				
Composite cognitive score	-0.08 (0.06)	-0.12 (0.08)	-0.25 (0.10)*	0.013*
MMSE (points)	22.6 (0.48)	0.47 (0.61)	-0.15 (0.78)	0.999
Stroop Test (seconds)	84.3 (2.31)	2.00 (2.95)	10.4 (3.78)*	0.010*
LDT (digits)	16.2 (0.51)	-1.04 (0.65)	-0.34 (0.83)	0.471
WLTI (pictures)	21.3 (0.48)	-1.07 (0.61)	-2.18 (0.77)*	0.004*
WLTD (pictures)	7.33 (0.23)	-0.30 (0.29)	-0.78 (0.39)*	0.037*
GDS-15 (points)	2.93 (0.21)	-0.05 (0.26)	0.55 (0.34)	0.158
ApaI (A/C)				
Composite cognitive score	-0.22 (0.07)	0.00 (0.08)	0.16 (0.10)	0.135
MMSE (points)	23.1 (0.51)	-0.32 (0.63)	-0.56 (0.76)	0.456
Stroop Test (seconds)	89.9 (2.45)	-2.65 (3.05)	-6.80 (3.68)	0.068
LDT (digits)	16.4 (0.53)	-1.40 (0.66)*	-0.06 (0.80)	0.737
WLTI (pictures)	19.8 (0.51)	0.49 (0.63)	1.61 (0.76)*	0.041*
WLTD (pictures)	6.83 (0.24)	0.23 (0.30)	0.44 (0.36)	0.222
GDS-15 (points)	3.42 (0.21)	-0.56 (0.26)*	-0.72 (0.32)*	0.019*
TaqI (A/G)				
Composite cognitive score	-0.07 (0.06)	-0.13 (0.08)	-2.94 (0.10)*	0.004*
MMSE (points)	22.7 (0.48)	0.55 (0.60)	-0.30 (0.78)	0.899
Stroop Test (seconds)	84.0 (2.29)	2.12 (2.92)	11.0 (3.80)*	0.008*
LDT (digits)	16.3 (0.50)	-0.96 (0.64)	-0.63 (0.84)	0.310
WLTI (pictures)	21.4 (0.47)	-1.15 (0.60)	-2.51 (0.78)*	0.001*
WLTD (pictures)	7.40 (0.23)	-0.37 (0.29)	-0.99 (0.37)*	0.009*
GDS-15 (points)	2.93 (0.20)	-0.06 (0.26)	0.60 (0.33)	0.135

*$p < 0.05$; SE – standard error; MMSE - Mini-Mental State Examination; LDT - Letter Digit Coding Test; WLTI - Word Learning Test Immediate Recall; WLTD - Word Learning Test Delayed Recall; GDS-15 - 15-item Geriatric Depression Scale

of depressive symptoms no differences were observed (Table 2). In contrast, carriers of the *ApaI* variant-allele tended to have less depressive symptoms than non-carriers during follow-up (p_{trend}=*0.019*) (Table 2). These differences were observed for both heterozygous (-0.56 points, 95 % CI:-1.07 to -0.04, *p=0.036*) and homozygous (-0.72 points, 95 % CI: -1.35 to -0.09, *p=0.026*) *ApaI* variant-allele carriers. In addition, these participants performed better, although not statistically significant, on tests measuring processing speed, attention and memory (Table 2).

The haplotype analyses revealed similar results as those with the individual polymorphisms. Carriers of at least one copy of haplotype 1 (baT), which contains the *ApaI* polymorphism, had less depressive symptoms (p_{trend}=*0.026*), and performed better, although statistically not significantly, on tests measuring attention, immediate memory and delayed memory compared to non-carriers (Table 3). The opposite was observed for carriers of haplotype 2 (BAt), which combines the variant alleles of *BsmI* and *TaqI* polymorphisms (Table 3). For all these associations, an allele dosage dependent effect was observed, which was more pronounced for haplotype 2 (BAt) carriers, who had mainly impairments in attention (p_{trend}=*0.008*), immediate memory (p_{trend}=*0.001*) and delayed memory (p_{trend}=*0.009*) (Figure 2).

Table 3. Cognitive functioning and depressive symptoms during follow-up dependent on the *VDR* haplotypes

	VDR haplotypes			
	0-copies Mean (SE)	1-copy Difference (SE)	2-copies Difference (SE)	p-trend
Haplotype 1 (baT)				
Composite score	-0.09 (0.07)	-0.01 (0.08)	0.13 (0.10)	0.215
MMSE (points)	22.5 (0.55)	-0.32 (0.62)	-0.60 (0.76)	0.424
Stroop Test (seconds)	84.3 (2.72)	-1.72 (3.01)	-5.78 (3.67)	0.124
LDT (digits)	15.3 (0.59)	-1.44 (0.66)*	-0.23 (0.80)	0.576
WLTI (pictures)	22.6 (0.56)	0.52 (0.62)	1.47 (0.76)	0.056
WLTD (pictures)	7.95 (0.27)	0.26 (0.29)	0.39 (0.36)	0.254
GDS-15 (points)	2.60 (0.24)	-0.48 (0.26)	-0.69 (0.33)*	0.026*
Haplotype 2 (BAt)				
Composite score	0.05 (0.07)	-0.13 (0.08)	-0.29 (0.10)*	0.004*
MMSE (points)	22.0 (0.53)	0.57 (0.60)	-0.29 (0.77)	0.912
Stroop Test (seconds)	79.3 (2.70)	2.12 (2.92)	10.7 (3.77)*	0.008*
LDT (digits)	15.2 (0.59)	-1.01 (0.64)	0.63 (0.83)	0.298
WLTI (pictures)	24.1 (0.55)	-1.15 (0.60)	-2.50 (0.77)*	0.001*
WLTD (pictures)	8.51 (0.26)	-0.37 (0.28)	-0.98 (0.37)*	0.009*
GDS-15 (points)	2.14 (0.24)	-0.03 (0.26)	0.56 (0.33)	0.147
Haplotype 3 (bAT)				
Composite score	-0.10 (0.05)	0.12 (0.09)	0.56 (0.28)*	0.032*
MMSE (points)	22.0 (0.40)	0.76 (0.68)	2.77 (2.25)	0.124
Stroop Test (seconds)	83.3 (2.05)	-3.17 (3.30)	-13.3 (10.5)	0.153
LDT (digits)	14.2 (0.44)	1.25 (0.72)	3.74 (2.29)	0.024*
WLTI (pictures)	22.9 (0.42)	0.76 (0.68)	3.84 (2.20)	0.074
WLTD (pictures)	8.05 (0.20)	0.32 (0.32)	2.13 (1.04)*	0.067
GDS-15 (points)	2.14 (0.18)	0.39 (0.30)	0.48 (0.94)	0.171

* $p < 0.05$; SE – standard error; MMSE - Mini-Mental State Examination; LDT - Letter Digit Coding Test; WLTI - Word Learning Test Immediate Recall; WLTD - Word Learning Test Delayed Recall; GDS-15 - 15-item Geriatric Depression Scale

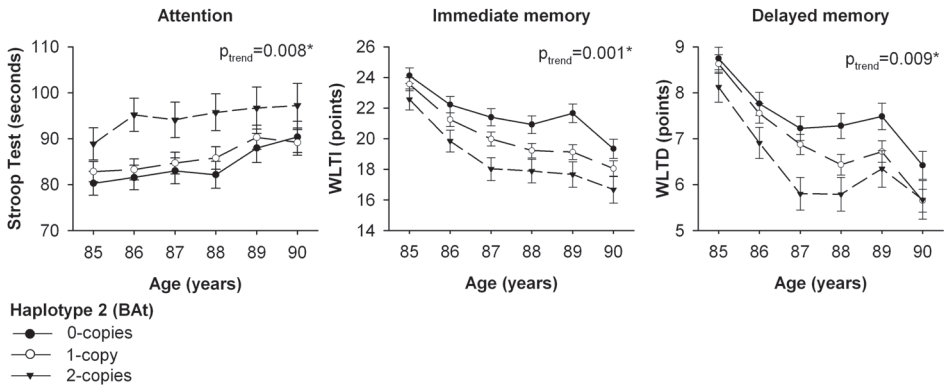

Figure 2. Differences in cognitive functioning between participants carrying 0-, 1- or 2-copies of the haplotype 2 (BAt). The p-value represents the overall mean difference in cognitive functioning during follow-up. * $p < 0.05$

In order to explore whether differences in calcium levels or selective survival have influenced the associations observed with cognitive functioning, we analyzed the relation between these phenotypes and *VDR* gene variants. In cross-sectional analyses at age 85 years, serum calcium levels were not associated with the *VDR* polymorphisms (data not shown), except the *Cdx-2* polymorphism. Homozygous (-0.05 mmol/l, 95 % CI: -0.10 to -0.00, *p=0.032*) but not heterozygous (0.001 mmol/l, 95 % CI: -0.02 to 0.02, *p=0.955*) *Cdx-2* variant allele carriers had lower serum calcium levels compared to non-carriers. No associations between the *VDR* haplotypes and calcium levels were observed. Likewise, the mortality risks did not differ between the different *VDR* polymorphism and haplotype carriers during mean 4.2-year follow-up period (data not shown).

Since in other studies the same polymorphisms and haplotypes as analyzed in this study have been associated with the risk of fractures, we assessed the influence of *VDR* polymorphisms and haplotypes on the incidence of fractures during follow-up. There were no differences in the incidence of fractures between the different polymorphism carriers (data not shown). However, the additional haplotype analyses revealed a trend for increased incidence of fractures for haplotype 1 (baT) carriers (1-copy HR: 1.46, 95 % CI: 0.86 to 2.49; 2-copies HR: 1.52, 95 % CI: 0.81 to 2.83).

Discussion

The main finding of this study is that genetic variance in the *VDR* gene contributes to differences in cognitive functioning and depressive symptoms in old age. An overall better performance on tests measuring attention, processing speed and memory, together with a lower prevalence of depressive symptoms were observed for carriers of *ApaI* variant-allele and of haplotype 1 (baT), which contains the *ApaI* variant-allele. In contrast, carriers of the *BsmI* and *TaqI* polymorphisms had impairments in attention and memory. Similar associations were observed

with haplotype 2 (BAt), which combines the variant alleles of *BsmI* and *TaqI*.

The research on the role of vitamin D in the human brain has so far focused mainly on the influence of vitamin D on mood disorders. Vitamin D deficiency is considered as a possible contributor to seasonal affective disorder (SAD), since SAD has been associated with winter months and sunlight deprivation (Rosenthal et al., 1984; Schlager et al., 1993; Spoont et al., 1991). In addition, there are studies showing associations between low vitamin D levels and mood disorders, accompanied with worse cognitive functioning (Lansdowne and Provost, 1998; Przybelski and Binkley, 2007; Wilkins et al., 2006). In accordance with the latter studies, we observed in this study that genetic variance in the *VDR* gene influences both cognitive functioning and depressive symptoms. From the specific domains of cognitive functioning, attention and memory were affected most. These two cognitive domains are most vulnerable and tend to decline constantly across adult lifespan, in contrast to cognitive abilities such as autobiographical memory and emotional processing, which stay unchanged throughout life (Hedden and Gabrieli, 2004). Our data suggest that carriers of the *BsmI* and *TaqI* polymorphisms are more susceptible for the age-related deterioration of cognitive functioning, whereas the *ApaI* polymorphism contributes to a protective effect.

In the assessment of overall influence of the *VDR* gene polymorphisms on cognitive functioning, we observed differences with composite cognitive score but not with MMSE. The composite cognitive score used in this study was calculated from four individual tests that have been designed to measure changes in specific domains of cognitive functioning. Therefore, the composite cognitive score might be more sensitive in detecting cognitive impairments than MMSE, which has been designed to assess global cognitive functioning, and contains only few items from the specific cognitive tests. It also might be that due to the 'ceiling' effect of the MMSE, mild impairments in cognitive functioning are not detectable (Houx et al., 2002).

There are several mechanisms through which vitamin D can affect mental performance. The downregulation of the expression of L-type voltage-sensitive calcium-channels (L-VSCC) by vitamin D in hippocampal neurons, has been shown to reduce the influx and excitotoxic effects of calcium to neurons (Brewer et al., 2001). The detrimental role of excessive calcium for memory formation and overall cognitive functioning is widely acknowledged (Sattler and Tymianski, 2000; Thibault et al., 2001; Veng et al., 2003). However, the differences in cognitive functioning between the *VDR* polymorphism and haplotype carriers were unlikely caused by increased or decreased calcium levels, since none of these polymorphisms and haplotypes were associated with calcium levels. However, it is unknown how well the peripheral calcium levels reflect those in the brain. The vitamin D endocrine system plays an essential role in overall calcium homeostasis and therefore we expected to see differences also in peripheral calcium levels within the polymorphism and haplotype carriers. Possibly, other brain specific functions of vitamin D are responsible for the observed effects. It has been shown that in the brain, vitamin D increases the production of neurotrophins, which support the survival of existing neurons and encourage the growth and differentiation of new neurons and synapses (Naveilhan et al., 1996; Neveu et al., 1994a; Neveu et al., 1994b; Saporito et al., 1994; Wang et al., 2000). These effects provide protection to, and diminish cognitive impairment underlying neurodegenerative disorders.

In previous studies, several polymorphisms and haplotypes in the *VDR* gene have been associated with bone mineral density and risk of fractures (Fang et al., 2005; Uitterlinden et al., 2004b). The risk haplotypes that have been identified include haplotype 1 (baT) and haplotype

2 (BAt), which were reported to contribute to increased and decreased risk of fractures, respectively (Fang et al., 2005; Uitterlinden et al., 2004b). In this study, we analyzed the same risk haplotypes, and observed a trend for increased risk of fractures for haplotype 1 (baT) carriers, which is in accordance with the other studies. However, for the same haplotype carriers we also observed better performance on test measuring cognitive functioning. An explanation for the apparent contradiction could be that people with better cognitive functioning are more active and therefore may face a higher risk for an incident fall and fracture. The lack of associations between the *VDR* polymorphisms and haplotypes and mortality suggest that selective survival has not influenced the associations observed with cognitive functioning. We speculate that the better cognitive functioning in the haplotype 1 (baT) carriers is due to a higher expression of the haplotype, since increased vitamin D levels have previously been associated with better cognitive functioning. The evidence for the functionality of haplotype 1 (baT), however, is contradictory. In one study it was reported that the haplotype 1 (baT) results in lower *VDR* gene expression and increased mRNA decay (Fang et al., 2005), whereas in another study it was shown that the haplotype 1 (baT) is overexpressed in human trabecular bone samples (Grundberg et al., 2007). It might also be that the associations observed in this study are resulted by other polymorphisms that are in LD with those analyzed in this study. Recently, several new polymorphisms in the *VDR* gene have been identified together with a complete description of the LD and haplo-block structure (Fang et al., 2005).

The strengths of the present study include the population-based sample of the oldest old with a high incidence of depression and cognitive decline, and the annual repeated assessment of depressive symptoms and the functioning of various cognitive domains. The limitations of the study include the lack of vitamin D levels, and information on environmental factors, such as (dietary) calcium and vitamin D intake, which could have influenced the associations. Another limitation is the ascertainment of incident fractures through self-reporting by a questionnaire. This might have led to ascertainment errors, but random errors would only underestimate associations. To our knowledge, this is the first and only report of a relationship between genetic variance in the *VDR* gene and cognitive functioning and depressive symptoms, and therefore until further replication, the possibility of a chance finding cannot be excluded.

In conclusion, our results show that genetic variance in the *VDR* gene influences cognitive functioning and the prevalence of depressive symptoms in old age.

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CHAPTER 5

SIRT1 Gene, Age-Related Diseases and Mortality. The Leiden 85-Plus Study

Maris Kuningas, Mariëlle Putters, Rudi G. J. Westendorp, P. Eline Slagboom and Diana van Heemst

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Abstract

The *Sir2* gene has been shown to regulate the lifespan of several model organisms. In mammals, the evolutionarily conserved homologue SIRT1 regulates neuroprotection, metabolism and cell survival in response to stress. Based on these data, we hypothesized that SIRT1 might influence the prevalence of age-related diseases and modify the lifespan of humans. In order to test this, we genotyped five single nucleotide polymorphisms (SNPs) in 1245 participants of the population-based Leiden 85-plus Study. *SIRT1* haplotypes were assessed and tested for association with the risks of mortality, metabolic profile, age-related diseases and cognitive functioning. These analyses revealed a trend for lower cardiovascular mortality for haplotype 2 and rs3758391 SNP carriers. In further analyses, this trend was not supported by intermediate phenotypes, albeit the rs3758391 T-allele carriers had better cognitive functioning. In conclusion, our results indicate a role for *SIRT1* in cognitive functioning, but the role in lifespan remains to be elucidated.

Introduction

Increased expression of Sir2 (Silent Information Regulator 2) either due to an extra copy of the gene or to caloric restriction, prolongs lifespan in various model organisms (Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001; Wood et al., 2004). In mammals, there are seven Sir2 homologues, of which SIRT1 (Sirtuin 1) is the most similar to Sir2 (Frye, 1999; Frye, 2000). In response to environmental signals, SIRT1 regulates metabolism and cell survival in various types of mammalian cells (Bordone and Guarente, 2005; Cohen et al., 2004; Yang et al., 2006). To date, it is unknown whether SIRT1 influences the prevalence of age-related diseases and modifies the lifespan of humans.

SIRT1 is a NAD+-dependent (nicotinamide adenine dinucleotide) protein deacetylase (Imai et al., 2000) and it regulates metabolism and cell survival through influencing gene silencing, and the activity of various transcription factors and co-regulators (Bordone and Guarente, 2005; Vaquero et al., 2004). It has been shown that activation of SIRT1 increases glucose tolerance and enhances insulin response to glucose in pancreatic β-cells (Bordone et al., 2006; Motta et al., 2004; Moynihan et al., 2005). Increased SIRT1 activity also promotes hepatic gluconeogenesis and inhibits glycolysis via *PGC1-a* during fasting (Rodgers et al., 2005). Furthermore, SIRT1 has an effect on fat metabolism, via inhibition of *PPARγ* (Picard et al., 2004). These findings suggest that increased SIRT1 activity results in a favorable metabolic profile, with decreased prevalence of diabetes and cardiovascular diseases. In addition, the role of SIRT1 in providing resistance to damage- or stress-induced apoptosis, may help to preserve organ function over time, although by doing so it may promote cancer (Hekimi and Guarente, 2003; Lim, 2006). Recent evidence also suggests a role for SIRT1 in neuroprotection and neurodegenerative disorders (Araki et al., 2004; Parker et al., 2005; Qin et al., 2006). Altogether, SIRT1 could influence lifespan through several ways. The involvement of *SIRT1* in human lifespan has been previously studied in a case-control study of elderly and young (Flachsbart et al., 2006). No differences in *SIRT1* allele and haplotype frequencies were observed between these groups. This, however, does not exclude the possibility that SIRT1 gene has an influence on human physiology and lifespan.

The aim of this study was to analyze the association between genetic variation in the *SIRT1* gene, and all-cause and cause-specific mortalities. In addition, metabolic profile, prevalence of age-related diseases, and cognitive functioning were tested in the participants of the prospective population-based Leiden 85-plus Study.

Participants and methods

Participants

The Leiden 85-plus Study is a prospective population-based study, in which all 85-year-old or older inhabitants of the city Leiden, The Netherlands, were invited to take part. The study design and data collection have been described elsewhere (der Wiel et al., 2002; Weverling-Rijnsburger et al., 1997). The study population consists of two cohorts, cohort '87 and '97, and all the study participants are of Caucasian origin. Cohort '87 includes 977 participants aged 85 or older,

enrolled between 1987 and 1989 (Weverling-Rijnsburger et al., 1997). Cohort '97 consists of 599 subjects, all members of the 1912-1914 birth cohorts, who were enrolled in the month of their 85th birthday between 1997 and 1999 (der Wiel et al., 2002). DNA was available for 682 participants from cohort '87 and for 563 participants from cohort '97. All participants were followed for mortality until August 1, 2005, with a mean follow-up period of 4.4 years. Primary causes of death were obtained from the Dutch Central Bureau of Statistics and categorized according to the 10th International Classification of Diseases (ICD-10). The Medical Ethical Committee of the Leiden University Medical Center approved the study and informed consent was obtained from all participants.

Metabolic profile in cohort '97

HbA1c (hemoglobin A1c), triglycerides, C-reactive protein (CRP) and high-density lipoprotein (HDL)-cholesterol concentrations were determined in serum using fully automated analyzers (Hitachi 747 and 911; Hitachi Ltd, Tokyo, Japan). Low-density lipoprotein (LDL)-cholesterol was estimated with the Friedewald equation (Friedewald et al., 1972).

Cardiovascular pathologies and diabetes in cohort '97

The prevalence and number of cardiovascular pathologies and diabetes were obtained from the participants' general practitioner or nursing home physician. For cardiovascular pathologies, also electrocardiograms were recorded (Macfarlane and Latif, 1996). Cardiovascular pathologies were classified as myocardial infarction, myocardial ischemia, stroke, arterial surgery and intermittent claudication (van Exel et al., 2002). Subjects were classified as having diabetes when they met at least one of the following criteria: 1) history of diabetes obtained from the general practitioner or the subject's treating physician; 2) use of sulfonylurea, biguanide, or insulin, based on information obtained from the subject's pharmacist; or 3) non-fasting glucose of ≥11.1 mmol/l.

Cognitive function and depressive symptoms in cohort '97

Overall cognitive functioning was measured with Mini-Mental State Examination (MMSE) (Folstein et al., 1975). From specific domains of cognitive functioning attention was assessed with Stroop Test (Klein et al., 1997), processing speed with Letter Digit Coding Test (LDT) (Houx et al., 2002) and memory with 12-Word Learning Test, which assesses immediate- (WLTI) and delayed recall (WLTD) (Brand and Jolles, 1985). The prevalence of depressive symptoms was assessed with 15-item Geriatric Depression Scale (GDS-15) (de Craen et al., 2003). The tests assessing specific domains of cognitive functioning could not be administered to 92 participants because of severe cognitive impairment (MMSE score < 19 points). All participants were visited annually for re-measurement of cognitive functioning and depressive symptoms during a mean follow-up period of 4.2 years. In addition to the specific tests, a composite cognitive score was calculated by converting the scores of the individual tests (Stroop Test, LDT, WLTI and WLTD) into a z-score ((individual level – mean level)/SD), and computing the average.

SNP selection and genotyping

The single nucleotide polymorphisms (SNPs) were selected from the public database of National Center for Biotechnology Information (NCBI; *http://www.ncbi.nlm.nih.gov*) to cover the *SIRT1* gene region (GeneID: 23411) in equally spaced intervals. The minor allele frequencies (MAF) of the polymorphisms had to be > 5 %. The selected SNPs were rs3758391 (promoter), rs3740051 (promoter), rs2236319 (intron), rs2273773 (exon) and rs3818291 (intron). All these SNPs were genotyped using the MassArray platform, according to the protocols of the manufacturer (Sequenom Inc., San Diego, CA, USA).

Statistical analysis

The program Haploview (Barrett et al., 2005) was used to estimate the SNPs' allele frequencies, test the genotypes for Hardy-Weinberg equilibrium, and estimate pair-wise linkage disequilibrium (LD) between the polymorphisms. Haplotypes and haplotype frequencies were calculated using SNPHAP software (*http://www-gene.cimr.cam.ac.uk/clayton/software*). The posterior probabilities of pairs of haplotypes per subject, as estimated by the SNPHAP, were used as weights in all analyses. The haplotype analyses approach used in this study assumes an additive effect of the haplotypes, and details of this approach have been described elsewhere (Wallenstein et al., 1998). CRP, triglyceride and HbA1c levels were not normally distributed and were ln-transformed. All-cause and cause-specific mortality risks with 95 % confidence intervals (CI) were calculated with Cox proportional hazard model, using left censoring to correct for the delayed entry into the risk set according to age. Associations between haplotypes and metabolic profile were analyzed using general linear model. Differences in the prevalence of cardiovascular pathologies and diabetes between the haplotypes were tested using binary logistic regression. The associations between cognitive functioning, depressive symptoms and haplotypes were tested with linear mixed model. All analyses were sex adjusted, except the analyses of cognitive functioning and depressive symptom, which were additionally adjusted for education. The analyses were performed with STATA version 9.0 (StataCorp LP, Texas, USA) and SAS version 8.2 (SAS Institute Inc., Cary, NC, USA) statistical software.

Table 1. Characteristics of the study subjects

	Leiden 85-plus Study	
	Cohort '87	Cohort '97
Number	682	563
Age (median, IQR)	89 (88-92)	85 (-)
Female (n, %)	491 (72 %)	375 (67 %)
Polymorphisms*		
rs3758391 (C/T)	0.33	0.36
rs3740051 (A/G)	0.07	0.07
rs2236319 (A/G)	0.07	0.07
rs2273773 (T/C)	0.07	0.08
rs3818291 (G/A)	0.13	0.13

IQR – interquartile range; *minor allele frequency

Results

All 1245 participants of the Leiden 85-plus Study were genotyped for the five SIRT1 polymorphisms (Table 1). The genotype frequencies of the SNPs were in Hardy-Weinberg equilibrium, and the allele frequencies were similar between the two elderly cohorts (Table 1). The polymorphisms were in strong linkage disequilibrium (D' 0.97-1.00), and gave rise to five different haplotypes of which four were common (frequencies > 5 %) and cumulatively accounted for 99.9 % of the haplotypes (Figure 1).

All-cause and cause-specific mortality risks were assessed after a mean follow-up period of 4.4 years. During that time 1001 (80 %) of the 1245 participants had died. From these 406 (41%) had died due to cardiovascular disease, 162 (16%) due to cancer, and 431 (43%) due to other causes. The cause of death was unknown for two participants. The mortality risk analyses revealed a trend for lower cardiovascular mortality (HR 0.82, 95 % CI: 0.66 to 1.01, *p=0.062*) for haplotype 2 carriers, compared to the reference haplotype (Figure 2). A similar trend was observed in the two Leiden 85-plus Study cohorts separately (data not shown). For the other haplotypes, no differences in all-cause or cause-specific mortality risks were observed (Figure 2).

In order to study further the role of *SIRT1*, we analyzed the associations between *SIRT1* haplotypes and metabolic profile, prevalence of cardiovascular diseases and diabetes. The data on these endpoints were available for 563 participants of the cohort '97. At baseline, no associations between the *SIRT1* haplotypes and various metabolic profile parameters were observed, except for LDL-cholesterol and haplotype 3. These haplotype carriers had 0.18 mmol/l lower (95 % CI: -0.34 to -0.02, *p=0.030*) LDL levels compared to the reference haplotype. In contrast, none of the *SIRT1* haplotypes were associated with the prevalence of cardiovascular pathologies or diabetes. For haplotype 2 carriers, non-significant trends for lower prevalence of arterial surgery (OR 0.82, 95 % CI: 0.41-1.65, *p=0.574*) and intermittent claudication (OR 0.76, 95 % CI: 0.36-1.60, *p=0.471*) were observed (Table 2).

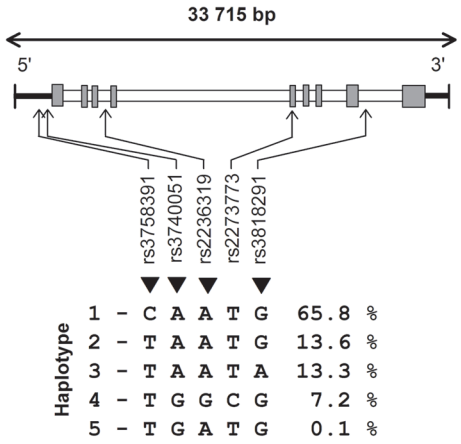

Figure 1. Structure and haplotypes of the *SIRT1* gene. The *SIRT1* gene is located in chromosome 10 and the coding region spans 33 715 bp. It contains nine exons, which are represented by gray boxes in the figure. The approximate positions of the SNPs are indicated with arrows. All SNPs were in strong LD and resulted in five haplotypes, of which four had frequencies > 5 %.

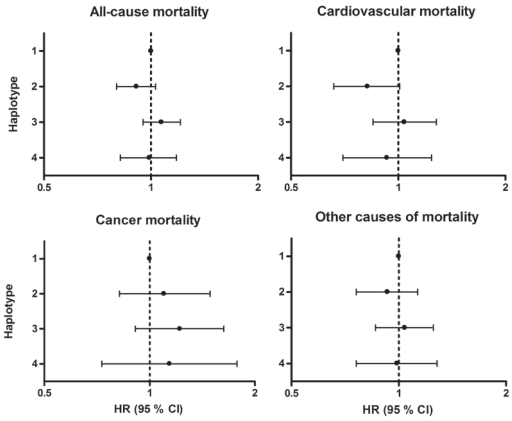

Figure 2. *SIRT1* haplotypes, all-cause and cause-specific mortality risks. Mortality risks were calculated in the combined cohort of the Leiden 85-plus Study (n=1245). All-cause and cause-specific mortality risks are presented as hazard ratios (HR) with 95 % confidence intervals (CI). The most common haplotype 1 was used as a reference group.

Table 2. *SIRT1* haplotypes, prevalence of cardiovascular diseases and diabetes at baseline in the Leiden 85-plus Study cohort '97 (n=563)

	Haplotype 1 OR (95 % CI)	Haplotype 2 OR (95 % CI)	Haplotype 3 OR (95 % CI)	Haplotype 4 OR (95 % CI)
CVD total (n=365)*	1 (ref)	1.17 (0.83-1.64)	1.11 (0.77-1.60)	0.84 (0.53-1.33)
Myocardial infarction (n=137)	1 (ref)	0.99 (0.69-1.44)	0.90 (0.60-1.36)	0.98 (0.58-1.67)
Myocardial ischemia (n=286)	1 (ref)	1.02 (0.74-1.40)	1.08 (0.77-1.51)	0.91 (0.58-1.44)
Stroke (n=57)	1 (ref)	1.07 (0.61-1.88)	1.14 (0.67-1.95)	0.90 (0.37-2.23)
Arterial surgery (n=37)	1 (ref)	0.82 (0.41-1.65)	0.98 (0.48-1.97)	0.75 (0.27-2.08)
Intermittent claudication (n=36)	1 (ref)	0.76 (0.36-1.60)	0.92 (0.43-2.00)	0.78 (0.29-2.09)
Diabetes (n=92)	1 (ref)	1.37 (0.89-2.11)	1.46 (0.93-2.28)	0.79 (0.40-1.55)

CVD – cardiovascular disease; OR - odds ratio; CI – confidence interval; * Participants with one or more cardiovascular pathology

In the cohort '97, cognitive functioning and the prevalence of depressive symptoms were assessed at baseline, age 85 years, and re-examined annually during a mean follow-up period of 4.2 years. Compared to the reference haplotype, there were no differences in cognitive functioning or in prevalence of depressive symptoms between the *SIRT1* haplotypes (data not shown).

In addition to haplotype analyses, univariate analyses with the individual polymorphisms were performed. From the five polymorphisms, associations with only one (rs3758391), which also resides in the haplotype 2, were observed. Heterozygous (HR 0.77, 95 % CI: 0.62 to 0.96, *p=0.018*) but not homozygous (HR 1.01, 95 % CI: 0.73 to 1.39, *p=0.965*) carriers of rs3758391 T-allele, had lower cardiovascular mortality risks. These differences were not attributable to changes in metabolic profile or in prevalence of age-related diseases (data not shown). In contrast, homozygous but not heterozygous carriers of the rs3758391 T-allele performed better on all tests measuring cognitive functioning (Table 3). These differences were most pronounced for immediate memory (2.26 points, 95 % CI: 0.62 to 3.89, *p=0.007*) and for delayed memory (1.06 points, 95 % CI: 0.29 to 1.84, *p=0.007*) (Table 3).

Table 3. *SIRT1* rs3758391 SNP, cognitive functioning and depressive symptoms during mean follow-up period of 4.2 years in the Leiden 85-plus Study cohort '97 (n=563).

	rs3758391			
	CC	CT	TT	
	Mean (SE)	Difference (SE)	Difference (SE)	p-trend
Composite cognitive score	-0.23 (0.06)	0.02 (0.08)	0.28 (0.11)*	0.031*
Global cognitive function (points)	22.7 (0.43)	0.24 (0.59)	0.63 (0.83)	0.443
Attention (seconds)	87.0 (2.12)	1.67 (2.90)	-2.28 (4.10)	0.823
Processing speed (digits)	15.7 (0.46)	-0.11 (0.63)	0.97 (0.88)	0.414
Immediate memory (pictures)	20.1 (0.43)	-0.10 (0.59)	2.26 (0.83)*	0.035*
Delayed memory (pictures)	6.86 (0.20)	0.02 (0.28)	1.06 (0.39)*	0.029*
Depressive symptoms (points)	2.97 (0.18)	0.18 (0.25)	-0.17 (0.36)	0.912

SE – standard error; * p-value < 0.05

Discussion

In this study, we tested the role of SIRT1 in age-related diseases, cognitive functioning and mortality in humans. The analyses of *SIRT1* haplotypes revealed a trend for decreased cardiovascular mortality for haplotype 2 carriers, and for the rs3758391 SNP carriers, which resides in the haplotype 2. None of these, however, were associated with metabolic profile or cardiovascular pathologies. In contrast, carriers of the rs3758391 polymorphism performed better on tests measuring cognitive functioning.

A specific role of SIRT1 in cell survival and in development of cancer has been proposed (Alcendor et al., 2004; Giannakou and Partridge, 2004; Luo et al., 2001). In this study, we found no associations between *SIRT1* haplotypes and cancer mortality, but we observed a trend for lower cardiovascular mortality for haplotype 2 carriers. This trend was observed in the combined, but also in the separate cohorts. Altogether, these observations are in accordance with the results from cell culture studies, where a protective effect of SIRT1 on cardiac myocytes has been demonstrated (Alcendor et al., 2004; Pillai et al., 2005). In addition, SIRT1 appears to be important for the development of heart, since *Sirt1* knock-out mice presented cardiac abnormalities (Cheng et al., 2003; McBurney et al., 2003). Based on these data, the lower cardiovascular mortality in the haplotype 2 carriers in our study population is in line with the expected functions of SIRT1. This implies that these *SIRT1* haplotype carriers might suffer less from cardiovascular diseases. In order to test that, we analyzed the prevalence of various cardiovascular pathologies dependent on *SIRT1* haplotypes. However, no associations were found, and also the parameters of metabolic profile, which underlie atherosclerosis, did not differ. For the latter, a beneficial profile was expected for the *SIRT1* haplotype 2 carriers. The lack of a consistent risk profile suggests that the association between the lower cardiovascular mortality and the *SIRT1* haplotype 2 could have arisen either due to other mechanisms or due to chance. It might also be that the beneficial effects of SIRT1 only appear in acute disease states, thereby decreasing the severity of outcomes from crisis events. This mode of action is consistent with the effects of SIRT1 on apoptosis.

Besides mortality and various intermediate phenotypes, we tested the role of SIRT1 in cognitive functioning and in prevalence of depressive symptoms. The involvement of SIRT1 in neurophysiological functioning has been discovered recently. Several studies have linked the SIRT1 protein and its biological activator, resveratrol, to axonal protection and survival of neurons (Araki et al., 2004; Parker et al., 2005; Qin et al., 2006). Axonal degeneration often precedes the death of neuronal cell bodies in neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Raff et al., 2002). As a result, impairments in cognitive functioning occur. In this study, we found no associations between *SIRT1* haplotypes and cognitive functioning and depressive symptoms. In contrast, a promoter polymorphism (rs3758391), which is the only variant allele in the haplotype 2, was associated with better cognitive functioning. From the specific domains of cognitive functioning, memory was the best preserved. These data, together with the evidence from recent literature, support a possible role of SIRT1 in the brain.

Our results are partly in accordance with a recent case-control study, where also no associations between *SIRT1* polymorphisms/haplotypes and lifespan were found (Flachsbart et al., 2006). In both studies, five polymorphisms from the *SIRT1* gene were analyzed, although only two were the same between the studies. However, besides analyzing individual polymorphisms we calculated haplotypes and tested their association with various phenotypes. The *SIRT1* gene is embedded in a region of strong LD (Supplementary Figure 1) and a haplotype-based approach enables to capture the majority of the genetic variation in the *SIRT1* gene. Since no associations with intermediate phenotypes were observed, we reason that the association between the rs3758391 SNP and cognitive functioning has arisen due to polymorphisms in LD with those analyzed in this study. These SNPs could reside in neighboring genes (DNAJC12 in 5' and HERC4 in 3') or in the regulatory regions of the *SIRT1* gene. We speculate that functional variability in the SIRT1 gene itself is constrained because it plays diverse but essential roles in human physiology.

In mammals, the *Sir2* gene has several homologues (Michishita et al., 2005) and perhaps one or more of the other SIRT family members (SIRT1-7) have bigger influences on lifespan. From this point of view, *SIRT3* gene which encodes a mitochondrial protein, has been implicated to play a role in human longevity (Bellizzi et al., 2005; Rose et al., 2003). In addition, recent evidence suggests a similar role for SIRT6, since *Sirt6*-deficient mice displayed genomic instability and premature aging-like phenotype (Mostoslavsky et al., 2006). Therefore, the analyses of other SIRT family members might shed more light into the regulation of human lifespan.

The strengths of the study include the possibility to estimate several phenotypes in one cohort, and the prospective analyses with high number of deaths during follow-up. A limitation of the study is the lack of data on the activity or levels of SIRT1, which would reflect the functionality of the polymorphisms analyzed. In addition, considering the number of tests performed, adjustment for multiple testing would eliminate all the statistically significant associations observed. Consequently, the results of this study are not exhaustive.

In conclusion, the results of this study provide evidence for a role of SIRT1 in cognitive functioning, but due to the lack of associations with intermediate phenotypes, the influence of genetic variation in the *SIRT1* gene on lifespan remains to be elucidated.

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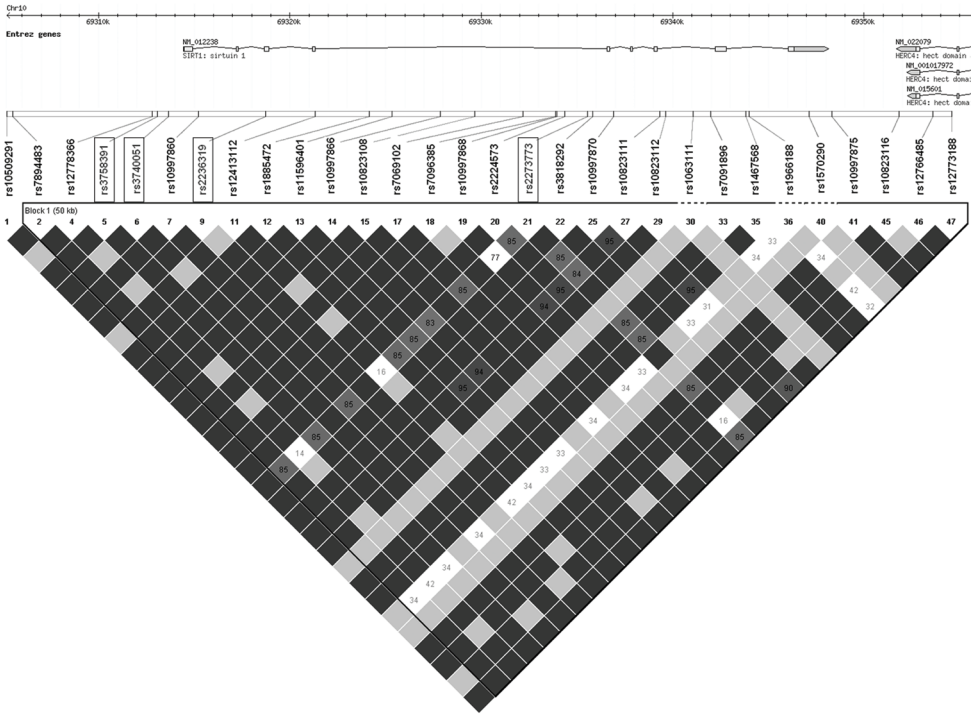
Supplementary materials



Supplementary Figure 1. Overview of the physical and genetic structure of the *SIRT1* gene region as generated by Haploview from the Caucasian HapMap data (release nr. 21a). The polymorphisms genotyped by the HapMap are shown in the top panel. The SNPs marked with boxes were analyzed in this study. The lower panel gives an overview of the linkage disequilibrium structure of the locus (D')

CHAPTER 6

Mental Performance in Old Age Dependent on Cortisol and Genetic Variance in the Mineralocorticoid- and Glucocorticoid Receptors

Maris Kuningas, Roel H. de Rijk, Rudi G. J. Westendorp, Jelle Jolles, P. Eline Slagboom and Diana van Heemst

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Abstract

Depression and cognitive decline have been associated with changes in circulating cortisol concentrations. Cortisol exerts its functions through mineralocorticoid (MR) and glucocorticoid (GR) receptors. However, data on the influence of variations in the *MR* and *GR* genes on depressive symptoms and cognitive functioning in older adults are scarce. Therefore, we explored the impact of MR-215G/C, MR-I180V, GR-ER22/23EK, GR-N363S and GR-*BclI* polymorphisms on these endpoints in the population-based Leiden 85-plus Study. This prospective study includes 563 participants aged 85 years and older, with a mean follow-up of 4.2 years. In this study, high morning cortisol levels (per 1 SD cortisol) associated with impairments in global cognitive functioning ($p=0.002$) at baseline (age 85). These impairments were mainly attributable to lower attention ($p=0.057$) and slower processing speed ($p=0.014$). Similar effects were also observed during follow-up (age 85-90), where participants with higher cortisol levels (per 1 SD cortisol) had impaired global cognitive functioning ($p=0.003$), as wells as impairments in attention ($p=0.034$) and processing speed ($p=0.013$). Changes in depressive symptoms were observed for the MR-I180V single nucleotide polymorphism (SNP), where during follow-up the prevalence of depressive symptoms was higher in the 180V-allele carriers ($p=0.049$) compared to non-carriers. Dependent on these polymorphisms, no differences in overall and in specific domains of cognitive functioning were observed. In conclusion, the MR-I180V SNP has a specific effect on depressive symptoms, independent from cognitive functioning and other polymorphisms in the *MR* and *GR* genes. In contrast, these genetic variants in the *MR* and *GR* genes do not influence cognitive functioning in old age.

Introduction

In response to a real or imagined threat the organism reacts by eliciting a stress-response in order to cope with the stressor. In humans, cortisol is the primary active stress hormone that mediates counter-responses to stress, aimed to re-establish homeostasis and coordinate behavioral adaptations (de Kloet et al, 1998, 2005; Munck et al, 1984). Cortisol exerts its effects through mineralocorticoid (MR) (NR3C2) and glucocorticoid (GR) (NR3C1) receptors, which are abundantly co-expressed in the neurons of limbic structures (Herman, 2003). MRs, which have ten times higher affinity for cortisol than GRs, determine the threshold or sensitivity of the stress system and thus the onset of the stress response (de Kloet et al, 2005). GRs, on the other hand, represent the slower mode that promotes the termination of the stress response (de Kloet et al, 2005). Balancing these two systems is essential for cell homeostasis, mental performance and health.

Various studies have shown that stress-responsiveness is highly variable among human subjects, and an inadequate stress-response increases vulnerability for disease. Changes in the stress hormone system have been shown to play a role in the development of depression (Belanoff et al, 2001b; Holsboer, 2000, 2001). In addition, changes in the circulating cortisol concentrations have been associated with impairments in various cognitive domains, including attention, perception and memory (Jameison and Dinan, 2001; Lupien et al, 2005; Newcomer et al, 1999). In terms of memory, excess cortisol levels have been shown to impair mainly declarative and not non-declarative memory (Lupien et al, 2005).

As MRs and GRs mediate the cortisol signal, variations in these genes may introduce changes in cortisol signaling dynamics, and thereby lead to changes in mood and cognitive function. In the *MR* gene, the 215G/C and I180V single nucleotide polymorphisms (SNPs) have been shown to change cortisol signaling in vitro (Arai et al, 2003), whereas in the *GR* gene, the ER22/23EK, N363S and *BclI* SNPs have been shown to change HPA-axis reactivity after a dexamethasone suppression test or a psychosocial stressor (Huizenga et al, 1998; van Rossum et al, 2002, 2003). In addition, the ER22/23EK variant has been associated with major depression in two studies (van Rossum et al, 2006; van West et al, 2006). To date, there are no publications that examine the role of these *MR* and *GR* variants in cognitive functioning and as yet no study has assessed the effects of variants in the *MR* gene on mood. Furthermore, a combined effect of the genetic variants in the *MR* and *GR* genes may have a different effect on depressive symptoms and cognitive functioning, owing to the interplay between these genes.

In this study, we examined the influence of cortisol levels and variants in the *MR* and *GR* genes on overall cognitive functioning, attention, processing speed, immediate and delayed memory, and on depressive symptoms in old age. All analyses were performed in the prospective population-based Leiden 85-plus Study. Since in old age cognitive decline and depressive symptoms are more prevalent, the effects of a lifelong exposure to changes in cortisol signaling owing to polymorphisms should be more easily detectable on these endpoints.

Participants and methods

Participants

The Leiden 85-plus Study is a population based prospective study of inhabitants of the city Leiden, The Netherlands. All 85 year old inhabitants were invited to participate. There were no selection criteria related to health or demographic characteristics. The study population consists of 599 participants, all Caucasians and members of the 1912-1914 birth cohorts, who were enrolled in the month of their 85th birthday between 1997 and 1999 (Bootsma-van der Wiel et al, 2002). For the present study DNA was available for 563 participants. The Medical Ethical Committee of the Leiden University Medical Center approved the study and informed consent was obtained from all participants or their guardian.

Cognitive function and depressive symptoms

Global cognitive functioning was assessed in all participants with the Mini-Mental State Examination (MMSE) (Folstein et al, 1975). Participants with a MMSE score 19 points or higher were subjected to additional tests to measure attention (Stroop Test) (Klein et al, 1997), processing speed (Letter Digit Coding Test, LDT) (Houx et al, 2002), immediate recall memory (Word Learning Test Immediate Recall, WLTI), delayed recall memory (Word Learning Test Delayed Recall, WLTD) (Brand and Jolles, 1985) and depressive symptoms (15-item Geriatric Depression Scale, GDS-15) (De Craen et al, 2003). The tests assessing specific domains of cognitive functioning were not administered in subjects with a MMSE score of 18 points or lower, because diminished reliability and validity in subjects with severe cognitive impairment. All participants were visited annually for re-measurement of cognitive functioning during a mean follow-up period of 4.2 years. Parallel versions of the tests were used. Details of cognitive testing are described elsewhere (Houx et al, 2002).

Cortisol measurement at baseline

All blood samples were drawn in the morning before 11.00 AM. The median time between venipuncture and centrifugation in our laboratory was 50 min (interquartile range: 30–70 min). Non-fasting plasma cortisol levels were determined by a fluorescence polarization immunoassay using the Abbott TDx (Abbott Laboratories, Abbott Park, IL, USA) according to the manufacturer's instructions. The within-assay coefficient of variation (CV) was below 5 % at different levels.

Possible confounders

Socio-demographic characteristics, such as sex and level of education were considered as possible confounders. Education was divided into two levels: a lower education level, including individuals without schooling or with only primary school education (less than 6 years of schooling), and those with a higher education level (6 years or more of schooling). Health related

correlates were assessed at baseline (age 85), and included drug use, obtained from the pharmacist's registers, and chronic diseases. Data on the use of oral corticosteroids were available for all participants. Chronic diseases included cardiovascular disease, diabetes mellitus, chronic obstructive pulmonary disease, arthritis, malignancy, dementia and Parkinson's disease. Data were obtained at baseline from a structured questionnaire and based on the diagnoses of the general practitioner.

Genotyping

The GR-ER22/23EK polymorphism consists of two single nucleotide polymorphisms (SNPs) in codons 22 (rs6189) and 23 (rs6190). The MR-I180V (rs5522), GR-ER22/23EK, GR-N363S (rs6195) and GR-BclI (van Rossum et al, 2003) variants were genotyped using an Assay-by-Design (Applied Biosystems, Foster City, CA), consisting of PCR primers and TaqMan MGB probes, on an ABI 7900 HT with real-time PCR (Applied Biosystems). MR-215G/C (rs2070951) was genotyped using the MassArray platform according to the protocols of the manufacturer (Sequenom, San Diego, CA).

Statistical analysis

Allele and genotype frequencies were calculated and analyzed for deviation from Hardy-Weinberg equilibrium using the χ^2-test. Cortisol levels were converted into z-scores ((individual level – mean level)/SD) in order to normalize the data and provide comparable estimates per 1-SD increase in cortisol level. The cross-sectional (age 85) and longitudinal (age 85-90) associations between baseline cortisol levels, cognitive functioning and depressive symptoms were analyzed using a linear regression and a linear mixed model, respectively. Differences in cortisol levels dependent on polymorphisms were assessed with a univariate general linear model. Associations between genotypes, cognitive functioning and depressive symptoms were analyzed using the linear mixed model, estimating the overall mean difference in cognitive functioning or depressive symptoms during follow-up. In multivariate analyses, the other polymorphisms were added to the model as independent covariates. The analyses of cognitive functioning were adjusted for sex and level of education, and the analyses of depressive symptoms were adjusted for sex and presence of chronic diseases. All these analyses were also performed with additional adjustment for cortisol levels or chronic diseases. The analyses were performed with SPSS statistical software, version 12.0 (Chicago, IL, USA).

Results

Baseline characteristics of the participants are shown in Table 1. From the 563 participants, 12 were excluded from the analyses owing to the use of corticosteroids. Global cognitive functioning was assessed in all participants using MMSE. Some 460 (83 %) scored 19 points or more on MMSE, and these subjects performed additional tests, measuring attention, processing speed, immediate and delayed recall memory, and depressive symptoms. All 551 subjects were genotyped for the MR-215G/C, MR-I180V, GR-ER22/23EK, GR-N363S and GR-*BclI* poly-

Table 1. Baseline characteristics of study participants

Number	551
Age[1]	85 (-)
Female (%)	365 (66 %)
Low level of education (%)	356 (65 %)
Chronic diseases	391/461
Cortisol (µmol/l)[1]	0.49 (0.15)
Mini-Mental State Examination (points)[1]	23.9 (6.27)
Mini-Mental State Examination ≥ 19 points (%)	460 (83 %)
Specific domains of cognition[1]	
Stroop Test (seconds)	82.4 (34.3)
Letter Digit Coding Test (digits)	17.1 (7.12)
Word Learning Test Immediate Recall (pictures)	23.9 (6.00)
Word Learning Test Delayed Recall (pictures)	8.65 (2.76)
15-item Geriatric Depression Scale (points)[1]	2.32 (2.44)
Polymorphisms (minor allele frequency)	
MR - 215G/C	0.47
MR - I180V	0.14
GR - ER22/23EK	0.03
GR - N363S	0.05
GR - BclI C/G	0.34

[1] Data presented as means with standard deviations (SD)

morphisms. The overall genotype distributions and resulting allelic frequencies of the variants were in agreement with the distribution predicted by the Hardy-Weinberg equilibrium, and in case of the GR gene similar to the frequencies observed in other studies. This is the first study reporting allele frequencies for the SNPs in the *MR* gene. Because of the low allele frequencies of the SNPs (Table 1), the heterozygous and homozygous minor allele carriers of all the polymorphisms were combined. However, for the MR-215G/C and GR-*BclI* variants, the analyses were also repeated using all genotypes, as these polymorphisms have higher allele frequencies.

Global cognitive functioning, attention, processing speed, memory and depressive symptoms were assessed at baseline and re-examined annually during a mean follow-up period of 4.2 years. During follow-up, there was a significant increase in depressive symptoms and a decline in cognitive functioning in all participants (all $p<0.001$). The influence of plasma cortisol levels on depressive symptoms and cognitive functioning was assessed at baseline (age 85) and also during follow-up (age 85-90). The cross-sectional analysis at age 85 revealed that higher cortisol levels (per 1 SD cortisol) were associated with impairments in overall cognitive performance. This was observed in the whole study population ($p=0.008$), as well as in the restricted sample (MMSE ≥ 19 points) ($p=0.002$) (Table 2). These differences were attributable to lower attention ($p=0.057$) and slower processing speed ($p=0.014$) (Table 2). Similar effects were also observed during follow-up (age 85-90), where participants with higher cortisol levels (per 1 SD cortisol) had impaired global cognitive functioning ($p=0.004$), attention ($p=0.034$) and processing speed ($p=0.013$) (Table 2). The overall difference in cognitive functioning and depressive symptoms

Table 2. Cortisol, cognitive functioning and depressive symptoms at baseline (age 85), and during the annual follow-up (age 85-90)

	Age 85 difference per 1 SD cortisol		Age 85-90 difference per 1 SD cortisol	
	Estimate (SE)	p-value	Estimate (SE)	p-value
All participants (n=551)				
Mini-Mental State Examination (points)	-0.67 (0.25)	0.008	-0.78 (0.27)	0.004
Restricted sample (n=460)[1]				
Mini-Mental State Examination (points)	-0.40 (0.13)	0.002	-0.37 (0.12)	0.003
Specific domains of cognitive function				
Stroop Test (seconds)	3.15 (1.65)	0.057	2.86 (1.34)	0.034
Letter Digit Coding Test (digits)	-0.79 (0.32)	0.014	-0.74 (0.30)	0.013
Word learning Test Immediate Recall (pictures)	-0.39 (0.29)	0.187	-0.38 (0.28)	0.186
Word learning Test Delayed Recall (pictures)	-0.21 (0.14)	0.129	-0.19 (0.13)	0.160
15-item Geriatric Depression Scale (points)	0.18 (0.12)	0.146	0.10 (0.13)	0.424

[1] Restricted to participants who scored on MMSE ≥ 19 points

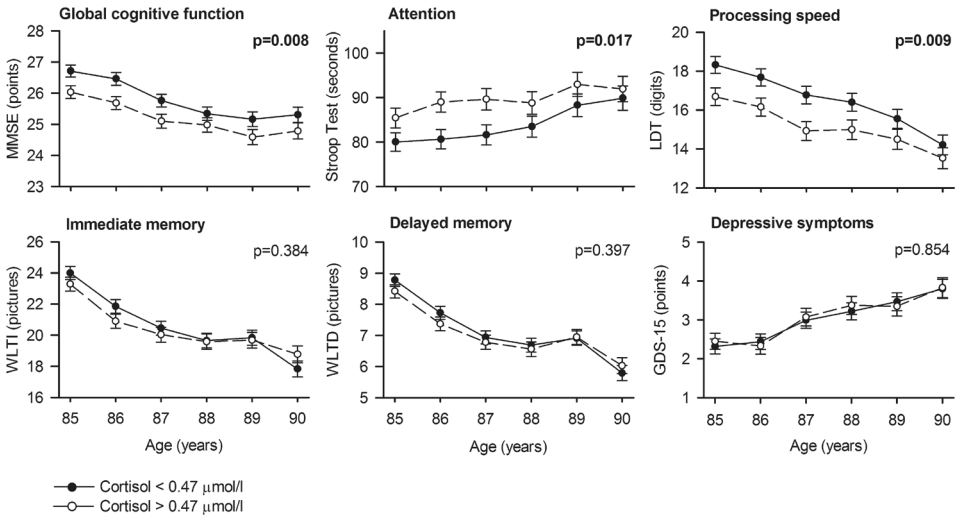

Figure 1. Cognitive functioning and depressive symptoms during the mean 4.2 year follow-up period in participants with low (<0.47 µmol/l) and high (>0.47 µmol/l) cortisol levels. The p-values represent the overall mean difference in cognitive functioning and mood during the follow-up between the two groups

Table 3. Cortisol levels (µmol/l) dependent on the MR and GR polymorphisms

	Non-carriers		Carriers		
	n	mean (SE)	n	mean (SE)	p-value
MR - 215G/C	135	0.52 (0.01)	376	0.48 (0.01)	0.008
MR - I180V	394	0.49 (0.01)	145	0.48 (0.01)	0.423
GR - ER22/23EK	504	0.49 (0.01)	33	0.49 (0.03)	0.895
GR - N363S	494	0.49 (0.01)	50	0.50 (0.02)	0.491
GR - *BclI* C/G	234	0.47 (0.01)	288	0.50 (0.01)	0.084

during follow-up dependent on cortisol levels (two groups based on median cortisol level 0.47 µmol/l) is shown in Figure 1.

The influence of *MR* and *GR* gene polymorphisms on plasma cortisol levels is shown in Table 3. Plasma cortisol levels were associated with the 215G/C variant in the *MR* gene, where the C-allele carriers had lower cortisol levels than non-carriers (*p=0.008*) (Table 3). In addition, for the GR-*BclI* C/G SNP, a trend for an increase in cortisol levels in G-allele carriers was observed (*p=0.084*) (Table 3).

Cognitive functioning and the prevalence of depressive symptoms were also analyzed dependent on the *MR* and *GR* gene polymorphisms. Differences in depressive symptoms were observed for the MR-I180V SNP, where carriers of the 180V-allele had more depressive symptoms than non-carriers (*p=0.049*) (Table 4, Figure 2). This difference increased after adjusting for cortisol levels (0.54 points, SE (0.27), and *p=0.043*). On the other hand, the analyses with cognitive functioning dependent on the polymorphisms revealed no differences, either in overall (Table 4) or in specific domains of cognitive functioning (data not shown). The results remained unaltered after the analyses with a multivariate model, or adjustment for depressive symptoms and cortisol concentrations (data not shown). The analyses using all the MR-215G/C and GR-*BclI* genotypes did not provide any extra information.

All the cognitive functioning analyses were repeated with additional adjustment for chronic diseases, as participants with higher cortisol levels had a higher prevalence of chronic diseases at baseline. This adjustment only marginally changed the results (data not shown).

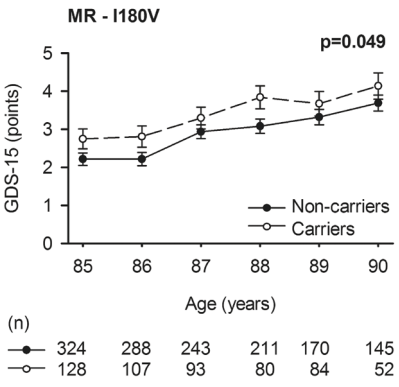

Figure 2. Prevalence of depressive symptoms in non-carriers and carriers of the MR-I180V polymorphism during the mean 4.2-year follow-up period. P-value represents the overall mean difference in the 15-item Geriatric Depression Scale (GDS-15) scores during follow-up between the two groups. (n) represents the number of participant

Table 4. Cognitive functioning and depressive symptoms during the mean 4.2 year follow-up period dependent on the *MR* and *GR* polymorphisms

	Non-carriers mean (SE)	Carriers mean (SE)	p-value
Mini-Mental State Examination (points)[1]			
MR - 215G/C	25.2 (0.25)	25.6 (0.15)	0.179
MR - I180V	25.5 (0.15)	25.5 (0.23)	0.926
GR - ER22/23EK	25.5 (0.14)	25.8 (0.48)	0.458
GR - N363S	25.5 (0.13)	25.3 (0.43)	0.776
GR - BclI C/G	25.5 (0.19)	25.4 (0.17)	0.809
15-item Geriatric Depression Scale (points)[1]			
MR - 215G/C	3.12 (0.24)	2.98 (0.15)	0.594
MR - I180V	2.90 (0.15)	3.42 (0.23)	0.049
GR - ER22/23EK	3.02 (0.13)	3.53 (0.48)	0.297
GR - N363S	3.04 (0.13)	2.55 (0.41)	0.248
GR - BclI C/G	3.26 (0.19)	2.89 (0.17)	0.133

[1] Restricted to participants who scored on MMSE ≥ 19 points

Discussion

In this study, we assessed the impact of cortisol levels and of variations in the *MR* and *GR* genes on cognitive function and depressive symptoms. The results revealed associations between high cortisol levels and cognitive impairment, but not with depressive symptoms. In contrast, the prevalence of depressive symptoms was dependent on a variation in the *MR* gene, where carriers of the 180V-allele had more depressive symptoms compared to the non-carriers. This association remained unchanged after taking into account the influence of the other polymorphisms in the *MR* and *GR* genes. Variations either in the *MR* gene or in the *GR* gene were not associated with cognitive function.

Similar to observations in other studies, we found an association between high cortisol levels and cognitive impairments. These differences were observed at baseline (age 85) and also during follow-up period (age 85-90). Impairments in global cognitive functioning, owing to high cortisol, were observed in the whole study population, and also in the restricted sample (MMSE ≥ 19 points). In the restricted sample, the estimates were without exception smaller however, they had increased significance. This implies that by excluding severely cognitively impaired participants, we lose some power but increase specificity, as these participants may have impaired cognition caused by other factors than high cortisol. From the specific domains of cognitive functioning, attention and speed of information processing were most affected, whereas for memory and mood only a modest influence was observed. Intriguingly, in literature high cortisol levels have been mainly associated with memory impairments and depression (Holsboer, 2000, 2001; Lupien et al, 2005). Most of these studies have been conducted with exogenous administration of glucocorticoids or with depressed patients, with higher cortisol concentrations than observed in this study. Therefore, it might be that cortisol levels higher than the average values in older

adults are necessary to affect memory function and depressive symptoms. However, the lack of associations could also be related to the single cortisol measurement, or to the tests used in this study. It has been shown, that not the acute basal cortisol levels, but rather the change in cortisol levels over time predicts cognitive impairments in aging (Belanoff et al, 2001a). In addition, it has been shown that in some cases, cortisol effects on cognitive performance are very difficult to detect with the use of standardized, less sensitive cognitive tests. Thus, it is plausible that the memory tests used in this study are not sensitive enough to detect cognitive impairments related to cortisol.

MRs have been implicated in the control of HPA-axis activity, as they determine the threshold of stress system's activation (de Kloet et al, 2005). To date, there is no information on how polymorphisms in the *MR* gene influence these functions. In this study, we found that plasma cortisol levels were lower in C-allele carries of the 215G/C SNP in the *MR* gene. As the 215G/C SNP precedes the start codon of the *MR* gene (Arai et al, 2003), it could interfere with the translational start site and thus, lead to a less efficient translation and change in MR-A and MR-B balance (Pascual-Le Tallec et al, 2004). However, the influence of the different *MR* isoforms on cortisol levels is not known. From the other *MR* polymorphisms, we found that the I180V amino acid change in the *MR* gene was associated with higher prevalence of depressive symptoms. It has been reasoned that this SNP changes MR's transactivational properties (Arai et al, 2003), which might change stress responsiveness and lead to increased vulnerability to develop depressive symptoms. On the other hand, carriers of the MR-I180V polymorphism did not display any deficits in overall or in specific domains of cognitive functioning, which suggests that the polymorphism specifically influences depressive symptoms and not cognitive functioning. The mechanisms behind cortisol mediated effects on cognitive functioning and depressive symptoms are most probably different, and modulated by different neuronal pathways and/or contextual conditions (Lupien et al, 2005).

Previously, it has been shown that the *GR* gene polymorphisms alter HPA-axis responsiveness and thereby cortisol sensitivity. The ER22/23EK variation has been associated with resistance to cortisol (van Rossum et al, 2002), whereas the N363S and *BclI* polymorphisms were shown to increase the sensitivity to cortisol (Huizenga et al, 1998; van Rossum et al, 2002, 2003). We reasoned, that the increased resistance to cortisol attained by the ER22/23EK variant would protect against the damaging effects of high cortisol levels on cognitive decline and depression, whereas increased cortisol sensitivity would have opposite effects. In this study, we found no influences of the analyzed polymorphisms on plasma cortisol levels, or on cognitive functioning. For depressive symptoms, only a trend for an influence by the ER22/23EK variant was observed. In contrast, the 22/23EK-allele carriers tended to have more depressive symptoms than non-carriers, without any deficits in cognitive functioning. The lack of a significant difference in depressive symptoms between the ER22/23EK carriers and non-carriers in our study may result from the low number of participants carrying the variant allele. Despite that, the trend observed in our study, is in accordance with two recent studies, showing an enrichment of the 22/23EK-allele in patients with major depression (van Rossum et al, 2006; van West et al, 2006). It could be that in our study the lack of a distinct influence of the *GR* gene SNPs on cognitive functioning and mood is a result of the non-stressful testing conditions. GRs are activated in response to stress and they promote the termination of the stress response and normalization of homeostasis (de Kloet et al, 2005). This suggests that the effects of the polymorphisms are only seen in response to stress or challenge, and that testing in basal conditions does not reveal any

differences in cognitive functioning and depressive symptoms.

The response to stress is modulated by the interplay between MRs and GRs, and a combined effect of genetic variants in these genes may have a different effect on depressive symptoms and cognitive functioning. In this study, we attempted to take into account the influence of genetic variability in both the *MR* and *GR* genes. However, the multivariate analysis did not add any extra information. The cortisol signaling cascade is complex and involves numerous chaperones, accessory proteins, co-regulators and interacting transcription factors that permit differentiation between the MR- and GR-mediated actions (De Bosscher et al, 2003; Pascual-Le Tallec and Lombes, 2005). For instance, recently it was found that increase in recurrence of depressive episodes is associated with a variant in a gene (FKBP5) that regulates GR activity (Binder et al, 2004). Therefore, not only genes directly involved in stress-induced signaling pathways, but genes regulating their activity also appear to be involved in the development of these disorders, and their actions should be analyzed in more detail.

Strengths of the present study include the population-based sample of the oldest old with high incidence of depression and cognitive decline, and the annual repeated assessment of depressive symptoms and various cognitive domains. A major limitation of the study is the one time cortisol measurement. Cortisol is secreted diurnally, and the single measurement of cortisol over an entire day of the circadian cortisol secretion, considerably weakens the value of this measure. Cortisol levels measured only once could be confounded by acute effects, and not reflect the basal concentration. It has been shown, that cortisol is relatively stable from day to day but it is not indisputably stable across the years. Therefore, the results on the predictability of baseline cortisol levels on cognitive decline over the years should be interpreted with care. Another limitation of the study is the lack of data on HPA-axis reactivity, or on other parameters that reflect the stress system's activity.

In conclusion, this is the first study looking into the influence of polymorphisms in the *MR* and *GR* genes on mood and cognitive functioning in older adults. We found an association between high baseline cortisol levels and impairments in cognitive functioning during follow-up. However, no differences in cognitive functioning were observed dependent on the polymorphisms in the *MR* and *GR* genes. On the other hand, the I180V variant in the MR gene influenced the prevalence of depressive symptoms, independently from cognitive functioning.

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CHAPTER 7

Genetic Variants in the Glucocorticoid Receptor Gene (*NR3C1*) and Cardiovascular Disease Risk. The Leiden 85-Plus Study

Maris Kuningas, Simon P. Mooijaart, P. Eline Slagboom, Rudi G. J. Westendorp and Diana van Heemst

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Abstract

Recently, the ER22/23EK, N363S and *BclI* polymorphisms in the glucocorticoid receptor (*GR*) gene have been linked to altered cortisol sensitivity and to cortisol-associated disorders. The aim of this study was to investigate the effect of these genetic variants in the *GR* gene on cardiovascular disease and mortality in elderly persons aged 85 years and over. In the population-based Leiden 85-plus Study, 552 participants were genotyped for the ER22/23EK, N363S and *BclI* polymorphisms, and the effects of the polymorphisms on metabolic profile, body composition, and on the prevalence of cardiovascular pathologies at baseline, were assessed. Allcause and cardiovascular disease mortality risks dependent on the SNPs were calculated after a 4.2-year follow-up. The analyses of metabolic profile revealed that carriers of the ER22/23EK polymorphism have higher HbA1C levels ($p<0.001$) and carriers of the N363S SNP have higher LDL cholesterol ($p<0.001$) and triglyceride concentrations ($p=0.03$), compared to the non-carriers. The only signficant association between genotype and body composition analyses was for height and the ER22/23EK polymorphism. Men carrying the ER22/23EK polymorphism were taller ($p=0.02$) compared to non-carriers. No associations with cardiovascular pathologies, allcause and cardiovascular mortality were observed for any of the polymorphisms. We conclude that, in spite the effect of the ER22/23EK and N363S SNPs on metabolism, these polymorphisms together with the BclI SNP, do not affect the risks of cardiovascular disease and survival at old age.

Introduction

In response to various stimuli, including stress, cortisol coordinates metabolic, endocrine, immune, and nervous system responses (Chrousos, 1995; Chrousos, 1998; de Kloet et al., 1998; McEwen, 1998; Munck et al., 1984). Cortisol exerts the majority of its functions through glucocorticoid receptors (GRs). Disturbances in cortisol signaling, resulting either from altered hormone availability or decreased/increased receptor-mediated signal transduction have been associated with a variety of phenotypes including cardiovascular disease, which is a major cause of death at old age.

It is known that sensitivity to cortisol varies considerably between individuals as demonstrated by the dexamethasone suppression test (Huizenga et al., 1998b). Therefore, the underlying susceptibility to cortisol-associated disorders may vary. A degree of inter-individual variation in responsiveness to cortisol is attributable to three polymorphisms in the *GR* gene (NR3C1). Recently an ER22/23EK variation in the *GR* gene was associated with a resistance to cortisol, and shown to result in a better metabolic and cardiovascular health profile, leading to increased survival rate (van Rossum et al., 2002; van Rossum et al., 2004a; van Rossum et al., 2004b). On the other hand, increased sensitivity to cortisol, related to the N363S (Huizenga et al., 1998a) and *BclI* (van Rossum et al., 2003) polymorphisms, has been shown to cause opposite effects. In middle-aged subjects, the N363S and *BclI* polymorphisms were associated with increased body mass index (BMI) and with several risk factors for atherosclerosis and coronary artery disease (Di Blasio et al., 2003; Lin et al., 2003; van Rossum et al., 2003). Together these results suggest that the *GR* gene plays an important role in modulating the susceptibility to cardiovascular disease. Futhermore, there are disparities in the published literature. For instance in some studies, associations between these three polymorphisms and metabolic parameters or body composition have been found (Di Blasio et al., 2003; Huizenga et al., 1998a; Lin et al., 1999; Lin et al., 2003; van Rossum et al., 2002; van Rossum et al., 2003; van Rossum et al., 2004b), whereas in other studies no association, or opposite effects have been observed (Dobson et al., 2001; Echwald et al., 2001; Panarelli et al., 1998; Rosmond et al., 2001; van Rossum et al., 2002; van Rossum et al., 2004a). However, the recent results with cardiovascular disease (Lin et al., 2003) and survival (van Rossum et al., 2004a) have never been replicated in elderly subjects. Therefore, the effect of the GR gene on cardiovascular disease and survival at old age remains to be elucidated.

In this study, we assessed the impact of the ER22/23EK, N363S and *BclI* variants in the *GR* gene on metabolic profile, body composition, and on the prevalence of cardiovascular pathologies. Also the risks of allcause and cardiovascular mortalities dependent on the polymorphisms were calculated. The study was carried out in the population-based Leiden 85-plus Study, using cross-sectional and prospective study designs.

Participants and methods

Participants

The Leiden 85-plus Study is a prospective population based study, in which all 85 year old in-

habitants of Leiden, The Netherlands were invited to take part. There were no selection criteria related to health or demographic characteristics. The study population consists of 599 subjects, all members of the 1912-1914 birth cohort, enrolled in the month of their 85th birthday between 1997 and 1999 (Bootsma-van der Wiel et al., 2002). For the present study DNA was available for 563 people. All participants of the Leiden 85-plus Study were followed for mortality until April 1, 2004. Primary causes of death were obtained from the Dutch Central Bureau of Statistics and categorized according to the 10th International Classification of Diseases (ICD-10). The Medical Ethical Committee of the Leiden University Medical Centre approved the study and informed consent was obtained from all participants. We also genotyped 370 blood donors (median age 32, inerquartile range (27-36)) from Leiden and surrounding areas for a cross-sectional analysis of genotype frequencies (Heijmans et al., 1999).

Metabolic profile and body composition at baseline

C-reactive protein, high-density lipoprotein (HDL)-cholesterol, triglycerides and HbA1c (hemoglobin A1c) concentrations were determined in serum using fully automated analyzers (Hitachi 747 and 911; Hitachi, Ltd, Tokyo, Japan). Low-density lipoprotein (LDL)-cholesterol was estimated with the Friedewald equation (Friedewald et al., 1972). Cortisol was determined by a fluorescence polarization immunoassay using the Abbott TDx (Abbott Laboratories, Abbott Park, IL, USA) according to the manufacturer's instructions. The within-assay coefficient of variation (CV) was below 5 % at different levels. Body weight (kg) and height (cm) were measured in all participants and body mass index (BMI, kg/m^2) was calculated. The measurement of height is often unreliable in elderly, and therefore also armspan (cm) was measured, which approximates to height at maturity.

Cardiovascular pathologies at baseline

The prevalence and number of cardiovascular pathologies were obtained from the participant's general practitioner or nursing home physician. In addition, electrocardiograms were recorded on a Siemens Siccard 440 and transmitted by telephone to the ECG Core Lab in Glasgow for automated Minnesota coding (Macfarlane and Latif, 1996). Cardiovascular pathologies were classified as: myocardial infarction, myocardial ischemia, intermittent claudication or stroke (van Exel et al., 2002).

Genotyping

The ER22/23EK variant consists of two single nucleotide polymorphisms (SNPs) in codons 22 (A/G, rs6189) and 23 (G/A, rs6190). The ER22/23EK, N363S (A/G, rs6195) and *BdI* (C/G) (van Rossum et al., 2003) polymorphisms were genotyped using an Assay-by-Design (Applied Biosystems), consisting of PCR primers and TaqMan MGB probes on an ABI 7900 HT with real-time PCR (Applied Biosystems). Amplification reactions and parameters were based on manufacturer's instructions.

Statistical analysis

Allele frequencies were calculated and analyzed for deviation from the Hardy-Weinberg equilibrium using the χ^2-test. Mean differences in the parameters of the metabolic profile and body composition dependent on the polymorphisms were assessed with the univariate general linear model. All continuous variables were normally distributed, except for the C-reactive protein and triglyceride levels, which were ln-transformed. Differences in the prevalence of cardiovascular pathologies between genotypes were tested using the binary logistic regression model adjusted for sex. Mortality was first estimated using the Kaplan-Meier method, followed by the calculation of sex adjusted mortality risks and 95 % confidence intervals (CI) for allcause and

Table 1. Baseline characteristics of the Leiden 85-plus Study

Number	552
Age	85
Female	366 (66 %)
Independently living	451 (82 %)
Metabolic profile	
Cortisol (mmol/l)	0.49 (0.15)
C-reactive protein (mg/l)	2.83 (4.26)
HDL cholesterol (mmol/l)	1.31 (0.40)
LDL cholesterol (mmol/l)	3.67 (0.97)
Triglycerides (mmol/l)	1.40 (1.58)
HbA1c (mmol/l)	5.79 (1.09)
Body composition	
BMI (kg/m^2)	
Men	26.3 (5.17)
Women	27.7 (4.74)
Weight (kg)	
Men	74.5 (15.2)
Women	67.5 (12.7)
Height (cm)	
Men	168.1 (6.49)
Women	156.2 (6.24)
Armspan (cm)	
Men	178.1 (7.31)
Women	162.5 (7.03)
Cardiovascular pathologies	
Myocardial infarction	134 (24 %)
Myocardial ischemia	280 (51 %)
Intermittent claudication	34 (6 %)
Stroke	56 (10 %)

Metabolic profile and body composition values presented as means (SD), except for C-reactive protein where geometric mean (SD) is presented

cardiovascular mortality with the Cox proportional hazard model. All analyses were performed with SPSS statistical software, version 12.0 (Chicago, Illinois, USA), with the exception of the mortality analyses, which were performed with STATA software, version 9.0 (Texas, USA).

Results

DNA was available for 563 participants, of which 11 were excluded due to the use of corticosteroids. The baseline characteristics of the remaining 552 participants are presented in Table 1. All study subjects were genotyped for the ER22/23EK, N363S and *BclI* polymorphisms with a genotyping error of lower than 5 %. Therefore the total number of analyzed participants was 540 for ER22/23EK, 548 for N363S and 526 for the *BclI* polymorphisms. The minor allele frequencies were 0.03, 0.05 and 0.34 for the ER22/23EK, N363S and *BclI* polymorphisms respectively. The overall genotype distributions and resulting allelic frequencies of the SNPs were in agreement with the distribution predicted by the Hardy-Weinberg equilibrium. In this study, the calculated allele and genotype frequencies did not differ between elderly and young subjects (n=370, median age 32 years)(data not shown).

At baseline, several parameters of the metabolic profile dependent on the ER22/23EK, N363S and *BclI* variants were assessed. The analyses revealed that carriers of the ER22/23EK variant have higher HbA1c levels (6.54 (0.19) vs. 5.74 (0.05), $p<0.001$) compared to the non-carriers (Table 2). For the carriers of the N363S polymorphism, higher concentrations of LDL cholesterol (4.18 (0.13) vs. 3.62 (0.04), $p<0.001$) and triglycerides (1.60 (1.06) vs. 1.39 (1.02), $p=0.03$) compared to the non-carriers were observed (Table 2). No associations between the metabolic profile and the *BclI* polymorphism were found. The analyses of body composition showed only differences in height for the ER22/23EK polymorphism, where men carrying the ER22/23EK variant were taller (171.9 (1.71) vs. 167.8 (0.50), $p=0.02$) compared to the non-carriers. The same trend was observed for women (Table 3).

The effect of the polymorphisms on the prevalence of cardiovascular pathologies was assessed at baseline. No associations with the prevalence of myocardial infarction, myocardial ischemia, intermittent claudication and stroke were found for the different polymorphisms (Table 4).

During the 4.2-year follow-up, 278 (50 %) of the participants had died, of which 115 (41 %) of deaths were due to cardiovascular disease. The Kaplan-Meier estimates of cumulative mortality indicated that the carriers of *BclI* polymorphism have lower allcause mortality during the follow-up compared to the non-carriers (Figure 1). The all-cause mortality risk estimate for the *BclI* variant was 0.85 (0.67-1.08). The cardiovascular mortality analyses revealed no differences in survival for any of the ER22/23EK, N363S and *BclI* polymorphism carriers (Figure 1). The results remained unaltered after repeating the analyses for men and women separately.

All the above mentioned analyses were also repeated with independently living participants (n=451), in order to implement the selection criteria used in other studies (van Rossum et al., 2003; van Rossum et al., 2004a). These analyses did not reveal additional associations, but confirmed those observed in the whole cohort.

Table 2. Metabolic profile and body composition dependent on the ER22/23EK (G/A), N363S (A/G) and *BclI* (C/G) polymorphisms

	ER22/23EK			N363S			*BslI* C/G		
	GG (n=507) mean (SE)	GA/AA (n=33) mean (SE)	p-value	AA (n=497) mean (SE)	AG/GG (n=51) mean (SE)	p-value	CC (n=236) mean (SE)	CG/GG (n=290) mean (SE)	p-value
Cortisol (mmol/l)	0.49 (0.01)	0.49 (0.03)	0.90	0.49 (0.01)	0.51 (0.02)	0.35	0.48 (0.01)	0.50 (0.01)	0.10
C-reactive protein (mg/l)	2.80 (1.06)	3.35 (1.30)	0.51	2.86 (1.07)	2.80 (1.22)	0.91	2.69 (1.09)	3.06 (1.09)	0.29
HDL cholesterol (mmol/l)	1.31 (0.02)	1.26 (0.07)	0.45	1.31 (0.02)	1.26 (0.05)	0.33	1.31 (0.03)	1.32 (0.02)	0.87
LDL cholesterol (mmol/l)	3.66 (0.04)	3.67 (0.17)	0.92	3.62 (0.04)	4.18 (0.13)	<0.001	3.72 (0.06)	3.63 (0.06)	0.29
Triglycerides (mmol/l)	1.40 (1.02)	1.32 (1.08)	0.47	1.39 (1.02)	1.60 (1.06)	0.03	1.40 (1.03)	1.39 (1.03)	0.81
HbA1c (mmol/l)	5.74 (0.05)	6.54 (0.19)	<0.001	5.81 (0.05)	5.72 (0.15)	0.60	5.78 (0.07)	5.80 (0.06)	0.84

Sex adjusted univariate general linear model. For C-reactive protein and triglycerides geometric means with standard errors (SE) are presented

Table 3. Body composition dependent on the ER22/23EK (G/A), N363S (A/G) and *BclI* (C/G) polymorphisms

	ER22/23EK			N363S			*BslI* C/G		
	GG (n=507) mean (SE)	GA/AA (n=33) mean (SE)	p-value	AA (n=497) mean (SE)	AG/GG (n=51) mean (SE)	p-value	CC (n=236) mean (SE)	CG/GG (n=290) mean (SE)	p-value
BMI (kg/m^2)									
Men	26.3 (0.42)	26.1 (1.40)	0.91	26.3 (0.41)	26.0 (1.39)	0.84	25.8 (0.59)	26.6 (0.54)	0.30
Women	27.8 (0.26)	27.7 (1.15)	0.99	27.7 (0.27)	27.5 (0.80)	0.77	27.2 (0.39)	28.1 (0.35)	0.06
Weight (kg)									
Men	74.3 (1.22)	77.9 (4.12)	0.40	74.7 (1.19)	72.8 (4.09)	0.65	73.4 (1.72)	75.2 (1.59)	0.43
Women	67.4 (0.70)	69.6 (3.07)	0.48	67.5 (0.72)	67.2 (2.11)	0.90	66.4 (1.05)	68.2 (0.94)	0.19
Height (cm)									
Men	167.8 (0.50)	171.9 (1.71)	0.02	168.3 (0.50)	167.2 (1.73)	0.56	168.4 (0.69)	167.8 (0.65)	0.48
Women	156.0 (0.34)	158.6 (1.47)	0.09	156.2 (0.35)	156.6 (1.04)	0.73	156.3 (0.52)	156.1 (0.46)	0.73
Armspan (cm)									
Men	177.8 (0.57)	180.6 (1.97)	0.18	178.3 (0.56)	176.5 (1.93)	0.36	178.7 (0.79)	177.6 (0.74)	0.31
Women	162.4 (0.39)	164.3 (1.66)	0.26	162.5 (0.40)	162.6 (1.17)	0.91	162.5 (0.58)	162.4 (0.52)	0.85

Univariate general linear model. Data presented as means with standard errors (SE)

Table 4. Risk of cardiovascular pathologies at baseline dependent on the ER22/23EK (G/A), N363S (A/G) and *BclI* (C/G) polymorphisms

	ER22/23EK		N363S		*BclI*	
	GG n=507	GA/AA n=33	AA n=497	AG/GG n=51	CC n=236	CG /GG n=290
Myocardial infarction (n=134)	1	1.35 (0.62-2.93)	1	1.25 (0.65-2.40)	1	0.91 (0.61-1.37)
Myocardial ischemia (n=280)	1	1.82 (0.87-3.78)	1	1.41 (0.78-2.52)	1	0.80 (0.57-1.13)
Intermittent claudication (n=34)	1	0.87 (0.20-3.86)	1	1.03 (0.30-3.52)	1	1.00 (0.49-2.04)
Stroke (n=56)	1	1.56 (0.58-4.24)	1	1.79 (0.79-4.05)	1	1.22 (0.69-2.16)

Sex adjusted binary logistic regression. Data presented as odds ratios (OR) with 95 % confidence intervals (CI)

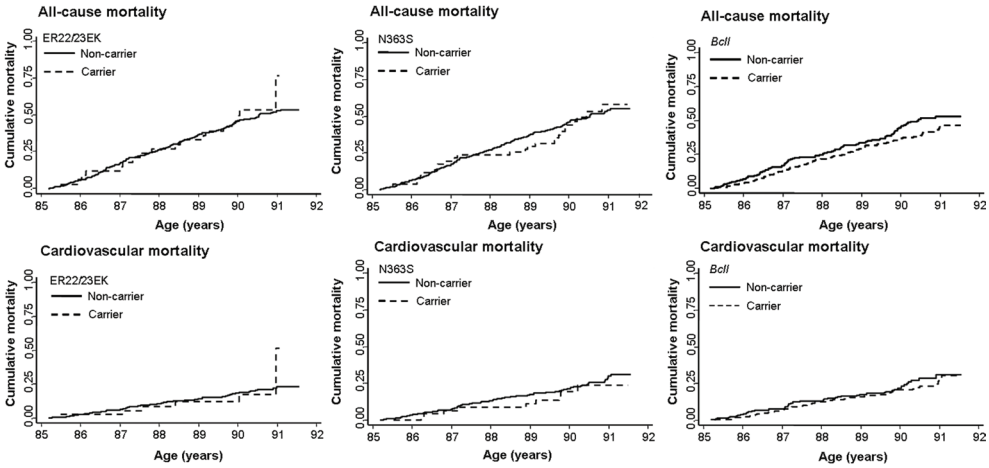

Figure 1. Kaplan-Meier curves of cumulative all-cause and cardiovascular mortality, dependent on the ER22/23EK, N363S and *BclI* genotypes in the 552 participants from age 85 years onwards.

Discussion

The aim of this study was to examine whether in old age polymorphisms in the GR gene (NR3C1) influence the prevalence of cardiovascular pathologies and survival due to differences in metabolic profile and body composition. The analyses revealed differences in metabolic profile for the ER22/23EK and N363S SNPs, and in body composition for the ER22/23EK variant but no associations with the prevalence of cardiovascular pathologies and lifespan.

Increased sensitivity to cortisol, accompanied with sub-optimal metabolic profile and increased risk for cardiovascular disease has been associated with the N363S and *BclI* polymorphisms (Di Blasio et al., 2003; Lin et al., 2003; van Rossum et al., 2003). In this study, we found no associations with the *BclI* polymorphism, but carriers of the N363S SNP had higher LDL-cholesterol and triglyceride levels, and a non-significantly higher prevalence for cardiovascular

diseases. The latter results are in accordance with another study in middle aged subjects (Lin et al., 2003), where N363S variant was associated with elevated cholesterol and triglyceride concentrations, and also with coronary artery disease. The lack of a relation between the N363S polymorphism and cardiovascular pathologies in our study population could be due to the fact that in the elderly high levels of LDL-cholesterol and triglycerides are not risk factors for cardiovascular disease anymore (Weverling-Rijnsburger et al., 1997; Weverling-Rijnsburger et al., 2003).

For the ER22/23EK variant, associations opposite of those expected were observed. It has been previously reported that elderly carriers of the ER22/23EK polymorphism have lower total cholesterol and LDL-cholesterol levels, lower fasting insulin concentrations, better insulin sensitivity and lower C-reactive protein levels, leading to a beneficial metabolic profile (van Rossum et al., 2002; van Rossum et al., 2004a). In this study, carriers of the ER22/23EK variant had higher levels of HbA1c compared to non-carriers, and a trend for higher C-reactive protein and LDL-cholesterol concentrations, which indicate a worse metabolic profile. Furthermore, no beneficial effects of the ER22/23EK polymorphism on the prevalence of cardiovascular pathologies, all-cause and cardiovascular mortality were found. These result are at odds with results from the previously published studies (van Rossum et al., 2002; van Rossum et al., 2004a). It could be, that the discrepancy between these studies has arisen due to the age difference of the subjects. In our study, participants aged 85-90 years were analyzed compared to subjects aged 67-77 years in the other studies (van Rossum et al., 2002; van Rossum et al., 2004a). The analyzed polymorphisms might have an effect before a certain age, and therefore the Leiden 85-plus cohort could consists of survivors. To test this, allele and genotype frequencies between the elderly and young participants in this study, and also between a young study group from the published literature (van Rossum et al., 2004b) were compared, revealing no differences. Thus, in contrast to an earlier report of an age-dependent enrichment for the ER22/23EK variation (van Rossum et al., 2002), no such enrichment was observed in our study.

Another possible source for differences could come from the participant's inclusion criteria. In order to apply similar inclusion criteria used in the other studies, only independently living participants were analyzed. However, this did not change our results, as well as stratification for gender. A more likely explanation could be that some of the observations have arisen by chance, leading to false negative or false positive associations. Recently it has been suggested that the role of genetic variations in common traits should be built up based on the evidence of many studies. Significant between-study diversity is frequent, and the results of the first study often correlate only modestly with subsequent research on the same association (Ioannidis et al., 2001).

The results of this study raise doubts whether the analyzed polymorphisms, shown to result in either resistance or increased sensitivity to cortisol (Huizenga et al., 1998a; van Rossum et al., 2002; van Rossum et al., 2003), are really functional. For the ER22/23EK variant, it has been reported that it does not alter the *in vitro* capacity to activate transcription (de Lange et al., 1997) and no altered response to cortisol was observed (Koper et al., 1997). However, a more recent study shows, that the transcriptional activity of the ER22/23EK carriers is decreased, due to higher expression of less transcriptionally active GR-A protein in the ER22/23EK carriers. It was proposed that this leads to the decrease in the sensitivity to cortisol (Russcher et al., 2005). For the other two polymorphisms, N363S and *BclI*, the functionality has not been established *in vitro*. On the other hand, the polymorphisms could be functional and result in altered hormone

sensitivity, which is counterbalanced by adaptive changes that attempt to normalize cortisol signaling. These adaptive changes may vary between individuals.

The strengths of this study are the ability to estimate several phenotypes in one cohort, and the prospective analysis with a high number of deaths, including the high number of cardiovascular deaths, during the follow-up. A weakness of the study is the lack of data on HPA-axis reactivity or on other parameters reflecting the stress system activity. However, in this aspect, we rely on the information presented in the published literature.

In conclusion, the results of this study show that in old age the ER22/23EK and N363S polymorphisms but not the *BclI* SNP in the *GR* gene have an influence on metabolic profile. However, the noticed differences in metabolic profile do not affect the prevalence of cardiovascular pathologies and the risks for all-cause and cardiovascular disease mortality at old age.

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CHAPTER 8

Impact of Genetic Variations in the *WRN* Gene on Age Related Pathologies and Mortality

Maris Kuningas, P. Eline Slagboom, Rudi G. J. Westendorp and Diana van Heemst

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Abstract

Mutations in the *WRN* gene lead to the Werner syndrome (WS), which resembles premature ageing. Here, we hypothesize that genetic variations in the *WRN* gene may also influence ageing-trajectories in the population at large. To test this hypothesis, we assessed the impact of the i1-C/T, L1074F and C1367R polymorphisms in the *WRN* gene on the occurrence of cardiovascular pathologies, on cognitive performance and on the risks of all-cause, cardiovascular and cancer mortalities in the population-based Leiden 85-plus Study. This prospective follow-up study includes 1245 participants aged 85 years and older, with a total follow-up of 5164 person-years. At baseline the risks of myocardial infarction, myocardial ischemia, intermittent claudication, arterial surgery and stroke dependent on the i1-C/T, L1074F and C1367R polymorphisms, did not vary between the different genotypes. In addition, no differences in cognitive functioning were observed, except for attention, where carriers of the 1367R allele performed worse compared to the 1367C homozygotes (94.2 (4.35) versus 84.8 (1.84), $p=0.04$). Mortality risks, calculated separately for all SNPs, were similar between the different genotype carriers of the i1-C/T, L1074F and C1367R polymorphisms, showing no evidence of altered survival. In conclusion, the i1-C/T, L1074F and C1367R polymorphisms in the *WRN* gene do not influence the ageing-trajectories and survival in the population at large.

Introduction

Werner syndrome (WS) is a segmental progeroid disorder with an autosomal recessive pattern of inheritance. Patients with WS exhibit a number of symptoms that resemble premature ageing. Characteristic clinical features of the syndrome include diabetes, osteoporosis, vascular diseases and high incidence of malignant neoplasms. Death usually occurs before the age of 50 years due to cancer or atherosclerosis (Martin, 1978; Salk, 1982). WS is caused by loss-of function mutations in the *WRN* gene. Since mutations in the *WRN* gene lead to accelerated ageing, it has been reasoned that polymorphisms in the *WRN* gene may also associate with age-related pathologies and thus influence ageing in the population at large.

The *WRN* gene encodes a nuclear protein with both helicase and exonuclease activities (Liu et al., 1999; Morozov et al., 1997; Mushegian et al., 1997). Evidence from several studies suggests that this protein is involved in the response to DNA damage during replication, recombination and transcription processes (Balajee et al., 1999; Webb et al., 1996). WRN is active in unwinding alternate DNA structures, such as DNA-RNA hybrids, triplexes and tetraplexes that may otherwise cause genomic instability (for review (Bachrati and Hickson, 2003; Opresko et al., 2003)). Genomic instability along with accumulation of damage and cellular senescence is commonly seen in WS cells (for review (Brosh, Jr. and Bohr, 2002; Macario and Conway, 2002)). Senescent cells go through alterations in gene expression patterns, which in turn have been shown to underpin several pathologies in tissues such as skin and vasculature (Minamino et al., 2004; Shelton et al., 1999). These processes are likely to lead to the disease pathologies seen in WS patients, and also during ageing in the population at large (Bird et al., 2003; Hasty et al., 2003). Since WS resembles accelerated ageing, it has been suggested that the *WRN* gene may also modulate the course of ageing in the population at large and play a role in the sensitivity or resistance to the development of age-related disorders. Only few studies have addressed this question, with contradictory results. Studies in Japanese have shown that a C1367R variation in the *WRN* gene is associated with myocardial infarction (Morita et al., 1999; Ye et al., 1997), whereas in Caucasians no associations with cardiovascular disease have been found (Bohr et al., 2004; Castro et al., 1999). An i1-C/T polymorphism, on the other hand, has been related with cognitive functioning (Bendixen et al., 2004), and for a L1074F SNP an age-dependent enrichment of the 1074L allele in Finnish and Mexican populations has been observed (Castro et al., 2000). The latter result suggests a beneficial effect on survival. These associations have been found in separate studies and to date there are no data on the influence of the C1367R polymorphism on cognitive function and survival, and no information of the i1-C/T and L1074F polymorphisms on cardiovascular disease risks in elderly.

The aim of this study was to assess the impact of the i1-C/T, L1074F and C1367R polymorphisms in the *WRN* gene on the occurrence of cardiovascular pathologies, on cognitive performance and on the risks of all-cause and cause-specific mortalities in the population at large. Since these polymorphisms are potentially functional (Bohr et al., 2004; Kamath-Loeb et al., 2004b), univariate analyses were performed. The study was carried out in elderly aged 85 years and older, using cross-sectional and prospective study designs. The use of elderly participants provides the best opportunities for determining the impact of genetic variations on ageing trajectories, since at that age the effects of truly functional variations should be most pronounced.

Participants and methods

Participants

The Leiden 85-plus Study is a prospective population-based study, in which inhabitants of Leiden, The Netherlands, aged 85 years or over, were invited to take part. There were no selection criteria related to health or demographic characteristics. The study population consists of two cohorts, cohort '87 and '97. Cohort '87 includes 977 participants aged 85 years and older, enrolled between 1987 and 1989 (Weverling-Rijnsburger et al., 1997). Cohort '97 consists of 599 subjects, all members of the 1912-1914 birth cohort, who were enrolled in the month of their 85th birthday between 1997 and 1999 (der Wiel et al., 2002). DNA was available for 682 participants from cohort '87 and for 563 people from cohort '97. All participants of the Leiden 85-plus Study were followed for mortality until April 1, 2004. Primary causes of death were obtained from the Dutch Central Bureau of Statistics and categorized according to the 10th International Classification of Diseases (ICD-10). The Medical Ethical Committee of the Leiden University Medical Center approved the study and informed consent was obtained from all participants. In addition, 247 young blood donors (aged 19-40 years) from Leiden were included for a cross-sectional comparison of genotype frequencies. To avoid population stratification due to geographic differences between the elderly and young, we restricted the young control population to those with either two Leiden-born parents or one Leiden-born parent and the other born within a 12-km distance from Leiden. Information regarding the birthplace of their grandparents was obtained from a written questionnaire (Heijmans et al., 1999).

Cardiovascular pathologies at baseline in cohort '97

The prevalence and number of cardiovascular pathologies were obtained from the participants' general practitioner or nursing home physician. In addition, electrocardiograms were recorded on a Siemens Siccard 440 and transmitted by telephone to the ECG Core Lab in Glasgow for automated Minnesota coding (Macfarlane and Latif, 1996). Cardiovascular pathologies were classified as: myocardial infarction, myocardial ischemia, intermittent claudication, arterial surgery and stroke (van Exel et al., 2002).

Cognitive function tests in cohort '97

Overall, cognitive function was measured with the Mini-Mental State Examination (MMSE). Individuals who scored equal to or above 19 points also performed tests measuring attention (Stroop Test) (Klein et al., 1997), processing speed (Letter Digit Coding Test) (Houx et al., 2002), immediate recall memory (Word Learning Test Immediate Recall) and delayed recall memory (Word Learning Test Delayed Recall) (Brand and Jolles, 1985). All participants were visited annually for re-measurement of cognitive functioning during a mean follow-up of 4.2 years. Parallel versions of the tests were used. Details of cognitive testing are described elsewhere (Houx et al., 2002).

Genotyping

The i1-C/T (rs2725335) and C1367R (rs1346044) variations were genotyped using an Assay-by-Design (Applied Biosystems), consisting of PCR primers and TaqMan MGB probes. Amplification reactions were made at standard conditions except for the following modifications. A qPCR core kit was used (Eurogentec) and a half of the amount of primers and probes. Real time PCR was performed on ABI 7900 HT (Applied Biosystems). The L1074F (rs2725362) polymorphism was genotyped by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS), using the Sequenom MassARRAYtm (Sequenom Inc.) methodology. Amplification reactions and parameters were based on the manufacturer's instructions

Statistical analysis

Allele frequencies were calculated and analyzed for deviation from the Hardy-Weinberg equilibrium using the χ^2-test. Differences in the prevalence of cardiovascular pathologies between genotypes were tested using the binary logistic regression model adjusted for sex. Associations between genotypes and cognitive functioning were analyzed using a linear mixed model, estimating the overall mean difference in cognition during the follow-up, adjusted for sex and level of education. Mortality was first estimated using the Kaplan-Meier method, followed by the calculation of sex adjusted mortality risks and 95 % confidence intervals (CI) for all-cause, cardiovascular and cancer mortality with the Cox proportional hazard model, using left censoring to correct for the delayed entry into the risk set according to age. For each polymorphism, hazard ratios (HR) were calculated using common allele homozygotes as the referent group. All analyses were performed with SPSS statistical software, Version 12.0 (Chicago, IL, USA), with the exception of the mortality analyses, which were performed with STATA software, Version 9.0 (StataCorp LP, TX, USA).

Results

All 1245 participants of the Leiden 85-plus Study and 247 young blood donors were genotyped for the i1-C/T, L1074F and C1367R polymorphisms. The location of the SNPs in the *WRN* gene and protein are indicated in Figure 1. The genotype and resulting allelic frequencies of the SNPs were in agreement with the Hardy-Weinberg equilibrium, except for the C1367R polymorphism in the cohort '97 (Table 1). In that case, a deficit of heterozygotes and an excess of both homozygote allele carriers were observed. The overall genotype distributions and resulting allelic frequencies of the SNPs were similar to these found in Caucasians (Bendixen et al., 2004; Castro et al., 1999; Castro et al., 2000). In this study, the calculated allele and genotype frequencies did not differ between the elderly and young subjects, showing no enrichment for any of the alleles (Table 1).

The prevalence of cardiovascular pathologies dependent on the i1-C/T, L1074F and C1367R polymorphisms, and the influence of these SNPs on cognitive functioning were assessed only in cohort '97. At baseline the risks of myocardial infarction, myocardial ischemia, intermittent claudication, arterial surgery and stroke dependent on the i1-C/T, L1074F and C1367R polymorphisms did not vary between the different genotypes (Table 2).

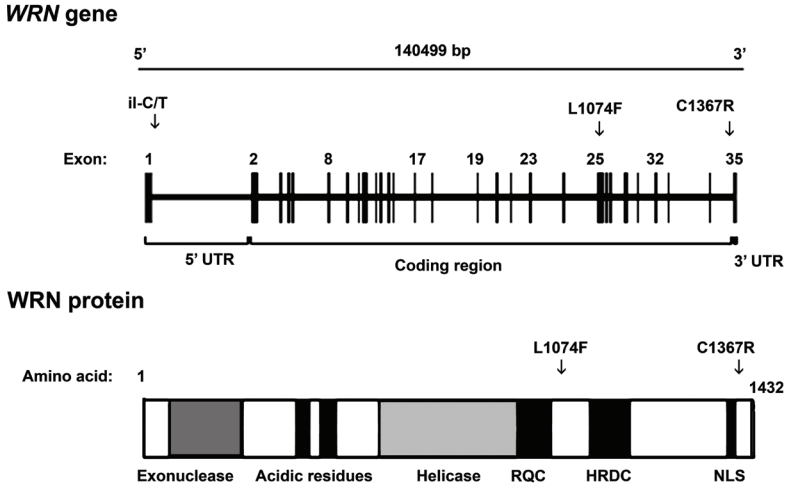

Figure 1. The *WRN* gene and protein structure with a graphic representation of the i1-C/T, L1074F and C1367R SNP localization. The positions of the SNPs in the *WRN* gene and protein are indicated with arrows. The *WRN* gene is located in chromosome 8 and it spans a genomic region of 140 kb, containing 35 exons. The WRN protein is 1432 amino acids long and has 5 functional domains: exonuclease domain, helicase domain, RecQ helicase domain (RQC), helicase and RNaseD-C-terminal (HRDC) domain and C-terminal nuclear localization signal (NLS). The WRN protein has in addition acidic residues with unknown function.

Cognitive functioning was measured at baseline and annually during a mean follow-up of 4.2 years. Global cognition, as measured with MMSE, was similar between the different genotype carriers of the i1-C/T, L1074F and C1367R polymorphisms (Table 3). From the specific domains of cognitive functioning, only differences in attention were observed for the C1367R polymorphism. Homozygous carriers of the 1367R allele had worse attention compared to the 1367C homozygotes (94.2 (4.35) versus 84.8 (1.84), $p=0.04$). The same trend was observed for the heterozygous 1367R allele carriers. The results remained unaltered after the adjustment for depressive feelings. No associations with processing speed, immediate and delayed memory were observed with the different genotypes (Table 3).

The influence of the i1-C/T, L1074F and C1367R polymorphisms on mortality risks was assessed separately for cohort '87 and cohort '97, and also in the combined cohort of 1245 participants (Supplementary Table 1). Since in some instances the mortality risk estimates found in cohort '87 were not replicated in cohort '97, the results for the combined cohort are presented. The higher number of participants in the combined cohort provides better statistical power and enables to calculate estimates that are more accurate. During the total follow-up of 5164 person-years, 957 (77 %) participants had died, of which 365 (38 %) from cardiovascular disease and 143 (15 %) due to cancer. The Kaplan-Meier estimates of cumulative mortality according to the genotypes of the different SNPs are presented in the Figure 2. According to the Kaplan-Meier mortality curves, it seems that carriers of the i1-CT genotype, and heterozygous and

Table 1. Characteristics of the study subjects

	Young control	Cohort '87	Cohort '97
Number	247	682	563
Age	19-40	84-100	85
Female	137 (56 %)	491 (72 %)	375 (67 %)
i1-C/T			
CC	223 (92 %)	586 (91 %)	489 (91 %)
CT	18 (8 %)	56 (9 %)	47 (9 %)
TT	-	-	-
Total	241	642	536
MAF	0.04	0.04	0.04
HWE	0.83	0.51	0.57
L1074F			
LL	66 (27 %)	202 (32 %)	175 (32 %)
LF	126 (51 %)	316 (51 %)	260 (47 %)
FF	55 (22 %)	105 (17 %)	117 (21 %)
Total	247	623	552
MAF	0.48	0.42	0.45
HWE	0.94	0.61	0.54
C1367R			
CC	139 (58 %)	329 (49 %)	316 (57 %)
CR	81 (34 %)	285 (42 %)	192 (34 %)
RR	21 (8 %)	58 (9 %)	50 (9 %)
Total	241	672	558
MAF	0.26	0.30	0.26
HWE	0.20	0.95	0.04

MAF-minor allele frequency, HWE- Hardy-Weinberg equilibrium

homozygous carriers of the 1367R allele have lower cancer mortality. Although not statistically significant, the mortality risk estimates of 0.56 (0.26-1.20) for i1-CT, 0.84 (0.59-1.20) for C1367R and 0.57 (0.27-1.17) for R1367R genotypes, show lower cancer mortality. The same trend was observed in cohort '87 and '97 (Supplementary Table 1). The other all-cause and cause-specific mortality risks, calculated separately for all polymorphisms, were similar between the different genotype carriers of the i1-C/T, L1074F and C1367R polymorphisms, showing no evidence of altered survival.

Discussion

The results of this study show that the i1-C/T, L1074F and C1367R polymorphisms in the *WRN* gene do not influence the occurrence of cardiovascular pathologies, cognitive performance and the risks of all-cause and cause-specific mortalities in a cohort of elderly aged 85 years and older.

Table 2. Risk of cardiovascular pathologies at baseline dependent on i1-C/T, L1074F and C1367R

	i1-C/T		L1074F			C1367R		
	CC	CT	LL	LF	FF	CC	CR	RR
		OR (95 % CI)		OR (95 % CI)	OR (95 % CI)		OR (95 % CI)	OR (95 % CI)
Myocardial infarction (n=103)	1	1.17 (0.56-2.46)	1	1.47 (0.89-2.43)	1.04 (0.55-1.97)	1	0.86 (0.54-1.39)	1.41 (0.69-2.89)
Myocardial ischemia (n=234)	1	0.51 (0.26-1.00)	1	1.22 (0.83-1.82)	1.53 (0.95-2.46)	1	1.02 (0.71-1.47)	0.60 (0.32-1.15)
Intermittent claudication (n=36)	1	2.26 (0.87-5.83)	1	0.91 (0.41-1.98)	0.90 (0.34-2.38)	1	1.64 (0.79-3.39)	1.57 (0.50-4.97)
Arterial surgery (n=37)	1	1.72 (0.62-4.75)	1	0.83 (0.38-1.78)	0.84 (0.32-2.20)	1	1.25 (0.60-2.59)	1.40 (0.45-4.40)
Stroke (n=57)	1	0.85 (0.29-2.47)	1	0.99 (0.52-1.90)	1.17 (0.54-2.51)	1	0.83 (0.45-1.51)	0.69 (0.24-2.04)

Sex adjusted odds ratios (OR) with 95 % confidence intervals (CI), estimated in the cohort '97

Table 3. Differences in various domains of cognitive function dependent on the i1-C/T, L1074F and C1367R genotypes during follow-up

	i1-C/T		L1074F			C1367R		
	CC mean (SE)	CT mean (SE)	LL mean (SE)	LF mean (SE)	FF mean (SE)	CC mean (SE)	CR mean (SE)	RR mean (SE)
Global cognitive function (points)	22.9 (0.31)	23.1 (0.93)	22.6 (0.49)	23.4 (0.40)	22.2 (0.60)	22.8 (0.38)	22.8 (0.47)	23.1 (0.90)
Attention (seconds)	87.3 (1.53)	83.3 (4.61)	88.4 (2.39)	87.1 (1.97)	84.6 (3.04)	84.8 (1.84)	88.9 (2.27)	**94.2 (4.35)**
Processing speed (digits)	15.5 (0.33)	16.7 (0.99)	15.3 (0.52)	15.7 (0.43)	16.3 (0.66)	15.8 (0.40)	15.6 (0.50)	15.3 (0.95)
Immediate memory (pictures)	20.3 (0.31)	21.2 (0.96)	20.5 (0.49)	20.5 (0.41)	20.1 (0.62)	20.4 (0.38)	20.3 (0.47)	20.2 (0.91)
Delayed memory (pictures)	7.00 (0.15)	7.47 (0.45)	7.05 (0.23)	7.05 (0.19)	6.97 (0.29)	6.99 (0.18)	7.00 (0.22)	7.14 (0.43)

Estimates represent the overall mean difference in cognitive function during the mean 4.2-year follow-up, dependent on genotypes in the cohort '97. The common allele homozygotes were taken as the referent group, and a significant difference ($p<0.05$) is indicated in bold. Analyses were adjusted for sex and education. SE – standard error

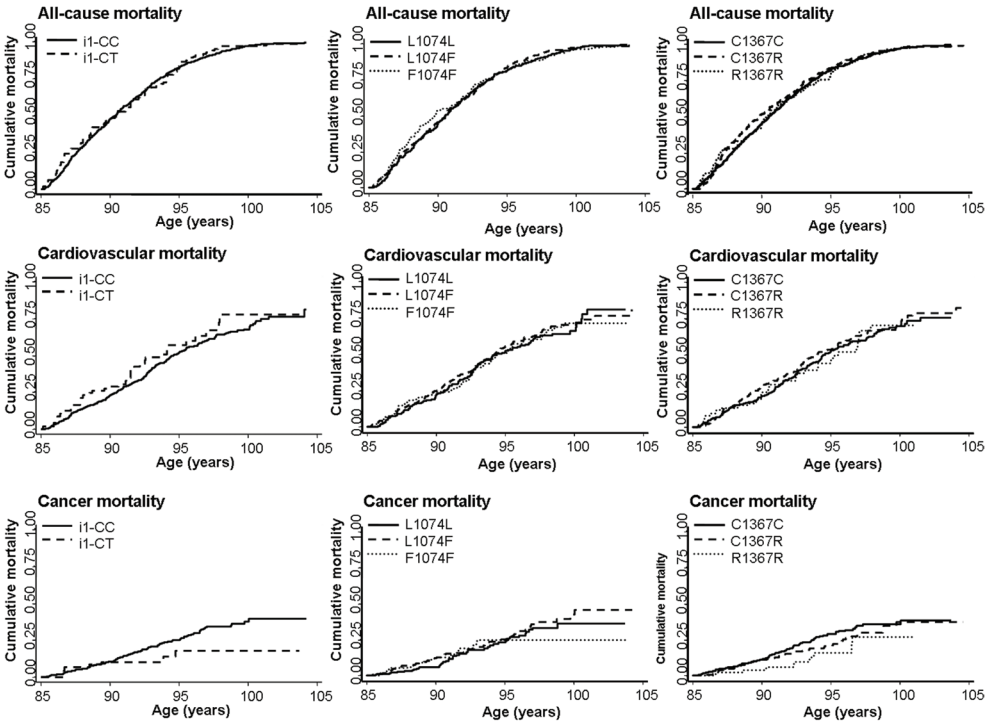

Figure 2. Kaplan-Meier curves of cumulative all-cause, cardiovascular and cancer mortality, dependent on i1-C/T, L1074F and C1367R genotypes in the combined cohort of 1245 individuals from age 85 years onwards

From the three polymorphisms polymorphisms analyzed in this study, the C1367R SNP was found to be out of Hardy-Weinberg equilibrium in cohort '97. Since all DNAs from cohort '87, '97 and from blood bank donors were genotyped simultaneously then a specific genotyping failure in cohort '97 is unlikely. Furthermore, 10 % of the samples were genotyped twice and for the C1367R SNP in cohort '97 the genotyping error was less than 1 %. Therefore, the marginal deviation from Hardy-Weinberg equilibrium could have been arisen by chance.

In this study, most major outcomes of atherosclerosis, including myocardial infarction, myocardial ischemia, intermittent claudication, arterial surgery and stroke, were assessed. None of the analyzed polymorphisms in the *WRN* gene associated with these pathologies. Furthermore, dependent on the polymorphism, either higher or lower risk estimates for the different cardiovascular pathologies were observed. In contrast, a consistent risk profile over these various outcomes of atherosclerosis was expected. Therefore, we concluded that the i1-C/T, L1074F and C1367R polymorphisms do not contribute to the risk of developing cardiovascular pathologies. For the C1367R SNP, these results are in line with recent studies in Caucasians (Bohr et al.,

2004; Castro et al., 2000), but at odds with findings in Japanese, where the carriers of the 1367R allele had lower risks for myocardial infarction (Morita et al., 1999; Ye et al., 1997). As already suggested by others (Bohr et al., 2004), the disparity in results might come from population differences, which is also supported by the fact that in the Japanese the minor allele frequency of the C1367R polymorphism is more than three times lower than in Caucasians. In the Japanese, the C1367R variation may also be in linkage disequilibrium with another, so far unidentified polymorphism that is not present in Caucasians.

Cognitive functioning dependent on the *WRN* gene i1-C/T and C1367R polymorphisms has been assessed previously only in one study (Bendixen et al., 2004). In that study, the T allele of the i1-C/T SNP was associated with better cognitive functioning in the elderly, and no associations with the C1367R SNP were found (Bendixen et al., 2004). The beneficial effect of the i1-T allele was only seen on the cognitive composite score and not on MMSE. In our study, carriers of the i1-T allele seemed to perform better on all the analyzed cognitive domains, however the differences did not reach statistical significance. A significant association on the other hand, was observed between the C1367R polymorphism and attention. Carriers of the 1367R allele had worse attention compared to the 1367C homozygotes. The other domains of cognition seemed to be unaffected. The positive association between the C1367R SNP and attention could be a chance finding. In this study we did not correct for multiple testing, however if such correction had been applied then the borderline significance would have disappeared. In order to exclude that the findings of this and the other study (Bendixen et al., 2004) between cognition and *WRN* gene polymorphisms are due to chance, corroboration in independent samples is needed. In WS patients, cognitive decline and dementia have not been described. It has been reasoned that the central nervous system may be less prone for damage due to the absence of mitotic activity during adult life. However subtle defects might emerge over time, if the WRN is important for neural stem cell function during adult life (Gage, 2002).

The influence of the *WRN* gene on mortality before the age of 85 years was examined in a cross-sectional design by comparing allele frequencies of the SNPs in elderly aged 85 years and older with those in young subjects. No differences were found in the allele frequencies between the elderly and young. With regard to the L1074F polymorphism, this finding is in contrast with a study showing an age-dependent enrichment of the 1074L allele in Finnish and Mexican populations (Castro et al., 2000). The latter indicates a beneficial effect on survival. To evaluate further the impact of the polymorphisms on lifespan, mortality risks after the age of 85 years, dependent on the SNPs, were assessed. The mortality risk estimates calculated for all-cause and cardiovascular mortality did not differ between the different genotype carriers of the polymorphisms. However, the observed trend for lower cancer mortality risks for the minor allele carriers of the i1-C/T and C1367R polymorphism needs to be studied in more detail, in order to make more profound conclusions. Taking together, the cross-sectional and prospective analyses provided no evidence for differential survival for any of these polymorphisms.

In WS patients, the observed pathologies and decrease in lifespan are attributable to the loss of a functional WRN protein. Polymorphisms in the *WRN* gene may also affect the functionality of the protein. The i1-C/T SNP is located in the first intron of the *WRN* gene. An intronic SNP may influence the splicing process or the stability of the mRNA, and thereby the amount of functional protein synthesized. However, for the i1-C/T polymorphism the effect has still to be established. The L1074F polymorphism is in exon 26, and in the vicinity of the RecQ C-terminal (RQC) domain. The C1367R SNP is located in exon 34, near to the nuclear localization signal

(Matsumoto et al., 1998). It has been shown that these polymorphisms result in subtle changes in the helicase/exonuclease activities of the WRN protein (Bohr et al., 2004; Kamath-Loeb et al., 2004a). The localization to the nucleus of the protein carrying the C1367R polymorphism appeared to be unaffected (Bohr et al., 2004). It can be reasoned that the differential functional effects caused by the polymorphisms in the *WRN* gene, are not sufficient for causing pathologies or influence the lifespan. However, another possibility is that the polymorphisms alter the functionality of the WRN protein, but due to compensatory mechanisms, no effects at phenotypic level are observed.

The first strength of this study is the elderly cohort, which is suitable for testing the effect of variations in the *WRN* gene on age-associated disease and mortality patterns in the population at large. The other strength is the possibility to estimate several phenotypes in one cohort, and the prospective analysis with a high number of events (deaths) during the follow-up. The latter results in a good power for the detection of effects on lifespan. The weakness of the study might be related to the selection of analyzed polymorphisms. The SNPs were selected based on their published associations, however there might be other, so far undetected functional polymorphisms in the *WRN* gene leading to changes in the pace of ageing. An approach to overcome this is to analyze, either separately and/or in a combination of haplotypes, a larger set of evenly distributed SNPs in the *WRN* gene, and determine their influence on the prevalence of age-associated diseases and lifespan.

In conclusion, the present study shows that the i1-C/T, L1074F and C1367R variations in the *WRN* gene do not influence the sensitivity or resistance to the development of age-related disorders and the course of ageing.

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Supplementary materials

Supplementary Table 1. All-cause and cause-specific mortality risks dependent on i1-C/T, L1074F and C1367R genotypes

	i1-C/T		L1074F			C1367R		
	CC	CT	LL	LF	FF	CC	CR	RR
		HR (95 % CI)		HR (95 % CI)	HR (95 % CI)		HR (95 % CI)	HR (95 % CI)
All-cause mortality								
Cohort '87	1	0.98 (0.74-1.29)	1	1.05 (0.88-1.26)	0.91 (0.71-1.15)	1	1.02 (0.87-1.19)	1.00 (0.75-1.32)
Cohort '97	1	1.06 (0.70-1.62)	1	0.99 (0.75-1.30)	1.25 (0.91-1.72)	1	1.14 (0.89-1.46)	1.05 (0.69-1.59)
Combined cohorts	1	0.99 (0.79-1.25)	1	1.03 (0.89-1.20)	1.01 (0.83-1.22)	1	1.06 (0.92-1.21)	1.02 (0.80-1.28)
Cardiovascular mortality								
Cohort '87	1	1.06 (0.68-1.65)	1	0.99 (0.74-1.33)	0.95 (0.65-1.40)	1	1.08 (0.83-1.40)	1.05 (0.67-1.66)
Cohort '97	1	1.31 (0.72-2.39)	1	1.31 (0.86-2.00)	1.11 (0.65-1.90)	1	1.25 (0.85-1.83)	0.90 (0.45-1.81)
Combined cohorts	1	1.13 (0.80-1.62)	1	1.09 (0.86-1.39)	0.99 (0.73-1.36)	1	1.14 (0.91-1.41)	1.00 (0.68-1.46)
Cancer mortality								
Cohort '87	1	0.45 (0.17-1.24)	1	1.00 (0.64-1.55)	0.56 (0.28-1.14)	1	0.95 (0.63-1.45)	0.67 (0.29-1.57)
Cohort '97	1	0.83 (0.26-2.69)	1	**2.38 (1.02-5.52)**	**2.85 (1.12-7.26)**	1	0.59 (0.30-1.17)	0.39 (0.09-1.62)
Combined cohorts	1	0.56 (0.26-1.20)	1	1.25 (0.85-1.84)	0.98 (0.58-1.65)	1	0.84 (0.59-1.20)	0.57 (0.27-1.17)

Sex adjusted hazard ratios (HR) and 95 % confidence intervals (CI), calculated with Cox proportional hazard model. The common allele homozygotes were taken as the referent group, and a significant difference ($p<0.05$) is indicated in bold

CHAPTER 9

Genes Encoding Longevity; from Model Organisms to Man

Maris Kuningas, Simon P. Mooijaart, Diana van Heemst, Bas J. Zwaan, P. Eline Slagboom and Rudi G.J. Westendorp

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Abstract

Ample evidence from model organisms has indicated that subtle variation in genes can dramatically influence lifespan. The key genes and molecular pathways that have been identified so far encode for metabolism, maintenance- and repair mechanisms that minimize age related accumulation of permanent damage. Here, we describe the evolutionary conserved genes that are involved in lifespan regulation in model organisms and have been studied for association with lifespan in humans. The accumulated data reveal that when moving up the evolutionary ladder, together with an increase of genome complexity, the impact of candidate genes on lifespan becomes smaller. The presence of genetic networks makes it more likely to expect impact of variation in several interacting genes to affect lifespan in humans. Extrapolation of findings from experimental models to man is further complicated as phenotypes are critically dependent on the setting in which genes are expressed while laboratory conditions and modern environments are markedly dissimilar. Finally, currently used methodologies may have only little power and validity to reveal genetic variation in the population. In conclusion, although the study of model organisms has revealed potential candidate genetic mechanisms determining ageing and lifespan, to what extent they explain variation in human populations is still uncertain.

Introduction

Over the last century, mean life expectancy in Western societies has increased dramatically (Oeppen and Vaupel, 2002). In Japan for instance, mean life expectancy has increased from fifty to eighty years in no more than six decades. It is unlikely that changes in the population genome over this time-period can explain for the observed increase in lifespan which is more likely to be attributable to the improvement of environmental conditions and medical care. The increase in mean life expectancy of the total population, however, has left the marked inter-individual variance in lifespan unaltered. Socio-economic factors can in part explain this phenomenon, but ample evidence suggests that genetic factors are also at play. Studies of twins and long-lived families have estimated that 20 to 30 per cent of the variation in human lifespan is determined by genetic factors, which impact becomes more important for survival at older ages (Herskind et al., 1996; Hjelmborg J. et al., 2006; Mitchell et al., 2001). Furthermore, siblings of centenarians have a significantly higher chance of becoming a centenarian themselves when compared to other members of their birth cohort (Perls et al., 2002). The survival benefits of family-members of these long-lived subjects are lifelong and persist up to the highest age-categories (Hjelmborg J. et al., 2006; Perls et al., 2002). Offspring of long-lived sibling pairs have a lower mortality risk already at middle age, whereas their spouses, with whom they have shared in part a common environment, do not show this survival benefit (Schoenmaker et al., 2006).

As for lifespan, ageing is under moderate genetic control influencing the rate at which stochastically induced damaged molecules accumulate. Such damage is caused by various endogenous and exogenous biological- and biochemical stresses. As a result, over the life course there is a constant rise in vulnerability of the body, leading to a continuously increasing risk of disease and death. Longevity and the maintenance of health in old age can be ensured via two principally different strategies that minimize the risk of permanent damage to occur i.e. by a decrease of environmental hazards or an increase of the durability of the body. Pathways that influence metabolism, maintenance and repair mechanisms, and prevent the accumulation of permanent damage thus represent key molecular candidates for the preservation of health and longevity.

Experiments in model organisms have demonstrated that a series of induced mutations in various genes that make up an integrated molecular pathway can dramatically increase lifespan. The most prominent example includes the *Caenorhabditis elegans daf-2* and *clk* double mutants that live nearly five times longer than wild-type worms (Lakowski and Hekimi, 1996). Most of the genes of model organisms are evolutionarily conserved and present in humans. Here, we will briefly review the genes and mechanisms that have been shown to regulate lifespan in model organisms, but limiting ourselves to those genes, which have human homologues, and have been studied for association with human health and/or longevity (Table 1). In addition, it is addressed whether we can expect to find single genes or molecular pathways that substantially affect lifespan in humans; whether the information obtained from model organisms can be translated to variation in lifespan in humans; and whether present genetic surveys are able to pick up this genetic variation.

The evidence

Insulin/IGF-1 signaling (IIS)

The first evidence for genetic regulation of lifespan came from studies with *C. elegans*. It was discovered that worms with mutations in the dauer formation (Daf) genes, such as *daf-2* and *age-1*, were able to bypass dauer formation, and become long-lived adults (Larsen, 2001). The molecular characterization of the *daf-2* and *age-1* genes revealed that these show homology to the mammalian genes encoding Insulin Receptor (IR) and Insulin-like Growth Factor 1 Receptor (IGF-1R) (Kimura et al., 1997), which are evolutionary conserved. Next, it was shown that, similar to *C. elegans*, reduced insulin signaling extends lifespan in *Drosophila melanogaster*. The increase in lifespan was observed for flies with mutated insulin-like receptor (InR) or its substrate (chico), and for flies with ablated insulin-producing cells (Giannakou and Partridge, 2007). In the latter case, the adult flies also exhibited increased storage of lipids and carbohydrates, reduced fecundity, and increased stress- and starvation resistance.

In vertebrates, the insulin signaling system is more complex and contains separate receptors for insulin (IR) and IGF-1 (Navarro et al., 1999). Data from mice indicate that both these receptors, IR and IGF-1R, are involved in lifespan regulation. The IGF-1 branch, which acts through growth-hormone-releasing hormone (GHRH), growth hormone (GH) and IGF-1, influences body composition and is involved in the regulation of gonadal function (Bartke, 2005). The long-lived Ames and Snell dwarf mice, which are deficient in growth hormone, thyroid hormoneand prolactin are infertile (Brown-Borg et al., 1996). A similar phenotype is observed for GH receptor knockout mice (Bartke, 2005). Furthermore, mice mutated for the IGF-1 receptor hint at a direct role for reduced IGF-1 signaling in mammalian longevity: Igf1r+/- females, but not males, exhibit a long-lived phenotype (Holzenberger et al., 2003). In contrast, complete disruption of the IR gene leads to insulin resistance, diabetes and shortened lifespan (Okamoto and Accili, 2003). Likewise, tissue specific IR knockout mouse models develop obesity, insulin resistance and impaired glucose regulation, with the exception of the fat-specific IR knockout mice (FIRKO) (Okamoto and Accili, 2003). These mice have reduced fat mass, are protected against age-related obesity, and live longer than their littermates. Taken together, the evidence in mouse models shows that reduced insulin/IGF-1 signaling can extend lifespan also in mammals.

In humans, there is some evidence that long-lived subjects, such as centenarians, have decreased plasma IGF-I levels and preserved insulin action, thus indicating that insulin responsiveness influences human longevity (Paolisso et al., 1997). For instance, a polymorphisms in the IGF-IR locus, that has been associated with lower plasma IGF-1 levels was shown to be enriched among Italian centenarians (Bonafe et al., 2003). This finding was not replicated in a prospective follow-up study of elderly Dutch subjects, but in the same study it was found that a polymorphism in the *GH1* gene, which controls IGF-1 activity, associates with longevity (van Heemst et al., 2005a). In addition, a combined effect of variation at the GH1, IGF-1 and IRS1 loci was observed, demonstrating an additive effect of multiple variants associated with reduced IIS signaling on human longevity.

Table 1. Selected example of genes identified to influence lifespan in model organisms (in alphabetical order)

Symbol	Gene name/description	Function	Organism*	Reference
age-1	phosphatidylinositol kinase	insulin signaling	C. elegans	(Morris et al., 1996)
Cat	catalase	antioxidant activity	D. melanogaster	(Orr and Sohal, 1994)
Chico	insulin receptor substrate	insulin signaling	D. melanogaster	(Clancy et al., 2001)
daf-2	insulin receptor-like gene	insulin signaling	C. elegans	(Kimura et al., 1997)
daf-12	nuclear hormone receptor	regulation of metabolic and developmental pathways	C. elegans	(Larsen et al., 1995)
daf-16	forkhead transcription factor	regulation of metabolic and developmental pathways	C. elegans	(Ogg et al., 1997)
Gh	growth hormone	insulin signaling, tissue proliferation	M. musculus	(Bartke, 2005)
Klotho	beta-glucuronidase	inhibits IIS signaling	M. musculus	(Kuro-o M et al., 1997)
Mei-41	phosphatidylinositol kinase	DNA repair	D. melanogaster	(Symphorien and Woodruff, 2003)
Pcmt	protein carboxyl methyl-transferase	protein repair	D. melanogaster	(Chavous et al., 2001)
p53	tumor protein p53	tumor suppression	M. musculus	(Tyner et al., 2002)
Sir2	NAD(+)-dependent deacety-lase	regulation of metabolism, stress resistance	S. cerevisiae	(Kaeberlein et al., 1999)
Sod1	superoxide dismutase	antioxidant activity	D. melanogaster	(Parkes et al., 1998)
Sod2	superoxide dismutase	antioxidant activity	D. melanogaster	(Sohal et al., 1995)

*Organism, in which the gene was first shown to influence lifespan

Klotho

The *Klotho* gene, which was identified in mouse models (Kuro-o M et al., 1997), encodes a mammalian specific hormone that negatively regulates the activity of IR and IGF-1R through repressing their autophosphorylation (Kurosu et al., 2005). In mice, genetic variation in the *Klotho* gene results in an early onset of various age-related disorders, including ectopic calcification, skin- and muscle atrophy, osteoporosis, atherosclerosis, and pulmonary emphysema (Kuro-o M et al., 1997). On the other hand, over-expression of Klotho in mice leads to inhibition of insulin and IGF1 signaling and increased lifespan (Kurosu et al., 2005). In humans, a haplotype allele called KL-VS, which contains six sequence variants that are in complete linkage disequilibrium, has been associated with *KLOTHO* expression and shown to be underrepresented in elderly individuals (Arking et al., 2002). Additional studies have demonstrated that the survival advantage is only present in heterozygous KL-VS allele carriers, whereas in homozygous allele carriers a disadvantage for HDL-cholesterol levels, systolic blood pressure, stroke, and longevity was observed (Arking et al., 2005).

Forkhead transcription factors

In *C. elegans*, the IIS pathway negatively regulates the activity of DAF-16, which is its main downstream target. The long-lived phenotype of the IIS mutants depends on the presence of an

active DAF-16 protein (Mukhopadhyay and Tissenbaum, 2007). In mammals, the DAF-16 homologues are forkhead transcription factors (FOXOs): FOXO1a, FOXO3a, FOXO4 and FOXO6 (Furuyama et al., 2000). Similar to DAF-16 in *C. elegans*, in mammals the FOXO proteins relay the effects of insulin on lifespan, influence fertility, and play a role in complex diseases such as diabetes (Carter and Brunet, 2007). In humans, only few studies have analyzed the role of FOXO proteins in the development of age-related diseases, fertility and lifespan. In most studies, no associations between genetic variance in the *FOXO1a* and *FOXO3a* genes and lifespan have been detected (Bonafe et al., 2003; Kojima et al., 2004). These findings, contrast with recent studies where genetic variance in the *FOXO1a* gene was linked to increased glucose levels (Karim et al., 2006; Kuningas et al., 2007a) increased risk of diabetes and decreased lifespan (Kuningas et al., 2007a). Support for these findings has come also from the Framingham Heart Study (personal communications). For genetic variance in the *FOXO3a* gene, associations were observed with increased risks of stroke and mortality, but not with fertility (Kuningas et al., 2007a).

DAF-12

In *C. elegans*, DAF-12 is a member of the evolutionarily conserved Nuclear Hormone Receptor (NHR) super-family (Mangelsdorf et al., 1995) and it has been implicated in dauer diapause, developmental timing, metabolism, fertility, and longevity. Current data have positioned DAF-12 downstream of the insulin- and germline signaling, as the long-lived phenotype of germline ablated mutants and of some IIS mutants depends on DAF-12 activity, but the exact position is still unknown (Rottiers and Antebi, 2006). In humans, the NHRs most similar to DAF-12 are the Liver X Receptors (alpha and beta), which have cholesterol breakdown products (oxysterols) as ligands. Upon activation, LXRs regulate various processes that result in cholesterol excretion from the body (Zelcer and Tontonoz, 2006). Recently, a common haplotype of the *LXRA* gene was associated with increased survival, predominantly due to lower mortality from cardiovascular causes and infection (Mooijaart et al., 2007a). A possible mechanism through which LXR could lead to the observed beneficial effects includes involvement of its target gene Apolipoprotein E (APOE). ApoE is an anti-atherosclerotic protein involved in the efflux of cholesterol from macrophages. Genetic variation in *APOE* has consistently been associated with cognitive decline and cardiovascular disease mortality. Moreover, we have recently shown that independent of genetic variation in *APOE*, high plasma apoE levels associate with increased risk of stroke (van Vliet et al., 2007), increased risk of cardiovascular mortality (Mooijaart et al., 2006) and decreased cognitive functioning (Mooijaart et al., 2007b). These data support previous observations that lipoprotein metabolism is critical for exceptional longevity. It has been shown that families of Ashkenazi Jewish centenarians have larger particles of HDL and LDL, which are associated with a decreased incidence of metabolic syndrome, cardiovascular disease and hypertension (Barzilai et al., 2003). Also in the Dutch Caucasian population, offspring of long-lived sibling pairs have larger LDL particles than their age-matched partners, again suggesting that larger LDL particles confer a survival benefit (Heijmans et al., 2006).

Sirtuins

The Sirtuins represent an evolutionarily conserved family of Silent Information Regulator 2

(Sir2) NAD-dependent protein deacetylases that interact with and influence the activity of various transcription factors and co-regulators (Bordone and Guarente, 2005). Increased expression of the *Sir2* gene, either due to an extra copy of the gene or to caloric restriction, prolongs lifespan in various model organisms (Haigis and Guarente, 2006). In mammals, there are seven Sir2 homologues (SIRT1-7), of which SIRT1 is the most closely related to Sir2 (Frye, 2000). In mouse models, SIRT1 and SIRT3 have been studied the most. SIRT1 has been associated with glucose and fat metabolism, stress resistance and cell survival (Haigis and Guarente, 2006), whereas SIRT3 regulates the activity of acetyl-CoA synthetase (AceCS), and thereby the entry of carbons from acetate into central metabolism (Haigis and Guarente, 2006). In humans, polymorphisms within *SIRT1* and *SIRT3* genes have been analyzed for association with age-related diseases and longevity. In case of *SIRT1*, no associations have been found (Flachsbart et al., 2006; Kuningas et al., 2007b), whereasof *SIRT3*, a G477T polymorphism and a variable number of tandem repeats (VNTR) have been associated with increased lifespan (Bellizzi et al., 2005; Rose et al., 2003). These results demonstrate that at least one member of the SIRT family is involved in human lifespan regulation.

Antioxidative enzymes

Antioxidative enzymes, such as catalase and superoxide dismutase (SOD), prevent damage from reactive oxygen species (ROS), but the evidence from model organisms on the beneficial effects of antioxidative enzymes on lifespan has been controversial. Studies with *D. melanogaster* have demonstrated that overexpression of CuZn-SOD (SOD1), Mn-SOD (SOD2), and catalase lead to lifespan extension (Orr and Sohal, 2003; Sohal et al., 1995). Additional experiments, however, showed that this effect depends on genetic background of the used lines (Orr and Sohal, 2003). Likewise, the extended lifespan of *C. elegans* by administration of synthetic SOD/catalase mimetics was shown to depend on laboratory conditions (Keaney and Gems, 2003; Melov et al., 2000).

In mammals, one catalase and three SOD genes have been characterized; SOD1, SOD2 and SOD3, of which Catalase and SOD2 seem to influence lifespan. In mice, disruption of the *SOD2* gene is lethal due to neurodegeneration and to damage to the heart (Li et al., 1995; Melov et al., 1998). In contrast, overexpression of *SOD2* leads to increased lifespan (Hu et al., 2007), as does overexpression of *Catalase* targeted to mitochondria (Schriner et al., 2005). Mice heterozygous for the mitochondrial form of *SOD2* showed high levels of DNA oxidation in multiple organs. In spite of their abnormally oxidized DNA, these animals showed no decline in lifespan and no acceleration in the hallmarks of aging, such as cataracts, immune dysfunction, and protein modifications (Van Remmen et al., 2003). These data suggest that mice can live reasonably long and healthy lives despite unusually high levels of oxidative damage.

The evidence for the role of antioxidative enzymes in the preservation of human health is not well established. It has been shown that RNA interference (RNAi) of SOD1 induces senescence in human fibroblasts (Blander et al., 2003), which suggests that SOD1 may play a role in the regulation of cellular lifespan. However, genetic variants in the *SOD1* gene have never been studied for that relationship. In contrast, genetic variants in the *SOD2* gene have been studied and associated with a number of phenotypes including increased risk for prostate- and breast cancer, immunosenescence profile, and DNA damage (Liu et al., 2004; Taufer et al., 2005), but

not with mortality (De Benedictis et al., 1998; van Heemst et al., 2005a). Likewise, no associations between genetic variants in the *Catalase* gene and mortality have been found (Christiansen et al., 2004).

Macromolecule repair mechanisms

Defects in mechanisms that repair damage to cellular components, such as DNA, proteins and membranes have been shown to reduce lifespan in various model organisms. Even though these mechanisms are evolutionarily conserved (Eisen and Hanawalt, 1999), systematic comparative genomic analyses across species have not been conducted. In addition, within species, there are many studies demonstrating detrimental effects of impaired repair systems on lifespan, but only few demonstrating beneficial effects of increased repair capacity on lifespan. The only evidence for the latter has come from experiments with *D. melanogaster*, where the absence of *mei-41* excision repair reduces lifespan, whereas flies with one- or two extra copies of the gene have significantly increased lifespan (Symphorien and Woodruff, 2003). Likewise, overexpression of protein carboxyl methyltransferase (*PCMT*), which is a protein repair enzyme, has been correlated with enhanced longevity in a temperature-dependent manner (Chavous et al., 2001). Both of these genes, *mei-41* and *Pcmt* have homologues in mammals, which are ataxia telangiectasia and Rad3 related (*ATR*) and *PCMT*, respectively. In mice, the disruption of the *ATR* gene leads to chromosomal fragmentation and early embryonic lethality (Brown and Baltimore, 2000), and in humans to a rare Seckel syndrome (Casper et al., 2004). The *Pcmt1*-null mice, on the other hand, display a fatal seizure disorder and retarded growth (Kim et al., 1999), and die at a mean age of 42 days (Lowenson et al., 2001).

Compared to the other repair mechanisms, DNA repair has been studied the most in relation to ageing and lifespan. The DNA repair deficient mouse models that have been generated, display a common phenotype of segmental premature ageing (progeria), or cancer predisposition, or both, and have a reduced lifespan (Hasty et al., 2003). Similarly, in humans, all mutations identified in DNA repair genes severely compromise health. For instance, mutations in the components of transcription coupled repair (TCR) have been associated with the premature ageing syndromes of Cokayne syndrome (CS) and Trichthiodystrophy (TTD) (Cleaver, 2005; Hoeijmakers, 2001). Likewise, mutations in the RecQ-like DNA helicase genes, *WRN*, *BLM* and *RecQ4* lead to the premature ageing syndromes of Werner-, Blooms-, and Rothmund–Thomson-Syndrome, respectively (Navarro et al., 2006). In contrast to the strong phenotypes associated with mutations in RecQ helicases, common polymorphisms in these genes do not seem to influence the aging-trajectories and survival in the general population (Bohr et al., 2004; Castro et al., 2000; Kuningas et al., 2006). The RecQ helicases are highly conserved throughout evolution, but in higher eukaryotes, the different homologues seem to have distinct functions because failure of one given RecQ gene cannot be complemented by another RecQ gene. These observations underpin the importance of DNA repair in all organisms. The key question that has yet to be answered is whether subtle variants in the DNA repair genes contribute to different lifespans and whether above average repair makes for a lifespan extension.

Cellular responses to damage

In response to unrepaired damage, cells trigger either apoptosis or cell cycle arrest. The most well known protein implicated in the maintenance of genomic stability is p53. Recently, p53 homologues were identified in *C. elegans* and *D. melanogaster*. In contrast to mammalian p53, which elicits apoptosis or cell-cycle arrest (Attardi, 2005), the p53 in *C. elegans* and *D. melanogaster* affects only apoptosis (Brodsky et al., 2004; Derry et al., 2001; Schumacher et al., 2001). Nevertheless, in all of these organisms, reduced p53 activity leads to lifespan extension (Bauer and Helfand, 2006). In mammals, this extension comes at the cost of increased cancer risk (Campisi, 2003). In humans, it has been shown that Pro/Pro carriers of the TP53 codon 72 polymorphism have a significantly lower apoptotic potential than Arg/Arg carriers, both in p53-inducible human cell lines (Dumont et al., 2003; Pim and Banks, 2004; Sullivan et al., 2004) and in normal diploid fibroblasts (Bonafe et al., 2004). Later on it was shown that despite an increased mortality from cancer, carriers of the same polymorphism have a significantly increased survival at old age in line with the experimental models (van Heemst et al., 2005b). Altogether, these observations support the hypothesis that reduced p53-mediated induction of apoptosis can have beneficial effects on lifespan if tumor formation can be avoided. This might hold true also for other genes that mediate cellular responses to damage.

Discussion

Experiments in model organisms have demonstrated that changes in genes can dramatically increase their lifespan. In some cases, mean and maximum lifespan is extended up to fivefold. The equivalent life extending effect in humans would result in an average lifespan of 400 years and maximum lifespan of over 600 years. Many of the pathways regulating lifespan in model organisms are conserved throughout evolution. Why then, have we not yet identified genetic determinants that could increase human lifespan by more than a few years? Are we looking at the right genes? Is it fair to expect such dramatic effects? Do we have the tools to observe genetic determinants of human lifespan?

Increased complexity

In vertebrate organisms novel genes and signaling components have appeared during evolution, contributing to increased complexity of the genomes (Long, 2001). Among several molecular mechanisms, gene duplication plays a major role in genome evolution (Britten, 2006; Long, 2001). Often, a mammalian genome contains several homologues of a single invertebrate gene with similar or distinct functions and expression patterns. This can hinder the assessment of the role of a specific candidate gene, since genetic variance in duplicated genes is likely to have less dramatic effects than in the original single gene in invertebrate organisms (Conant and Wagner, 2004; Gu et al., 2003). Furthermore, since vertebrates have acquired pathways that are more diverse, the homologous genes in phylogenetically distant animals could be involved in different pathways.

The genomes of vertebrate organisms also contain genes, which have no homologues in

invertebrates. The appearance of novel genes, or "add-ons", probably contributed to the arise of elaborate systems that regulate the activity of the conserved or "shared" genes (Levine and Tjian, 2003). The GH control over IGF-1 and the regulation of IR and IGF1-R activity by KLOTHO have arisen relatively recently in vertebrate evolution (Forsyth and Wallis, 2002; Mian, 1998). Genetic variance in these genes has been associated with lower IIS signaling and with increased lifespan, corroborating the findings from invertebrates. However, in these organisms this phenotype was attained by modifying the "shared" genes. This demonstrates that in humans, we should not limit ourselves to studying the evolutionarily conserved genes that have been implicated in lifespan regulation in invertebrates, but also include, and maybe even focus on, "add-ons" that regulate their activity. Taken together, the increased complexity of signaling systems in vertebrates adds robustness to the signaling pathways, which is the ability to maintain its functions despite changes in its components or environment (Lenski et al., 2003; Soyer and Bonhoeffer, 2006). In that respect, lifespan regulation can be regarded as a complex genetic trait, for which it is unlikely that single alterations in the genetic machinery will have dramatic effects. Furthermore, genes that have appeared later in evolution, "add-ons" are interesting candidates to determine longevity in vertebrate organisms.

Environmental influences

Environmental influences have played a major role in shaping and patterning the genomes of all organisms throughout evolutionary history. Changes in environment can lead to different expression of genotypic information, and thereby complicate the comparability of results between model organisms and humans. Most research on model organism has been performed under laboratory conditions, where temperature, presence of pathogens, food availability and population density are tightly controlled. In most cases these conditions poorly mimic the evolutionary niche in which the genes come to expression and it may therefore be questioned how well the results obtained in these condition are applicable to species under "natural" conditions (Clancy et al., 2001; Marden et al., 2003; Walker et al., 2000).

Even for humans, the environment in which the genome effectively evolved has changed. The genes that were originally selected for survival in adverse environments are now expressed under completely new, affluent conditions. For instance, the IIS system was selected and fine-tuned in times when food abundance and famine alternated. The genotypes that increased the efficiency to store energy in times of abundance and use these storages in times of famine had a survival advantage. In modern Western societies, where food is constantly abundant these "thrifty genotypes" lead to increased prevalence of storage diseases, such as obesity and diabetes (Neel, 1962). This reinforces the idea that our population genome has been optimized to increase fitness under adverse conditions whereas this need not to be the case under modern affluent conditions, the outcome of these new interactions are both unknown and unpredictable. Taken together, caution should be taken in extrapolating results on genetic variation obtained in model organisms to the human situation, since the environments in which the genes come to expression are often markedly different. Moreover, research into human ageing should include various environmental conditions that can explain for different phenotypes despite an equal genetic background

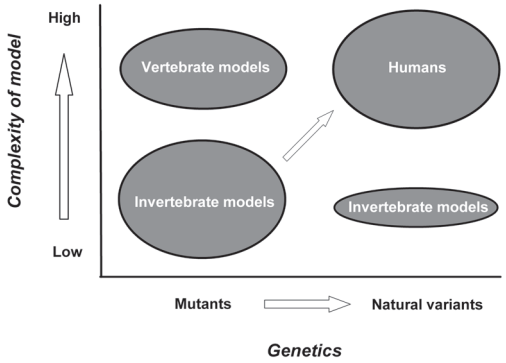

Figure 1. The amount of information obtained from different organisms on genetic mechanisms on ageing and longevity. The amount of available information on the effect of genetic variation on life span in organisms of different complexity is indicated by the size of the four quadrants. As can be seen, in humans most information on the effect of genetic variation on longevity is being obtained by studying naturally occurring variants, while in model organisms, most information on the effect of genetic variation on longevity has been obtained by studying the effect of mutations in invertebrate models, and, to a lesser extent, in vertebrate models. In contrast, only few studies have analyzed the contribution of standing natural genetic variation on lifespan in model organisms. The direct translation of the importance of candidate genes identified in mutant invertebrate models for variation in human life span is complicated by two possible discrepancies. The first includes the influences of mutations and standing (subtle) genetic variations in the candidate loci and the second possible discrepancy includes the differences in genome complexity between the organisms.

Mutants versus natural genetic variants

The majority of candidate genes of longevity have been identified by studies with mutant model organisms, such the worm, fruit fly and mouse models. These approaches are extremely powerful to disentangle biological pathways. However, to date it is largely unknown to what extent these mutations affect fitness in natural environments and whether these candidate loci contain genetic variation, which would contribute to phenotypic variance for lifespan in natural populations (Figure 1). These questions are of importance since not all candidate loci with major effects on longevity in laboratory conditions may exhibit variation in natural populations. Currently, only few studies have tried to disentangle this question. For instance, it has been shown that the long-lived mutant fruit fly methuselah (*mth*) underperforms in most cases under conditions that resemble more a natural situation (Baldal et al., 2006). This illustrates that the mth locus would never have been identified to influence lifespan if natural populations of the fruit fly would have been analyzed. A similar observation is obtained for the *Chico* variant that outlives the wild type under food affluence but becomes short lived when exposed to food restricted conditions (Clancy et al., 2002). In case of humans, most information on the effect of genetic variation on longevity is being obtained by studying naturally occurring variants in candidate genes identified in mutant model organisms. Hence, the discrepancy between the data from

mutant model organism and standing genetic variance in natural populations contributes to the difficulty of translating results from model organisms to humans (Figure 1). Therefore, to facilitate translation of the results from mutant model organisms to humans, the analysis of standing variation in the same loci in natural populations of model organisms should be encouraged.

Methodological considerations

The lack of very strong effects of the evolutionarily conserved genes in humans can have other reasons than those suggested above. Some of these include study design and methodology in human studies. Most of the genetic association studies of longevity have been performed in case-control settings, where genotype- or allele frequencies between elderly and a younger population are compared. The main advantage of case-control study design is that cases are readily obtainable and can be efficiently genotyped and compared with control populations. Cases can be nonagenarians, centenarians or long-lived sibpairs. By collecting long-lived sibpairs instead of long-lived singletons, an enrichment of genetic factors contributing to longevity in this population can be expected, while the likelihood of having reached a long life because of exceptional environmental conditions or chance is lower. The difficulty of case-control studies is in selecting controls. In longevity studies, the ideal control group should be composed of participants from the same birth cohort who were not long-lived, to minimize the effect of environmental differences caused by cohort differences. However, obtaining such a control group is impossible in a retrospective study design. Such case-control studies are only possible if one is studying in long-lasting population surveys. The longest ongoing studies (e.g. Framingham Heart Study) now allow a difference of 30 years between subjects that survived and those that did not. However, in most studies so far, controls have been selected from the general population of younger generations. This, can lead to biases where allele frequency differences between cases and controls can appear as an association, even if they only reflect the results of changes to the source population due to changes in environment, migratory history, gender differences, or other independent processes (Cardon and Palmer, 2003; Manolio et al., 2006). An alternative approach would be prospective cohort studies, which suffer less from population stratification but are more expensive and time-consuming (Manolio et al., 2006).

Another consideration for the methodology includes the selection and analysis of genetic variants in the candidate genes. Commonly, a selected number of polymorphisms from the coding region of candidate genes are analyzed, leaving aside genetic variants in regulatory regions. In addition, besides analyzing the individual polymorphisms only few studies have undertaken haplotype analyses. Given the amount of information that has recently become available through the International HapMap project, polymorphisms that tag common haplotypes can easily be identified. The analysis of haplotypes can be more powerful since this analysis captures the joint effect of all unknown gene variants that are in linkage disequilibrium with the markers forming the haplotype (Johnson et al., 2001).

Finally, similar to other association studies, also the results from longevity association studies have been rarely replicated. Explanations for lack of reproducibility include poor study design, small sample size, incorrect assumptions about the underlying genetic architecture, and over interpretation of the data. In addition, for a number of associations (mainly negative) no replication has been undertaken, leaving open the reproducibility. Replication of even negative

results is necessary, since the lack of associations in the first study could likewise have been due to poor study design, population stratification or other reasons. Therefore, before discarding a candidate gene from the list of possible candidates, replication in different cohorts with more thorough genetic analysis is necessary.

Future directions

Pathway analyses and epigenetic variation

Given the current feasibility of high throughput genotyping and increasing knowledge on cellular mechanisms, pathway analyses instead of analyzing individual loci separately could be performed. The appropriate tools are likely to be available soon, since the analysis of complex traits, which are under the influence of multiple and possibly interacting genes, has become a subject of new statistical methodological research (Kristensen et al., 2006). However, besides genetic variation, other mechanisms influence the expression of genomic information. For instance, epigenetic modifications, which are differences in gene expression that cannot be accounted for by changes in the primary DNA sequence, have a significant impact on gene function, and may explain how iso-genetic organisms are phenotypically very distinct. Likewise, a different level of transcriptional and post-transcriptional control through RNA interference, or other mechanisms, can account for phenotypic differences. These and other modifications contribute to the differences in lifespan between and within species, and this type of information from model organisms and humans is essential for the extrapolation of results.

Genes and pathways for future

A number of interesting candidate genes, with or without homologues in model organisms remain to be investigated in humans. The most interesting and so far not very thoroughly analyzed genes in respect to longevity include those involved in fertility. In model organisms, fertility and lifespan are closely linked (Partridge et al., 2005). In *C. elegans*, ablation of germline precursor cells of the gonad abolishes reproduction and extends lifespan (Hsin and Kenyon, 1999), as do mutations that reduce germline proliferation (Arantes-Oliveira et al., 2002). In *D. melanogaster*, a reduction in fecundity extends lifespan in females (Sgro and Partridge, 1999) and long-lived heterozygous chico mutant females exhibit reduced fecundity, with the homozygotes being almost sterile (Clancy et al., 2001). In mice, Ames and Snell dwarfs are long-lived and sterile (Bartke, 2005). The observational studies in historical human populations living under pre-affluent conditions have provided similar evidence. In the English and Finnish aristocracy, women with the longest lifespan had the smallest number of offspring (Korpelainen, 2000; Westendorp and Kirkwood, 1998). Despite this evidence, the genetic determinants for the trade off between fertility and lifespan are unknown.

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CHAPTER 10

Samenvatting/Summary in Dutch

Samenvatting

Genetische factoren spelen een belangrijke rol bij variatie in de levensduur van mensen. In de afgelopen jaren zijn, vooral in modelorganismen, een aantal genen geïdentificeerd die betrokken zijn bij langlevendheid. Een aantal van de geïdentificeerde genen zijn interessante kandidaten voor studie naar de snelheid van veroudering en langlevendheid bij de mens.

Hoofdstuk 1 bevat een algemene inleiding met een overzicht van de kandidaatgenen die in dit proefschrift zijn onderzocht in relatie met aan leeftijds gerelateerde ziekten en langlevendheid. Alle onderzoeken die in dit proefschrift worden beschreven zijn uitgevoerd binnen de Leiden 85-plus Studie, een prospectief bevolkingsonderzoek onder de oudste ouderen in de stad Leiden.

Hoofdstuk 2 beschrijft het onderzoek naar de effecten van genetische variatie in de evolutionair geconserveerde genen *FOXO1* en *FOXO3a* op de prevalentie van aan leeftijd gerelateerde ziekten en langlevendheid. De humane *FOXO1a* en *FOXO3a* genen zijn homoloog aan het *daf-16* gen in *Caenorhabditis elegans*. Deze genen coderen voor transcriptiefactoren die zowel bij de mens als bij *C. elegans* het belangrijkste doelwit zijn van een door insuline/IGF-1 aangestuurd signaaltransductie-systeem. Eerder onderzoek heeft uitgewezen deze signaalroute de activiteit van Daf-16 negatief beïnvloeden. Een lager signaal en hogere Daf-16 activiteit zijn geassocieerd met een langer leven in verschillende modelorganismen. Bij de mens zijn zowel FOXO1 als FOXO3a betrokken bij verschillende cellulaire functies, zoals metabolisme, cel differentiatie en reparatie van DNA. Bovendien speelt FOXO1a een specifieke rol bij het effect van insuline op de glucoseproductie in de lever. FOXO3a speelt een rol bij de vruchtbaarheid in vrouwen. De analyses van genetische variatie in de *FOXO1a* en *FOXO3a* genen lieten zien dat deze genen ook in de mens bij het reguleren van levensduur betrokken zijn. De genvariaties in *FOXO1a* bleken geassocieerd met een hoger risico op mortaliteit, dat verklaard kon worden door een verhoogde sterfte door diabetes. De ouderen die drager waren van een van deze genvariaties hadden ook verhoogde HbA1C concentraties, een uiting van een slechtere glucose huishouding en *diabetes mellitus*. Tevens bleken dragers van een variant in het *FOXO3a* gen een verhoogd sterfterisico te hebben, in het bijzonder aan hart- en vaatziekten. Ook het risico op een hartinfarct was groter. Anders dan verwacht werd er géén relatie gevonden tussen varianten van het *FOXO3a* gen en metabole parameters of vruchtbaarheid. Deze bevindingen tonen aan dat genetische variatie in *FOXO1a* en *FOXO3a* invloed heeft op levensduur.

In hoofdstuk 3 en 4 wordt respectievelijk de rol van *LXRA* en die van *VDR* op de levensduur onderzocht. Beide genen zijn humane homologen van het *daf-12* gen in *C. elegans*. In *C. elegans* is Daf-12 betrokken bij de regulatie van de overgang naar het stress-resistente "dauer" stadium, dat wordt geïnduceerd in reactie op nadelige omgevingsinvloeden, zoals voedseltekort. Mutaties in het *daf-12* gen kunnen leiden tot de vorming van wormen die geen "dauer" stadium meer kennen en wormen die permanent in het "dauer" stadium verblijven. Deze wormen zijn respectievelijk lang- en kortlevend. Onze genetische analyses van het *LXRA* gen in de mens toonden aan dat een variant van dit gen met een lager risico op mortaliteit is geassocieerd, gekenmerkt door een verminderde sterfte aan infectieziekten. Dragers van deze genvariant hadden ook hogere concentraties van het apolipoproteine E, waarvan de transcriptie (deels) wordt gereguleerd door LXRA, en ook door hogere concentraties van triglyceriden. Deze resultaten wijzen erop dat genetische variatie in het *LXRA* gen via metabole processen invloed heeft op de

levensduur van de mens. In hoofdstuk 4 laten we zien dat we géén associaties tussen genetische variatie in het *VDR* gen en levensduur konden aantonen. Variaties in dit gen bleken wel geassocieerd met cognitieve functies en met symptomen van depressie, hetgeen suggereert dat het *VDR* gen een rol speelt in cognitieve achteruitgang.

In hoofdstuk 5 worden de resultaten beschreven van onderzoek aan het *SIRT1* gen. Dit gen is een van de humane homologen van het *Sir2* gen van de gist *Saccharomyces cerevisiae*. Eerder onderzoek aan modelorganismen heeft aangetoond dat overexpressie van het *Sir2* gen leidt tot een langer leven. In zoogdieren is SIRT1 betrokken bij het behoud van neuronen, bij metabolisme, en bij de resistentie van cellen tegen stress. Wij hebben onderzocht of genetische variatie in *SIRT1* in de mens invloed heeft op sterfte, cognitieve functies en aan leeftijd gerelateerde ziekten. De resultaten lieten zien dat een variant van dit gen geassocieerd is met een lager risico op mortaliteit als gevolg van hart- en vaatziekten en met cognitieve functies. Ondanks deze relaties met het optreden van aan leeftijd gerelateerde ziekte werd géén relatie gevonden met de verschillende parameters van het metabole profiel. Deze resultaten kunnen wijzen op een rol van *SIRT1* in cognitieve functies, maar omdat een associatie met het metabole profiel ontbrak is de relatie met langlevendheid onzeker.

In hoofdstuk 6 hebben we de invloed van het stresshormoon cortisol en van genetische variaties in de mineralocorticoid receptor (*MR*) en de glucocorticoid receptor (*GR*) genen op cognitief functioneren en depressieve symptomen bij ouderen geanalyseerd. Cortisol speelt, via de *MR* en *GR*, een belangrijke rol in de coördinatie van reacties op stress. Aan stress gerelateerde ziekten zoals depressie gaan vaak samen met overmatige secretie van cortisol. Verder beïnvloedt cortisol ook het cognitief functioneren. Het vermogen om adequaat met stress om te gaan en een behoud van een goede geestelijke gezondheid zijn essentieel voor een lang leven. In onze studies waren hogere cortisol concentraties geassocieerd met een achteruitgang van globale cognitieve functies bij aanvang van de studie en gedurende de follow-up. De achteruitgang van de cognitieve functies kon met name worden toegewezen aan een verminderde concentratie, en aan een verlaagde cognitieve snelheid. Er werd echter geen invloed van *MR* en *GR* gen varianten op cognitieve functies geobserveerd. In tegenstelling tot de cognitieve functies was de prevalentie van depressie symptomen van verschillend bij dragers van een of meerdere *MR* gen varianten. Deze resultaten tonen aan dat cortisol een belangrijke rol speelt in cognitief functioneren en dat genetische variatie in het *MR* gen het voorkomen van symptomen van depressie beïnvloedt.

Eerder onderzoek heeft uitgewezen dat veranderingen in het cortisol signaal ook het risico op hart- en vaatziekten beïnvloeden. Hart- en vaatziekten zijn de voornaamste doodsoorzaak onder ouderen. In hoofdstuk 7 wordt de invloed geanalyseerd van *GR* gen varianten op het risico op hart- en vaatziekten en op mortaliteit. De resultaten lieten zien dat varianten van het *GR* gen het metabole profiel beïnvloeden, maar niet het risico op hart- en vaatziekten en sterfte op hoge leeftijd. De invloed van genetische variaties in het *MR* gen op het optreden van aan leeftijds gerelateerde ziekte en mortaliteit moet nog onderzocht worden.

In 1996 werd het *WRN* gen geïdentificeerd, dat gemuteerd is bij het Werner syndroom. Het Werner syndroom is een zeldzame aandoening, die gepaard gaat met een versnelde, vroegtijdig optredende veroudering. De vraag is of subtielere variatie in het *WRN* gen zou kunnen leiden tot een versnelde en/of vroegtijdig optredende veroudering. Daarom hebben we in hoofdstuk 8 de invloed van veel voorkomende varianten in het *WRN* gen op veroudering en langlevendheid in de algemene populatie onderzocht. Onze analyses toonden géén verschillen in het risico

op hart- en vaatziekten, niet in cognitieve functies en niet in het risico op sterfte. In onze studiepopulatie vonden wij dus géén aanwijzingen dat het *WRN* gen invloed heeft op variatie in levensduur.

In Hoofdstuk 9 worden de resultaten van al deze onderzoeken samengevat en in een breder perspectief besproken. De studies laten zien dat kandidaatgenen uit experimentele modellen in de meeste gevallen aantoonbaar het metabolisme en of het optreden van aan leeftijdsgerelateerde ziekten en levensduur bij de mens beïnvloeden. Echter, in vergelijking met de modelorganismen zijn de effecten bij de mens veel kleiner. Dit zou verklaard kunnen worden door de grotere complexiteit van het humane genoom, of omdat de interacties tussen genen en de omgeving bij de mens complexer zijn. Desalniettemin blijkt een strategie waarbij kandidaatgenen uit experimentele modellen voor langlevendheid in de mens worden geanalyseerd een belangrijke bijdrage te kunnen leveren aan de identificatie van biologische mechanismen die belangrijk zijn voor het optreden van aan leeftijd gerelateerde ziekten en een lange levensduur.

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Kuningas M, Mooijaart SP, van Heemst D, Zwaan BJ, Slagboom PE, Westendorp RGJ. Genes encoding longevity; from model organisms to man. *Submitted for publication*

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Curriculum vitae

Maris Kuningas was born on July 21, 1979 in Kuressaare, Estonia. She spent her childhood in Võhma, where she also went to school. After her graduation from the secondary school in 1997 she continued her education in Tartu University, Estonia, at the department of Biotechnology and Biomedicine. She obtained her Bachelor in Science (BSc) degree in Biotechnology and Biomedicine in 2001. At the same department she obtained her Masters' in Science (MSc) degree in Molecular Biomedicine in 2003. From 2004 to 2007 she was a PhD student at the department of Gerontology and Geriatrics of the Leiden University Medical Center, The Netherlands. Since January 2007 she is employed as a PostDoc at the department of Gerontology and Geriatrics of the Leiden University Medical Center on the project "LifeSpan" (FP6 036894), which is an EU funded Network of Excellence that integrates research into development and ageing.