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Impact of genetic variations in the *WRN* gene on age related pathologies and mortality

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Abstract

Mutations in the *WRN* gene lead to the Werner syndrome (WS), which resembles premature aging. Here, we hypothesize that genetic variations in the *WRN* gene may also influence aging-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. Also 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 aging-trajectories and survival in the population at large.

Keywords: *WRN* gene; i1-C/T, L1074F and C1367R polymorphisms; Cohort

1. 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 aging. 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 aging, it has been reasoned that polymorphisms in the *WRN* gene may also associate with age related pathologies and thus influence aging 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 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 aging in the population at large (Bird et al., 2003; Hasty et al., 2003). Since WS resembles

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accelerated aging, it has been suggested that the *WRN* gene may also modulate the course of aging 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., 2004), 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 aging trajectories, since at that age the effects of truly functional variations should be most pronounced.

2. Subjects and methods

2.1. Study population

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 (Bootsma-van 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 1 April 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).

2.2. 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).

2.3. 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).

2.4. 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/ionization 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.

2.5. 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 (TX, USA).

3. 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 Fig. 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

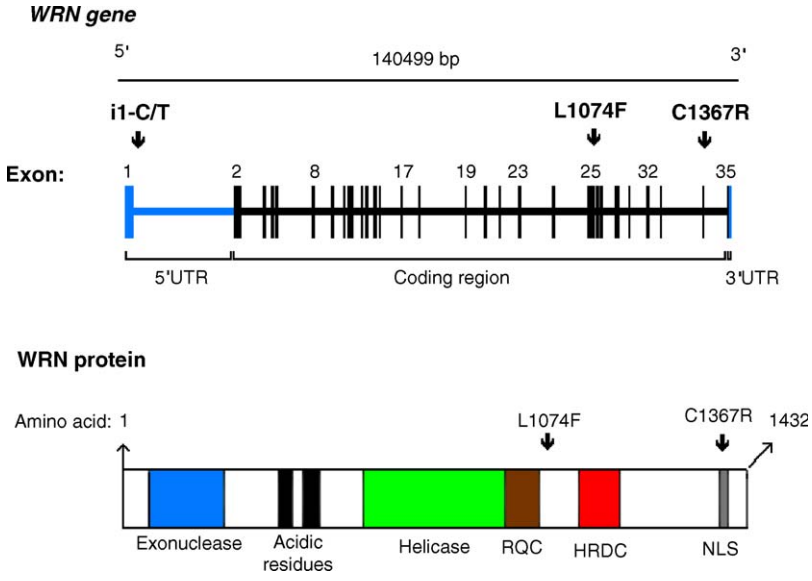

Fig. 1. The *WRN* gene and protein structure with a graphic representation of the i1-C/T, L1074F and C1367R SNP localization. The positions of the SNPs in the *WRN* gene and protein are indicated with arrows. The *WRN* gene is located in chromosome 8 and it spans a genomic region of 140 kb, containing 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.

and resulting allelic frequencies of the SNPs were similar to these found in Caucasians (Bendixen et al., 2004; Castro et al., 1999, 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).

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. Values in parenthesis are in percentage.

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).

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 '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 more accurate estimates. During the total follow-up of 5164

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

	i1-C/T		L1074F			C1367R		
	CC	CT[a]	LL	LF[a]	FF[a]	CC	CR[a]	RR[a]
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.
[a] OR (95% CI).

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[a]	CT[a]	LL[a]	LF[a]	FF[a]	CC[a]	CR[a]	RR[a]
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. S.E.: standard error.
[a] Mean (S.E.).

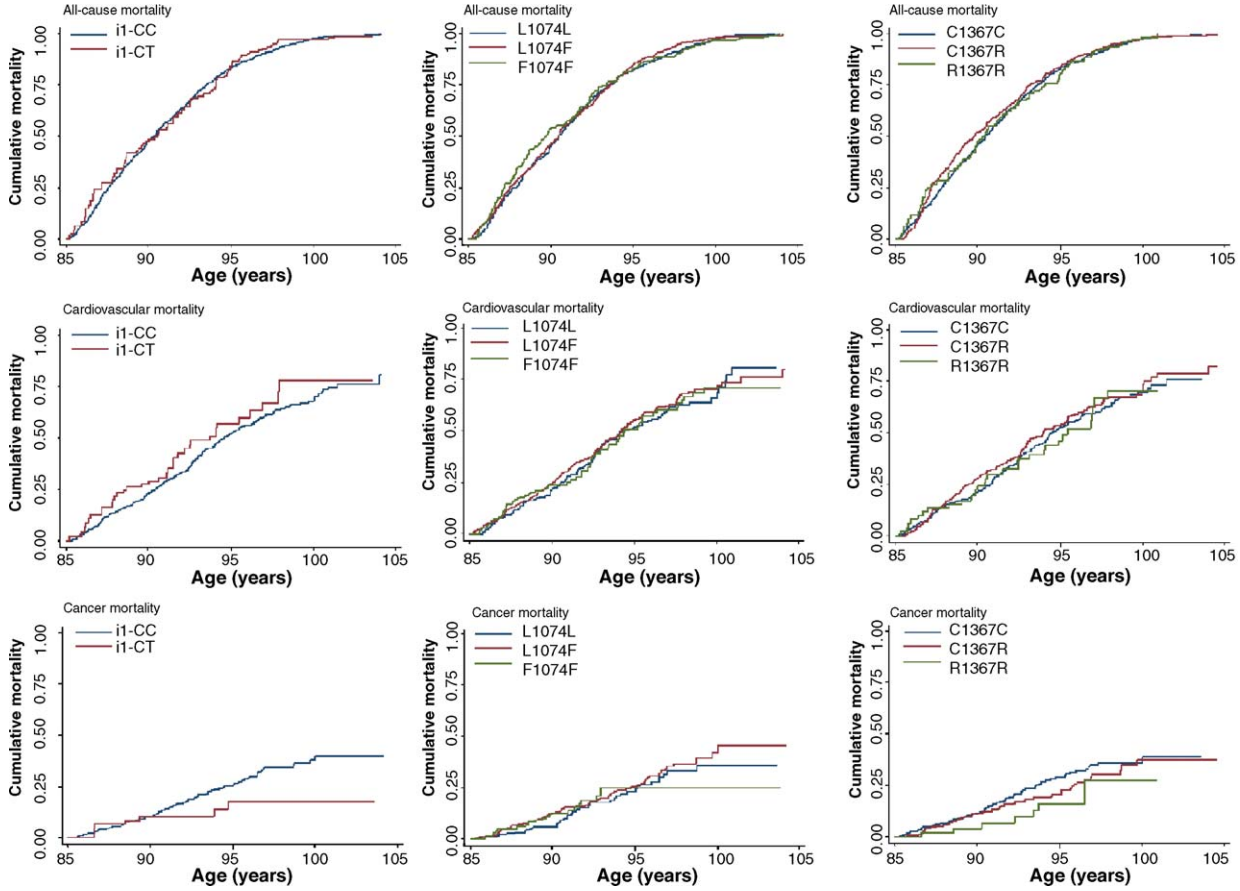

Fig. 2. Kaplan–Meier curves of cumulative all-cause, cardiovascular and cancer mortality, dependent on i1-C/T, L1074F and C1367R genotypes in the combined cohort of 1245 individuals from age 85 years onwards.

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 Fig. 2. According to the Kaplan–Meier mortality curves, it seems that carriers of the i1-CT genotype, and heterozygous and 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.

4. 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.

From the three 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., 2004). 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 aging. 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 aging.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mad.2005.11.005.

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