A study into genes encoding longevity in humans

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CHAPTER 2

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

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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).

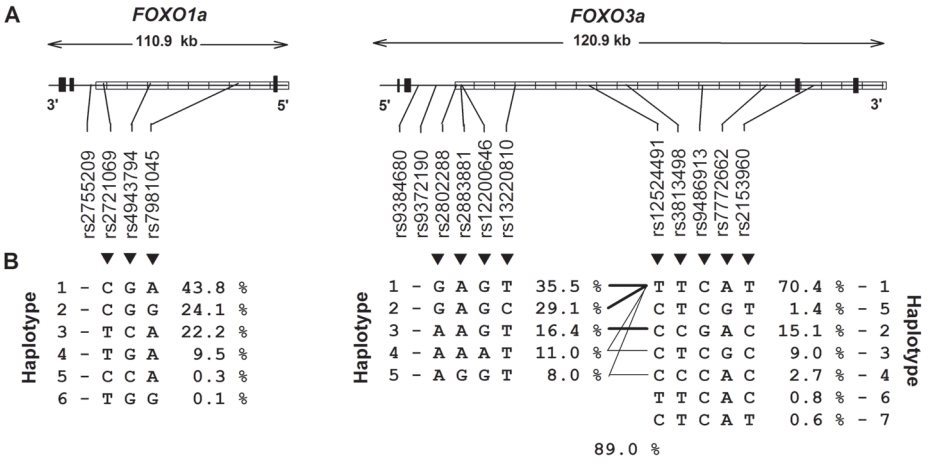

Figure 1. *FOXO1a* and *FOXO3a* gene structure, LD and haplotypes. (a) Genomic structure of *FOXO1a* and *FOXO3a* genes, where exons are represented by black boxes, and introns and intragenic regions by lines. The long striped horizontal box indicates the extent of LD based on the Hapmap data. Long vertical 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)