Common gene variants and mortality in the population at large

Proefschrift

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Stellingen behorend bij het proefschrift 'Common gene variants and mortality in the population at large'.

1. Homozygotie voor een variant van het gen dat codeert voor methyleentetrahydrofolaatreductase (MTHFR) verhoogt het risico op kanker in de algemene bevolking (dit proefschrift).

2. De factor V Leiden mutatie is niet geassocieerd met sterfte in de algemene bevolking (dit proefschrift).

3. Als genfrequenties verschillen tussen jonge en oude mensen, moet uitgesloten worden dat dit een gevolg is van regionale verschillen in genfrequenties of van preferentiële selectie van gezonde ouderen, voordat de conclusie wordt getrokken dat deze bevinding een associatie met mortaliteit weerspiegelt (dit proefschrift).

4. Genvarianten die geassocieerd zijn met een verhoogd sterfterisico in de algemene bevolking, zijn veelal een risicofactor voor meer dan één leeftijdsgerelateerde ziekte.

5. Verhoogde niveaus van homocysteïne in plasma zijn geen oorzakelijke risicofactor voor hart- en vaatziekten.

6. Koppelingsonderzoek zal een bescheiden rol spelen bij het ontrafelen van de genetica van complexe ziekten.

7. De kwaliteit van de fenotypische gegevens is bepalend voor het succes van genetisch onderzoek.

8. De identificatie van genetische oorzaken van complexe ziekten zal richting geven aan de ontwikkeling van preventieve maatregelen die bestaan uit aanpassingen in omgevingsfactoren.

9. Binnen de levenswetenschappen vindt een verschuiving plaats van hypothesegedreven onderzoek naar beschrijvend onderzoek als gevolg van de introductie van technologieën die in korte tijd zeer grote hoeveelheden gegevens genereren.

10. Het poldermodel, waarin iedereen een beetje gelijk moet krijgen, is een alibi voor de politiek om pijnlijke, maar heldere keuzes uit de weg te gaan.

11. Bij het formuleren van stellingen voor een proefschrift is er slechts beperkte ruimte voor het berijden van stokpaardjes.

Leiden, 2 november 2000
Bas Heijmans

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Chapter 1

General introduction

1.1 Introduction

Human life expectancy at birth has increased dramatically over the past one and a half centuries, particularly in Western countries. Initially, this was the result of major reductions in childhood and early adult mortality, owing to improving standards of living, public-health measurements and medical progress, but since 1950 it is also the result of a decline in mortality rates at older ages.[1] Consequently, elderly individuals constitute a substantial and rapidly growing part of the population. The proportion of the population aged 65 years and over is projected to increase from 16% in 2000 to 30% in 2050 in Western European countries. The group aged 80 years and over is expected to increase even more, from 4% to 12% (table 1). These demographic developments stress the urgency of research into the biological causes of ageing and, particularly, its consequences, the occurrence of age-related diseases and mortality.

In humans and other species with repeated reproductive periods, ageing refers to the accumulation of changes with time that manifests itself in a progressively increasing probability of death.[2] Most of these changes will be degenerative; some may arise to compensate for a gradual loss of homeostasis, but will ultimately be inadequate.[2] These changes are found at all levels of organismal organisation. Examples are the accumulation of aberrant molecules and alterations in hormone status and organ function. The degenerative changes may eventually promote the development of a broad range of diseases that eventually lead to mortality.

Although most organisms are affected by ageing, the rate of ageing is highly variable. This is clearly demonstrated by the large differences in maximum lifespan between species. Also within a species, however, differences in the rate of ageing appear to exist. The identification of determinants of the rate of ageing in humans may provide important clues to the prevention and treatment of age-related diseases. So far such specific determinants have proven to be elusive. Even well-established risk factors for age-related diseases in middle age, such as high cholesterol levels and smoking, are of minor importance in old age.[3-5] It has become apparent, however, that the inter-individual variation in lifespan and the risk of age-related diseases is influenced by genetic factors.[6-8] The fast technological advancements in molecular genetics and genomics over the past decade provide new tools to unravel these genetic influences.

Table 1. Projected percentage of individuals aged 65 and 80 and over based on US Census Bureau International DataBase (http://www.census.gov/ipc/www/idbagg.html; updated 10-5-2000).

	65+			80+		
	2000	**2025**	**2050**	**2000**	**2025**	**2050**
World	6.9%	10.6%	16.3%	1.2%	2.1%	4.6%
Western Europe	16.3%	22.8%	29.7%	3.7%	6.6%	11.8%
The Netherlands	13.6%	21.7%	26.4%	3.2%	5.4%	10.7%

Already, associations between common gene variants and the risk of complex age-related diseases such as cardiovascular disease, type 2 diabetes and dementia are being identified at a high rate. Only very few studies, however, are designed to investigate the influence of gene variants on fatal disease and overall mortality. Establishing which variants contribute to mortality in the population at large may reveal those with a major relevance to overall disease risk, and might point at critical biological pathways in human ageing. Moreover, these studies might help to determine which of the dramatically growing number of gene variants potentially affecting disease risk have more major effects that warrant in-depth studies. Therefore, a study was initiated to search for common gene variants that affect mortality in the population at large. The results of these studies are described in this thesis.

1.2 Evolutionary theories of ageing

Evolutionary theories describe the origin of ageing and provide insights into the nature of genetic factors that contribute to ageing and mortality in humans. The concepts and predictions of these theories will be briefly summarised.

In the wild, mortality in age-structured populations is largely determined by environmental hazards, such as accidents, predators, drought, starvation and infectious diseases. Consequently, fewer individuals will be alive at progressively older ages, irrespective of the impact of ageing. Young parents - and not old parents with the genetic make-up that permitted long survival - will thus be the main contributors to the gene pool of the successive generations. The result is that the power of natural selection to discriminate between alleles with beneficial and deleterious effects gradually declines with age. The declining force of natural selection in old age has been shown mathematically[9,10] and forms the basis of evolutionary theories of ageing.

Two of these evolutionary theories are based on population-genetic mechanisms. Medawar (1952)[11] postulated that there is no restriction on the accumulation of germ-line mutations contributing to disease and death only late in life since there is little or no selection to eliminate such mutations from the population. In more general terms: as a result of the declining force of natural selection in old age, gene variants with late-acting deleterious, neutral or beneficial effects are allowed to exist simultaneously in a population. In the theory of antagonistic pleiotropy, Williams (1957)[12] predicted the occurrence of alleles with pleiotropic effects so that they have favourable effects early in life, but deleterious consequences later. Such genes will be favoured by natural selection and spread in the population because of their early fitness benefits, whereas their delayed side-effects are disregarded. In contrast, the early fitness effects of the late-acting deleterious mutations envisioned by Medawar are presumed to approach neutrality.

Kirkwood (1977)[13,14] developed a third evolutionary theory of ageing, the disposable soma theory, which is compatible with the mechanism of antagonistic pleiotropy but deals with networks of genes regulating general physiological processes rather than single genes. It emphasises the importance of trade-offs between the allocation of energy in reproduction and growth versus maintenance of the soma. On the one hand, too low an investment in the prevention and repair of somatic damage and the soma disintegrates before the end of expected lifespan in the wild, thus wasting a chance of

producing additional progeny. On the other hand, an investment in maintenance that is higher than needed to survive the expected lifespan in the wild goes to waste as well, as it could have been allocated to reproduction and growth. As a result, species with a short life expectancy in the wild due to the large impact of environmental hazards would display fast maturation, early fecundity and high reproduction rate, but poorly developed somatic maintenance mechanisms and low maximum life span, whereas species with a relatively long life expectancy would have efficient somatic maintenance mechanisms and high maximum lifespan but would mature slowly and display a low reproduction rate. In both cases, however, fitness is maximised at a level of investment in somatic maintenance that is less than would be required for indefinite survival without ageing.

These theories have been tested extensively in animal studies. The mutation accumulation theory of ageing states that natural selection does not suppress the dispersion of mutations with late-acting deleterious effects and that such mutations may thus reach high frequencies owing to genetic drift. In contrast, mutations with deleterious effects early in life will remain rare and are likely to disappear from the population owing to selection. Consequently, the contribution of genetic factors to variation in life history traits is predicted to increase with age. Indeed, in male *Drosophila melanogaster*, the genetic variability of 3-week mortality rates was greatly increased at very late ages.[15]

Other studies on *D. melanogaster* revealed trade-offs between fertility and longevity, which support the concepts of antagonistic pleiotropy and the disposable soma. The direct selection of flies on the basis of a long lifespan, which was performed at low larval densities, resulted in a lower production of progeny.[16] The authors proposed that the underlying genes are involved in the relative allocation of lipids to reproductive activities and somatic maintenance.[16].

The importance of somatic maintenance mechanisms is indicated by studies comparing different species demonstrating that long-lived species have more effective anti-oxidant defence mechanisms[17,18] and higher capacity to repair DNA[19-22] than short-lived species. Additional support comes from different mutants of the nematode *Caenorhabditis elegans* that have a 50% to more than 100% increased life expectancy[2] and concomitantly exhibit an increased resistance to oxidative stress and UV radiation[2] and express higher levels of superoxide dismutase (SOD),[19,23] which is a main enzyme involved in anti-oxidant defence.

Very interesting evidence for a central role of somatic maintenance mechanisms in ageing was obtained in *D. melanogaster*. Transgenic animals overexpressing both Cu/Zn *SOD* and catalase live 34% longer than controls,[24] while a 40% lifespan extension was achieved with the expression of human *SOD1* exclusively in the motor neurons.[25] Both studies, however, utilised highly inbred laboratory strains. Therefore, it has been suggested that the transgenes may merely complement some strain-specific vulnerability (G.M. Martin, personal communication).

Empirical evidence in simple animal models thus supports evolutionary theories of ageing. Interestingly, data are now becoming available that the concepts from these theories might also apply to the ageing of mammals. Mice deficient in the gene encoding

the $P66^{shc}$ adaptor protein gene, which is putatively involved in the stress apoptotic response, live up to 25% longer than wild-type mice,[26] which favours the role of somatic maintenance in mammalian ageing. Especially mammalian models of ageing allow pathological examinations, which might help to characterise the relevance of these models to human ageing.

A very promising method to identify genes involved in ageing, is the study of changes in gene expression profiles. The comparison of gene expression profiles in mouse skeletal muscle of young and old mice revealed age-related changes in expression of genes involved in stress response, energy production and biosynthesis.[27] Most of these changes were either completely or partially prevented by caloric restriction. It should, however, be noted that such changes may have been a reflection of changes in cell content of the muscle rather than changes in gene expression patterns of individual cells.[28] Human fibroblast cell lines from young, middle aged and old donors differed in the expression profile of 61 out of 6300 genes investigated.[29] The genes of which the expression pattern was altered were involved in remodelling of extra-cellular matrix proteins, inflammation and, in particular, cell-cycle control. Many of these changes were also observed in cell lines from children with the progeroid syndrome Hutchinson Gilford.

1.3 Genetic influence on variation in human lifespan

Evolutionary theories of ageing stress the role of genetic factors in ageing and thereby in the variation in lifespan. In the next section, the contribution of genetic factors to the inter-individual variation in lifespan between humans will be explored.

Mortality of human and other ageing populations may be divided in three phases, which is illustrated by data on mortality rates and survival of the Dutch 1891-1900 birth cohort in figure 1.[30] After the high mortality in the years immediately after birth (~20%; nowadays, <1% in Western European countries), mortality remained relatively low until the age of 55-60 years (premature mortality). Next, mortality started to accelerate owing to the impact of age-related diseases (old-age mortality). Approaching the maximum human lifespan, however, the increase in mortality started to decelerate (extreme longevity). As firstly proposed by Slagboom,[31,32] a different set of genes and gene variants with distinct characteristics might contribute to mortality in each of the three phases.

1.3.1 Genetics of premature mortality

Mortality before the age of 55-60 years can generally not be attributed to the presence of major ageing-related changes (figure 1). During this phase, causes of death vary greatly and include accidents and rare diseases. A study of adult adoptees[33] indicated a considerable genetic influence on mortality before the age of 50 years. There are a large number of mendelian genetic diseases that may lead to premature death. The mutations underlying these diseases are typically rare owing to the strong negative selection, recent in origin and highly penetrant. The high penetrance, the low occurrence of phenocopies and the availability of multiple generations make mapping of genes harbouring the mutations relatively straightforward using pedigree-based linkage studies. The field of

human genetics has, consequently, been very successful in identifying genes underlying mendelian disorders causing premature disease and mortality.

Mendelian genetic disorders cause a wide range of unrelated pathologies. Some of them have direct implications for ageing and disease in the population at large because they are rare, early-onset variants of common age-related pathologies. The elucidation of these diseases with a relatively simple aetiology may serve as a starting point to unravel

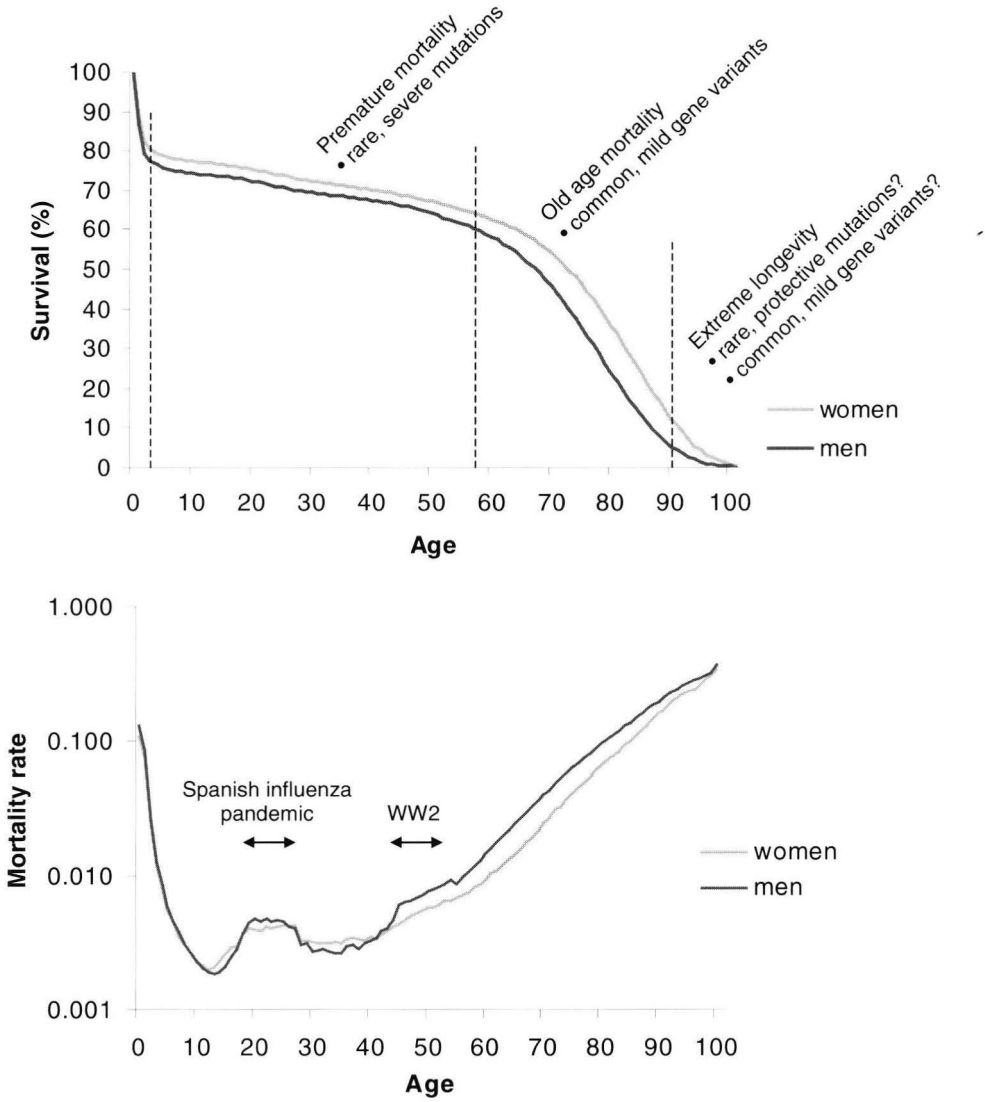

Figure 1. Survival and mortality rates of the Netherlands 1891-1900 birth-cohort (n = 1,583,490) according to gender. The proportion of men surviving to the age of 65, 85 and 100 years was 51.9%, 11.4% and 0.2%, respectively. For women these figures were 58.3%, 21.4% and 0.6%, respectively.

the vastly more complex late-onset forms. Examples are mutations in the LDL-receptor that cause familial hypercholesterolaemia[34,35] and frequently lead to premature death from myocardial infarction if not treated. Dissecting the molecular mechanism of familial hypercholesterolaemia thus revealed a pathway critical to the occurrence of cardiovascular disease at older ages, which is related to milder deviations.

Mutations that cause progeroid syndromes are of particular interest because multiple features of ageing are accelerated in affected individuals. Werner syndrome[36] patients, for example, suffer from premature skin ageing (including premature greying and loss of hair), insulin resistance, osteoporosis, atherosclerosis and cancer. The median age of death of these patients is 47 years and the commonest cause of death is myocardial infarction. However, the distribution of cancers is specific for the Werner syndrome and patients do not develop dementia. It is nevertheless striking that a mutation in a single gene can bring about such a variety of seemingly ageing-related pathologies. The Werner locus has been identified as a member of the recQ helicase family[37] and may participate in DNA replication, recombination, repair or transcription.[38] Interestingly, common alleles of the Werner gene have been associated with the risk of myocardial infarction in the Japanese population[39] suggesting that variation at this locus may contribute to age-related disease in the population at large.

1.3.2 Genetics of mean lifespan

After the age of approximately 55-60 years, the impact of ageing becomes apparent in the exponentially accelerating mortality (figure 1), which is fuelled by the massively rising incidence of major age-related pathologies, such as cardiovascular disease, cancer, dementia and type 2 diabetes. Owing to the very high mortality rate in old age, this phase is the major determinant of mean lifespan in Western countries where childhood and early adult mortality are low. There is evidence for a significant contribution of genetic factors to mortality in this phase. Family studies generally showed moderate correlations in lifespan between parent and offspring (0.01-0.15).[6,40,41] The resemblance in family studies, however, is caused by genetic as well as environmental factors. To disentangle genetic and environmental contributions, large twin studies have been performed using Danish, Swedish and Finnish twins. The joint analysis of these 31,000 twin pairs indicated that up to 50% the variation in lifespan could be attributed to genetic factors.[8] In accordance with the heritability of lifespan, genetic factors have a major impact on cardiovascular disease[42,43] and its risk factors,[44-47] and on type 2 diabetes.[48] The genetic component of variation of cognitive and physical ability in old age is high and may even increase with age.[1,49] These latter findings are compatible with a significant contribution of late-acting deleterious mutations. The development of cancer, however, appears to be mainly influenced by environmental factors.[50]

The genetic component of variation in lifespan is highly complex and its dissection strains current theoretical and technological possibilities. Late-onset diseases underlying the acceleration of the mortality rate are multifactorial and are anticipated to be the consequence of interactions between relatively small genetic and environmental effects. Likely candidates to explain part of the genetic component of old-age mortality are gene variants that lead to relatively modest deleterious alterations in gene function or

expression. Such variants may exert a negative influence on health status only after a long period of exposure to these deleterious effects or are only deleterious in combination with a condition that occurs late in life. As the frequency of these variants is not restricted by selection but the result of genetic drift, they may attain high frequencies in the general population and, consequently, have major public health consequences despite their mild effects. Simulation studies indicated that gene variants with a frequency of 10% generally arose more than 100,000 years ago and variants with a frequency of 50% are frequently even more than 200,000 years old, and thus predate the origin of *Homo sapiens sapiens* 100-150 thousand years ago.[51] A substantial part of the late-acting deleterious gene variants underlying the variation in human lifespan may therefore be universal for all human populations. A relatively common variant that was proposed to fit in the category of late-acting deleterious mutations is the apolipoprotein E (*APOE*) ε4 allele[2] (frequency in African Americans, Caucasians and Japanese: ~20%, ~15%, ~10%[52]), which is associated with the risk of dementia[52] and cardiovascular disease.[53-56]

On theoretical grounds, genetic variation with antagonistically pleiotropic effects may be a less important determinant of the variation in lifespan between individuals. Beneficial effects early in life are under control of powerful positive selection and selection tends to diminish genetic variation. The variation-reducing effects of natural selection may especially be profound since the same early benefits are likely to apply to evolutionarily related species. Antagonistically pleiotropic genes may, therefore, already have evolved in an ancient ancestral species of *Homo sapiens*. A study of an historical data set from the British aristocracy, however, revealed that women who died at an age of 80 years or over had a smaller number of progeny than women who died between the age of 50 to 80 years, which would be compatible with the presence of trade-offs between fertility and lifespan in humans.[57] This outcome would be in accordance with the presence of such trade-offs in *D. melanogaster*.[16] Although a larger study on the relation between the number of progeny and age at death in the Icelandic population did not indicate such trade-offs,[58] they were observed among a German population but only among those who were suffering economic deprivation.[59] The extent to which genetic factors contributed to these trade-offs observed in human populations remains to be established. Examples of specific genes with antagonistically pleiotropic effects have not yet been identified. Candidates are genes encoding inflammatory factors. A potent inflammatory response is vital in the defence against pathogens and may influence reproductive success,[60] but chronic inflammation appears to be a common component in the development of cardiovascular disease,[61] dementia[62-64] and diabetes.[65] Another possible example is allelic variation at the androgen receptor locus. Functionally robust alleles due to comparatively small numbers of exonic CAG repeats have been associated with an earlier age of onset and possibly a more virulent form of prostatic carcinoma.[66] One could imagine that alleles with robust androgen receptor function evolved for the enhancement of reproductive fitness.

To identify genes harbouring functional variants that contribute to mortality, cross-sectional and prospective studies are being performed. In cross-sectional studies, the frequency of gene variants is compared between old and young populations. A

decreased frequency in populations of octo- and nonagenarians indicates that the gene variant is associated with excess mortality at ages around the mean lifespan. In prospective studies, an elderly cohort is followed over a period of time and the association of gene variants with mortality is directly assessed. Studies into major age-related pathologies have revealed large numbers of gene variants that potentially affect mortality. So far the mortality effects of only a limited number of gene variants implicated in cardiovascular disease, dementia, cancer and immune-system related diseases were studied in cross-sectional designs (table 2). These first generation studies could not yet be conclusive as to the contribution of these variants to mortality because most studies were relatively small and most gene variants were tested in a single population only. Moreover, data from prospective studies are generally lacking. The most extensive data are available on variation at the *APOE* locus. Both cross-sectional and prospective studies indicate that the ε4 allele, particularly in homozygous state, confers an increased risk of mortality (table 2). The *APOE* ε4 allele is a risk factor for both cardiovascular disease[53-56] and dementia.[52] An association with multiple pathologies may turn out to be a common feature of gene variants that contribute to mortality.

1.3.3 Genetics of extreme longevity

Remarkably, after human populations reach the age of 90-95 years, the increase in mortality decelerates (figure 1) and may even level off at the age of 110 years.[1] This feature is not unique for humans and has been observed in populations of ageing *Drosophila* species and nematodes, and is not even restricted to living organisms: the survival of automobiles exhibits the same pattern, suggesting that mortality deceleration may be a general property of complex systems.[1] Mortality deceleration in ageing human populations is generally attributed to heterogeneity. In any population, some individuals are frailer than others due to innate or acquired weaknesses. Frail individuals tend to suffer higher death rates, leaving a robust subset of survivors relatively resistant to mortality. A shift in the distribution of causes of death suggests that extreme survivors are relatively resistant to cancer; cardiovascular diseases, however, remain the number one cause of death.[67] In addition, the rate at which the prevalence of dementia increases with age falls at older ages and the prevalence remains constant after the age of 95 years.[68]

The possibility that genetic factors play a marked role in attaining extreme longevity was suggested by a comparison of survival rates of siblings of centenarians with siblings of persons who died at the age of 73 years.[69] Siblings of centenarians had a 4-fold higher survival rate to ages older than 85 years. Again, there is little insight into the specific gene variants underlying these findings. Persons who attain extreme old ages might simply lack the genetic risk factors for late-onset diseases that cause mortality in the population at large. However, a more important role of genes in attaining extreme old ages would also be compatible with the presence of a limited number of major protective gene effects. The protective effects of such gene variants should then extend over more than one disease or slow down the rate of ageing in general. If such major protective variants exist, they apparently have a low frequency in the population. This might reflect the effect of trade-offs between extreme longevity and a decreased Darwinian fitness.

Table 2. Overview of studies assessing the association between common gene variants, mortality and extreme longevity using cross-sectional and prospective study designs.

Gene and variant and implicated disease		Cross-sectional studies		Prospective studies
		Octo- and nonagenarian studies	Centenarian studies	
Cardiovascular disease				
ACE	intron16I/D	$-_{ba}^{161}\ -_{bc}^{162}$	$+_{ac}^{70}\ -_{cb}^{168}$	
AGT	Met235/Thr		$-_{cb}^{71}$	
APOB	XbaI		$-_{ca}^{70}\ -_{cc}^{\star 169}$	
APOE	ε2/ε3/ε4	$+_{bc}^{\star 163}\ -_{bc}^{162}$	$+_{ac}^{70}\ +_{ca}^{71}\ +_{cc}^{72}$	$+_{a}^{74}\ -_{a}^{176}\ +_{a}^{77}\ -_{a}^{73}\ +_{a}^{75}\ -_{a}^{76}$
F5	Arg506/Gln		$-_{ab}^{170}\ -_{ca}^{171}\ -_{ca}^{\star 172}$	
F7	Arg353/Gln	$-_{cc}^{164}$	$-_{cb}^{168}\ -_{cc}^{\star 173}$	
FGB	-455G/A		$-_{cb}^{168}\ -_{cc}^{\star 173}$	
ITGB3	Leu33/Pro		$-_{cb}^{168}\ -_{cb}^{174}$	
LPA	isoforms		$\pm_{cc}^{175}$	
MTHFR	Ala222/Val	$-_{aa}^{\star 165}\ +_{ca}^{166}\ -_{bc}^{162}\ -_{bb}^{167}$	$+_{aa}^{168}\ -_{cb}^{\star 173}$	
PAI1	-675(4G/5G)		$-_{cb}^{168}\ +_{cc}$	
TPA	intron8I/D		$-_{cb}^{168}$	
Dementia				
ACE	intron16I/D	(see cardiovas. dis.)		
APOE	ε2/ε3/ε4	(see cardiovas. dis.)		
Cancer				
CYP2C19	*2,*3		$-_{ba}^{177}$	
CYP2D6	*3,*4,*5		$-_{aa}^{178}\ -_{ba}^{177}$	
GSTM1	gene deletion		$-_{aa}^{178}$	
MTHFR	Ala222/Val	(see cardiovas. dis.)		
NAT2	*5,*6,*7,*14A		$-_{aa}^{178}$	
P53	Arg72/Pro		$-_{cb}^{\star 179}$	
General/Other				
HFE	Cys282/Tyr	$-_{aa}^{180}$		
HLA	many alleles	$+_{aa}^{181}\ +_{cb}^{182}$	$+_{aa}^{183}$	
mtDNA	several alleles		$+_{aa_{78}}\ +_{bb}^{\star 79}$	
WRN	Cys1367/Arg		$+_{bb_{72}}\ -_{cc}$	

Legend

+ indicates association, **–** indicates absence of association, **±** indicates trend.

a, b and c indicate number of old subjects and controls, a: n≥300, b: 200≤n<300, c: 100≤n<200. Example: ab = more than 300 old subjects and between 200-300 controls.

* Study included healthy old subjects only.

Abbreviations genes

ACE = angiotensin I-converting enzyme *AGT* = angiotensinogen *APOB* = apolipoprotein B *APOE* =apolipoprotein E *CYP2C19* = cytochrome P450 2C19 *CYP2D6* = cytochrome P450 2D6 *F5* = factor V *F7* = factor VII *FGB* = fibrinogen β *GSTM1* = glutathione *S*-tranferase μ *HFE* = haemochromatosis gene *HLA* = human leukocyte antigen *ITGB3* = platelet glycoprotein IIIα *LPA* = apolipoprotein (a) *mtDNA* = mitochondrial DNA *MTHFR* = 5,10-methylenetetrahydrofolate reductase *NAT2* = arylamine *N*-acetyltransferase 2 *P53* = tumour protein p53 *PAI1* = plasminogen activator inhibitor 1 *TPA* = tissue type plasminogen activator *WRN* = Werner helicase

Unfortunately, the search for such major gene effects is hampered by the small number of candidate genes. The disposable soma theory suggests the involvement of genes that contribute to somatic maintenance mechanisms, such as defence against oxidative damage (for instance *SOD*, catalase and glutathione peroxidase) and systems that safeguard genomic stability (for instance DNA-repair genes).

Analogous to studies of gene variants contributing to mortality around the mean lifespan, gene variants are studied in extremely long-lived individuals and compared with populations of young individuals. In practice, centenarians are selected for such studies. The majority of studies investigated disease-associated variants (table 2). The studies performed thus far do not yet suggest which pathways are critical in extreme longevity. Deviating *APOE* genotype distributions were found in both French[70] and Finnish[71,72] centenarians. However, follow-up studies in cohorts of different ages indicated that the *APOE* locus exerts its effects on mortality only before the age of 70-80 years.[73-77] *APOE* genotypes do thus not discriminate between persons who die around the mean lifespan and extremely long-lived individuals. There also was an association of variation in mitochondrial DNA with extreme longevity in French[78] and Italian[79] centenarians. This is an intriguing finding in view of the long-standing hypothesis that mitochondria play a role in the process of ageing.[80]

It is important to note that the interpretation of genetic studies in centenarians may be complicated. A mathematical model predicting trajectories of mortality-associated genotypes suggested that, at first, the frequencies of these genotypes decrease with age, as is to be expected, but start to rise rapidly after the age of 95 years and will exceed the frequency that was present in early age.[81] This would mean that, when relying on centenarian studies, protective effects might be ascribed to deleterious gene variants. This may, however, be an infrequent phenomenon in practice in view of the assumptions the authors used for obtaining these results. Particularly the assumption that genotypes associated with increased old-age mortality are also associated with greatly increased mortality rates in the young may be criticised since strong early deleterious effects are likely to be affected by natural selection. A more important complication of centenarian studies may be that since centenarians are rare, groups of centenarians will always display considerable geographical and thus genetic heterogeneity. This severely hampers the collection of an appropriate young control population.

1.4 Common gene variants and cardiovascular disease

All in all, there is ample evidence that genetic factors contribute to the inter-individual variation in lifespan. Specific gene variants affecting human ageing and disease may be revealed by studies of extreme longevity or mortality in the population at large. Centenarian studies may reveal the presence of rare gene variants that exert protective effects on ageing in general. Investigations of mortality in the population at large may be better suited to investigate the influence of susceptibility genes for common diseases.

The studies described in this thesis focus on cardiovascular diseases because their occurrence is a crucial aspect of human ageing and was shown to have a significant genetic component.[42,43] Atherosclerosis is a primary cause of cardiovascular disease. The development of atherosclerosis is largely restricted to primates[82] and is a universal

feature of human ageing. The first signs of atherosclerosis can already be demonstrated in over 70% of children aged 12-14 years.[83] In old age, no individual is free of atherosclerosis. This may explain why cardiovascular diseases are the main cause of death in both developed (48%) and developing (23%) countries.[84] Moreover, atherosclerosis is an important risk factor for other major age-related pathologies such as dementia.[85] Although the development of atherosclerosis is universal, important inter-individual differences exist that ultimately determine the risk of cardiovascular disease. These differences include the rate at which atherosclerosis progresses, the tendency of atherosclerotic plaques to develop complications such as plaque rupture, and the capacity to counter these complications. Common gene variants with late-acting deleterious effects may significantly contribute to the genetic component of this variation and thereby to mortality in the population at large.

1.4.1 Candidate genes

There are many genes that may be hypothesised to harbour common variants that affect mortality through their effects on cardiovascular disease risk (i.e. candidate genes). Although only few studies have assessed the influence of such gene variants on mortality rate, variants of 50 genes have already been tested for their contribution to the risk of cardiovascular disease in more than 300 studies. A comprehensive overview of the results of these studies is provided in the appendix (page 133) and includes information on the cardiovascular disease-related pathway in which the gene is involved. In this section, it will be explained why the genes tested were considered to be candidate genes for cardiovascular disease.

A number of candidate genes are derived from studies into rare, severe mendelian genetic disorders that are strongly associated with premature atherosclerosis and myocardial infarction. The majority of genes underlying these disorders were found to affect lipid metabolism such as the LDL-receptor gene underlying familial hypercholesterolaemia[34,35] and the recently identified ATP-binding cassette-1 gene underlying Tangier disease.[86-88] Genes from other pathways in which mutations can cause aggressive premature atherosclerosis include the cystathionine β-synthase and methylenetetrahydrofolate reductase genes, which cause greatly elevated plasma levels of the amino-acid homocysteine,[89,90] and the Werner helicase gene.[37,38] The number of genes that have been identified to underlie such mendelian genetic disorders is, however, small (<15).[91] Also, the associations with premature cardiovascular disease are generally relatively weak when taking into account the severe effects of the mutations on gene function.[91]

Other candidate genes have emerged from current insights into the pathogenesis of atherosclerosis and its complications. In view of the crucial role of this knowledge in the identification of genetic risk factors for cardiovascular disease, a brief summary will be given of the pathogenesis of atherosclerosis and its complications as it has been reconstructed from cell culture experiments, animal models and pathological studies in humans. An overview of pathways and specific factors that are hypothesised to play a critical role in the pathogenesis of atherosclerosis and its complications is given in figure 2.

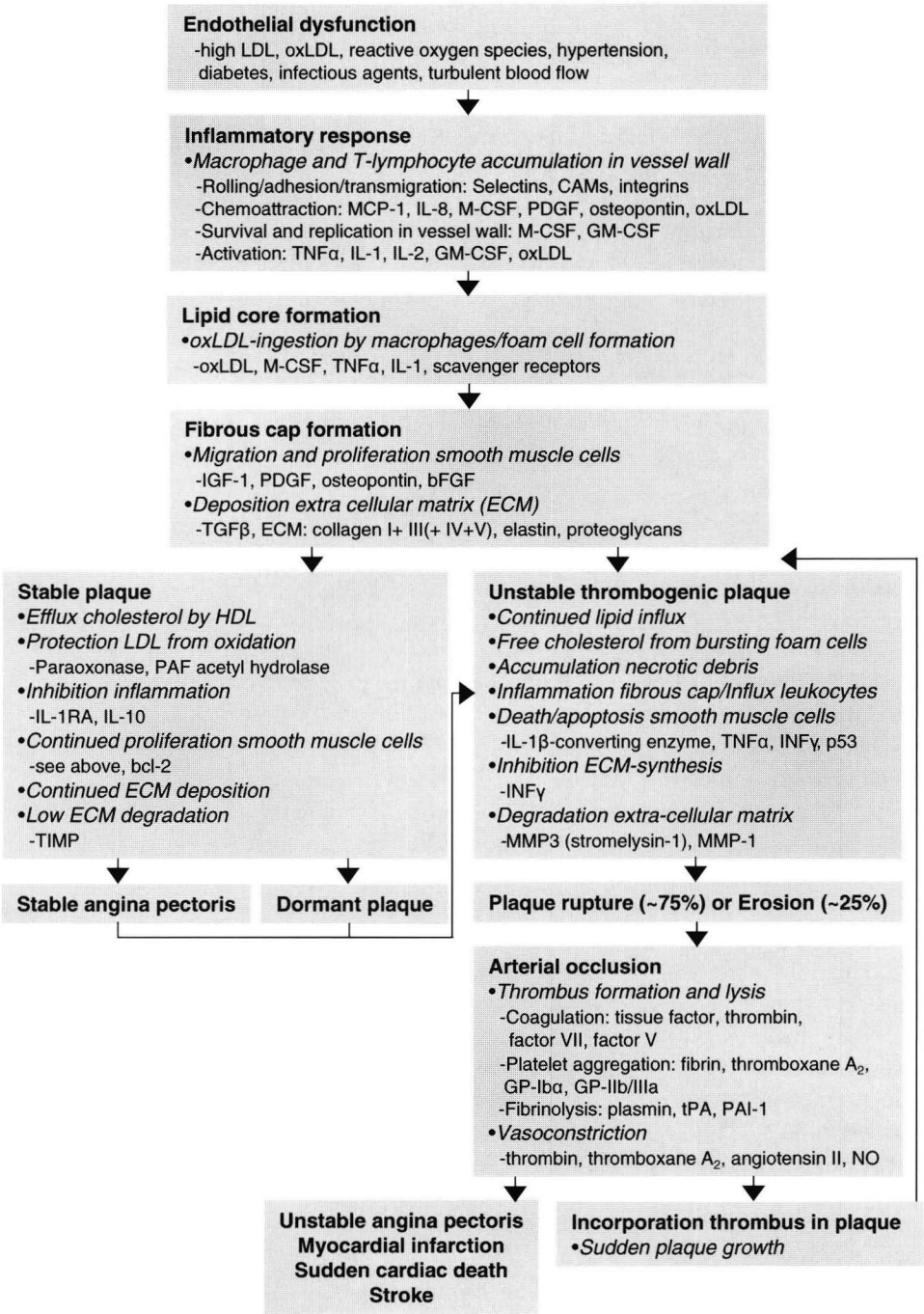

Figure 2. Overview of the pathogenesis of atherosclerosis and its complications.

According to the response-to-injury hypothesis,[61,92] dysfunction of the arterial endothelium is the first step in atherogenesis and may be caused by factors such as elevated and modified low-density lipoprotein (LDL), reactive oxygen species or hypertension. In response to the injury, the endothelium initiates an inflammatory response by recruiting and activating monocytes/macrophages, T-lymphocytes, smooth muscle cells and platelets. However, if the injurious factor is chronic, the inflammatory response will be unsuccessful in neutralising the cause of the injury and, consequently, continues indefinitely, which eventually leads to atherosclerosis. This involves two main steps. First, a sub-endothelial lipid-core develops as LDL particles that have been oxidised by endothelial cells, smooth muscle cells and macrophages are being ingested by macrophages. Second, a fibrous cap is produced that covers the lipid core and consists of migrated and proliferated smooth muscle cells, which have synthesised an extra-cellular connective tissue matrix.

The growth of the atherosclerotic plaque will cause a partial obstruction of the artery. The size of the plaque, however, appears to be of minor clinical important since it is a poor predictor of a cardiovascular event.[93,94] In half of myocardial infarction cases, the atherosclerotic plaque underlying the event caused less than 50% stenosis. More important determinants of its clinical relevance are the stability and thrombogenicity of the atherosclerotic plaque.[95,96] A typical stable atherosclerotic plaque is characterised by a relatively small lipid core covered by a thick fibrous cap that provides strength to the lesion (figure 3). Stable plaques are thought seldom to cause fatal cardiovascular events. On the other hand, unstable plaques are prone to rupture and may trigger the formation of an artery-occluding thrombus owing to their vulnerability to mechanical and haemodynamic forces. The characterisation of ruptured unstable plaques in autopsy studies showed that they in general consist of a large thrombogenic lipid-core, a thin fibrous cap and abundant inflammation, which may constitute the major cause of

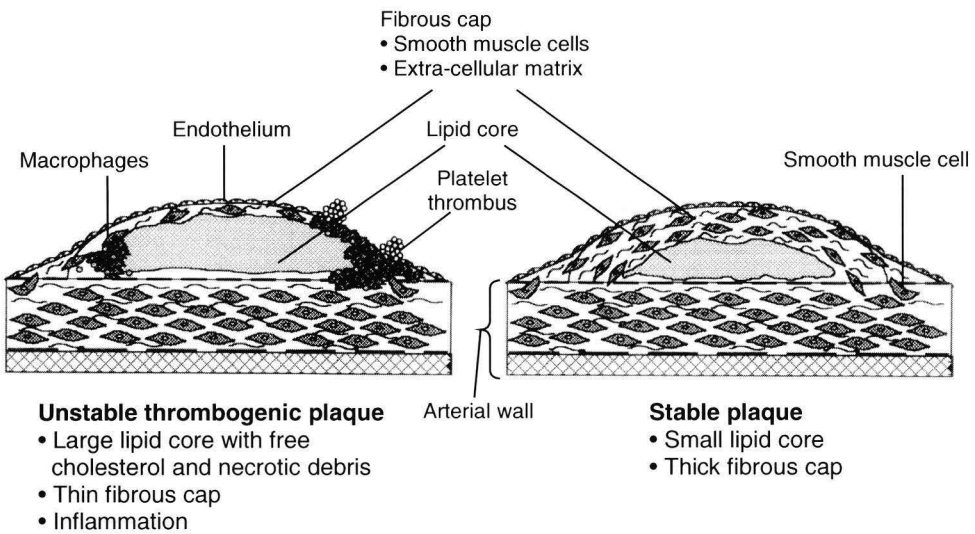

Figure 3. Schematic representation of a stable and unstable atherosclerotic plaque.

weakening of the fibrous cap (figure 3).[95,97,98] Plaque ruptures underlie more than 75% of acute myocardial infarctions[99,100] and sudden cardiac deaths.[101] In women and at younger ages, however, superficial erosion of a fibrous cap at the site of inflammation appears to be a common additional event that triggers thrombus formation (up to 25-30%).[100,102] Ischaemic strokes are mostly precipitated by the formation of a thrombus in the atherosclerotic carotid artery or aortic arch that becomes detached and occludes a distal artery in the brain.[103]

The time an artery is occluded by a thrombus and distal tissues are deprived of oxygen determines the severity of the clinical outcome. For example, the cessation of myocardial perfusion for more than one hour causes transmural necrosis of the myocardium involved and, often, death. The persistence of the arterial occlusion is dependent on factors such as the size of the thrombus, which is determined by the extent of the plaque damage, the thrombogenicity of the lipid core and a systemic procoagulant state, and other mechanisms including the activity of the fibrinolytic system, the composition of the thrombus (platelet-rich plaques are less susceptible to fibrinolysis) and the extent of local vasoconstriction exacerbating the occlusion.[96] An additional determinant of clinical outcome is the presence of collaterals that can partly take over the blood flow.[96]

1.4.2 Common gene variants and cardiovascular disease risk

As mentioned in the previous section, laboratories world-wide have been testing common variants in candidate genes as risk factors for cardiovascular disease since the early 1990s. The large majority of genes investigated were selected on the basis of their putative role in the pathogenesis of cardiovascular disease. Now, ten years later, variants of more than 35 genes have been found to be associated with the risk of cardiovascular disease in one or more studies (see appendix). Many of these variants were also linked to altered gene function or expression, or altered levels of the gene product in plasma. It is important to note that these associations do not prove that the variants are functional themselves. They may serve as markers for yet unknown functional variants that reside on the same haplotype as the neutral variant that was investigated.

Despite the accumulation of data from more than 300 studies, many questions remain as to the relevance of the studied gene variants to disease risk. This is illustrated by the overview of studies that have been performed thus far that is shown in the appendix. It is by no means possible to unequivocally confirm or reject a role for any of the pathways hypothesised to contribute to the development of cardiovascular diseases. Even more, the contribution to disease risk of most of the gene variants themselves is still ambiguous, including those studied in many patient populations. To a large extent this may be attributed to the fact that studies differed greatly in size, investigated different clinical phenotypes, age groups and sexes, varied in the representativeness of the control population and included individuals with diverse genetic and environmental backgrounds. Unfortunately, it is difficult if not impossible to ascribe different findings of individual studies to one of these factors.

1.5 Outline of thesis

The aim of the studies described in this thesis is to identify common gene variants that affect mortality in the population at large. All gene variants that were selected to be investigated in these studies were either functional or consistently associated with altered plasma levels of gene products to increase the prior probability of finding associations. Furthermore, variants were chosen to cover various pathways implicated in the development of cardiovascular disease, which might allow the relative importance of these pathways in the pathogenesis of cardiovascular disease to be ranked. Finally, priority was given, first, to variants that were also implicated in other pathologies since these might have a stronger impact on mortality, and, second, to variants for which the association with cardiovascular disease risk was still inconclusive. If gene variants are associated with mortality in the population at large, elaborate studies as to their role in disease may be justified despite equivocal previous data.

To establish the presence or absence of associations between common gene variants and mortality in the population at large, cross-sectional and prospective studies were performed based in the Leiden 85-plus Study,[4] a population-based study in which all subjects aged 85 years and over living in Leiden were invited to take part. Leiden is a city with about 120,000 inhabitants in the western part of The Netherlands and at the entry date, 1 December 1986, the cohort of inhabitants aged 85 years and over comprised 1258 persons. 977 of these eligible persons participated (94% of living individuals and 78% of the initial cohort), 221 died before enrolment and 60 persons refused participation. Sufficient cell material was available from 666 subjects for genetic studies. The loss of 311 subjects for the genetic studies is unlikely to have resulted in selection bias since participants from whom DNA was available and those from whom it was not, were very similar with respect to cardiovascular risk factors and health status, except that they were on average 5 months older. Moreover, the 10-year survival rate of both groups was similar.

The study design is outlined in figure 4. In the cross-sectional design, the frequencies of gene variants among these old persons and a young population were compared. The inference of a lower frequency in old age is that the gene variant is associated with increased probability to die prior to the age of 85 years, while an increased frequency indicates a protective effect. It was ensured that the old and young populations had the same genetic background by comparing only old persons born in Leiden (55% of complete cohort) to young persons whose families originated from the Leiden region (n = 250). The young population consisted of blood donors who had either two Leiden-born parents or had one Leiden-born parent and one born within a 12-km distance of Leiden. In addition, information regarding the birthplace of their grandparents was obtained.

Mortality after the age of 85 years was investigated in a prospective study. All participants were followed for mortality over a 10-year period and data were collected on specific causes of death. Since this design is not distorted by geographical variations in genotype distribution, the complete cohort was included in the analyses (55% born in Leiden, 45% born elsewhere in The Netherlands).

In the next sections, the genes investigated in this thesis will be discussed.

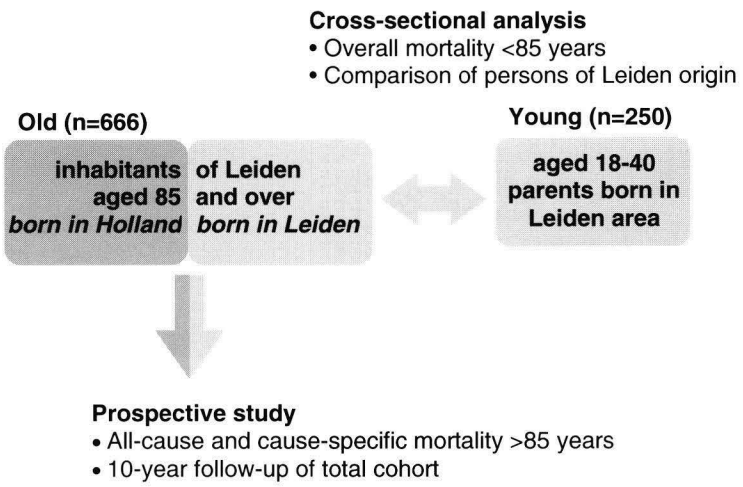

Figure 4. Study design.
The old subjects are participants of the Leiden 85-plus Study and the young subjects are blood donors.

1.5.1 Methylenetetrahydrofolate reductase (MTHFR) – Chapters 2 and 8

Severe mutations in the key enzymes of the folate metabolism and the linked methionine/homocysteine metabolism, namely methylenetetrahydrofolate reductase (*MTHFR*) and cystathionine β synthase, lead to aggressive premature atherosclerosis.[89,90] This was attributed to the 10 to 50 times increased plasma homocysteine levels among patients with these mutations. Homocysteine has been suggested to cause endothelial damage and oxidative stress,[104] which are both pivotal factors in the pathogenesis of atherosclerosis. However, the relevance of these studies to the situation *in vivo* has been challenged because up to 100 times the physiological concentration of homocysteine was needed to observe these effects and the effects are not specific for homocysteine.[105,106] As an alternative explanation, homocysteine has been proposed to disturb intra-cellular methylation of proteins and DNA because homocysteine is in equilibrium with S-adenosyl-homocysteine, which is a potent inhibitor of methylation reactions. In support of this hypothesis, the $p21^{ras}$ protein was found to be hypomethylated in vascular endothelial cells that were exposed to physiological concentrations of homocysteine.[107]

Further epidemiological studies reported that cardiovascular disease patients have, on average, mildly elevated plasma homocysteine levels[108] suggesting that less severe disturbances of the folate and methionine/homocysteine metabolisms may also contribute to disease risk. This stimulated a search for common, milder variants of the *MTHFR* gene. An Ala222/Val variant was identified that, in homozygous state, is associated with a 70% decreased enzyme activity[109] and a 25% increased plasma homocysteine level.[110] Smaller initial studies provided evidence that the mutation was associated with an increased risk of cardiovascular disease,[111,112] but later studies could, in general, not confirm this result (see appendix).

The folate and methionine/homocysteine metabolisms may also play a role in the development of cancer. Homocysteine is formed when the methyl-group of methionine is transferred to DNA and other molecules. A disturbed methionine/homocysteine metabolism might result in an undermethylation of DNA and, consequently, an increased mutation rate[113] or the expression of proto-oncogenes.[114] Interestingly, homozygosity for the common *MTHFR* variant was indeed associated with decreased levels of DNA methylation.[115] Moreover, 5,10-methylenetetrahydrofolate, which is converted in the 5-methyl form by MTHFR, is used in nucleotide synthesis.[116] The association of the MTHFR variant with the risk of colorectal cancer[117,118] and acute leukaemia[119] would accord with this hypothesis.

1.5.2 Apolipoprotein E (APOE) – Chapter 3

Apolipoprotein E (APOE) is a constituent of most lipoprotein particles (chylomicrons, VLDL, IDL and HDL) and is the ligand for the LDL and VLDL receptors as well as the LDL receptor-related protein. The common *APOE* ε4 allele is associated with elevated levels of cholesterol and triglycerides in plasma,[120] which are both risk factors for cardiovascular disease. In agreement with this observation, the ε4 allele confers an increased risk of myocardial infarction (see appendix). Moreover, the ε4 allele is even more strongly associated with the risk of vascular and Alzheimer's type dementia.[52,85] The biological mechanism underlying this association is not known. Hypotheses include a differential effect of *APOE* alleles on amyloid deposition,[121] tangle formation,[122] neuronal plasticity[123] and cholinergic function[124]. In addition, the ε2 allele was indicated to confer a decreased risk of Alzheimer's type dementia.[52] Two recently identified variants in the *APOE* promoter[125,126] were found to influence transcriptional activity *in vitro*[125] and enable a further characterisation of the role of the locus in disease.

1.5.3 Paraoxonase (PON1) – Chapter 4

Oxidised LDL particles play a central role in atherogenesis.[127,128] They cause dysfunction of the endothelium and, in contrast to unmodified LDL, can be ingested by macrophages present in the vessel wall, which leads to the formation of a lipid core. Factors influencing the oxidation of LDL are thus prime candidates for modifying cardiovascular disease risk. Evidence is accumulating that the enzyme paraoxonase protects LDL from oxidation.[129-131] Two common, functional variants of the paraoxonase gene (*PON1*) have been identified that lead to a Met55/Leu and a Gln192/Arg amino-acid substitution.[132-135] However, the impact of these variants on cardiovascular disease risk is still equivocal (see appendix).

1.5.4 Tumour necrosis factor α (TNFA) – Chapter 5

The cytokine tumour necrosis factor α (TNFα) is a pivotal mediator of the inflammatory response and implicated in numerous pathologies. TNFα may contribute to the pathogenesis of cardiovascular disease by, for example, stimulating foam cell formation, T-lymphocyte activation and expression of matrix metalloproteinases that destabilise the plaque by degrading the extra-cellular matrix.[61] Also, TNFα plays a key role in obesity-

linked insulin resistance, a major risk factor for cardiovascular disease, by interfering with the insulin-induced kinase activity of the insulin-receptor.[136-138] Furthermore, it has been suggested that inflammation promotes Alzheimer's type dementia,[62-64] although a specific role of TNFα has not been established. Finally, TNFα may affect the outcome of infectious diseases.

Polymorphisms at the TNF locus that have been implicated in the risk of various diseases include a -308G/A polymorphism in the promoter of the *TNFA* gene, which was indicated as affecting transcriptional activity,[139-141] and the short tandem repeats TNFa (14 alleles) and TNFc (2 alleles).

1.5.5 Factor V (F5) – Chapter 6

An Arg506/Gln mutation in the gene encoding coagulation factor V, referred to as factor V Leiden, causes a poor response to activated protein C,[142,143] the primary inhibitor of coagulation, and is consistently associated with an increased risk of venous thrombosis.[144,145] The factor V Leiden mutation might confer excess risk of mortality due to its contribution to pulmonary embolism, the potentially lethal complication of venous thrombosis. The mutation might also contribute to the formation of larger thrombi after the rupture of an atherosclerotic plaque, thereby extending the period distal tissues are deprived of oxygen.

1.5.6 Angiotensin I-converting enzyme (ACE) – Chapter 7

Angiotensin I-converting enzyme (ACE) can induce vasoconstriction by catalysing the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor, and by degrading bradykinin, a vasodilator. Vasoconstriction may severely complicate the occlusion of an artery by a thrombus that is formed as a result of a plaque rupture.[96] In addition, angiotensin II promotes smooth muscle cell growth, which might promote the stability of atherosclerotic plaques. An *alu* repeat insertion/deletion polymorphism in intron 16 of the *ACE* gene accounts for 15%-25% of the variation in plasma activity,[146,147] although it is likely to be a neutral variation. Extensive searches for the actual functional variant supposedly in linkage disequilibrium with the I/D variant were thus far not successful.[148-150] The polymorphism gained much attention in view of the efficacy of ACE-inhibitors in the treatment of hypertension and hypertrophy. Despite the relatively large number of studies that have been performed (see appendix), its relevance to cardiovascular disease risk is still a matter of intense debate.[151,152] This was fuelled by the finding that the putative deleterious D/D genotype was more frequent among centenarians,[70] suggesting beneficial effects in extreme old age.

1.5.7 Plasminogen activator inhibitor 1 (PAI1) – Chapter 7

Plasminogen activator inhibitor-1 (PAI-1) is the primary inhibitor of fibrinolysis. The importance of fast lysis of a thrombus in the event of plaque rupture is eminent and underscored by the highly beneficial effects of administering plasminogen activators to patients with an acute myocardial infarction. A 4/5-guanine-tract polymorphism in the *PAI1* promoter affects binding of putative transcription factors, expression[153-155] and

plasma level[153,154,156] and thus possibly fibrinolytic capacity. The relation of the promoter polymorphism with cardiovascular disease remains uncertain (see appendix).

1.5.9 Gene-gene interactions

Gene-gene and gene-environment interactions are suspected to play a major role in disease. On the basis of the function of the genes measured here, the presence of several gene-gene interactions may be hypothesised. First, there are potential intra-genic interactions between three functional variants measured in the *APOE* gene and two in the *PON1* gene. Furthermore, interactions may exist between *TNFA* and *PAI1* variants, since TNFα is a key component of the obesity-linked elevation of PAI-1[157] and compromises insulin-receptor activity,[136-138] and studies of *PAI1*-deficient mice suggested a causal role for PAI-1 in circulating insulin levels[158] possibly due to its involvement in proteolytic processes responsible for the activation of proinsulin. Finally, it would be interesting to test for an interaction between the *PAI1* and the *ACE* gene variants since both are associated with PAI-1 levels in plasma.[159,160] To be able to test for gene-gene interactions large sample sizes are needed. Even in the most favourable case – the potential interaction between the *ACE* and *PAI1* variants – only about 6% (25%x25%) of individuals will be homozygous for both putative risk genotypes. Our study was generally not large enough (666 old and 250 young persons) to have sufficient power for testing gene-gene interactions.

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Chapter 2

Mortality risk in men is associated with a common mutation in the methylenetetrahydrofolate reductase gene (*MTHFR*)

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Abstract

An elevated level of homocysteine in plasma is associated with the occurrence of cardiovascular disease. A common Ala-to-Val mutation in the methylenetetrahydrofolate reductase gene (*MTHFR*) is associated with an elevated level of plasma homocysteine. We studied the possible detrimental effects of the *MTHFR* mutation on mortality.

Within a population-based study in the city of Leiden, the Netherlands, we first compared the *MTHFR* genotype distribution among 365 elderly subjects aged 85 years and over born in Leiden, and 250 young subjects aged 18 to 40 years whose families originated from the same geographical region. Second, the complete cohort of 666 subjects aged 85 years and over was followed over a period of 10 years for all-cause and cause-specific mortality and stratified according to *MTHFR* genotype.

The frequency of the *MTHFR* mutation was significantly lower in the elderly subjects than in the young (0.30 and 0.36, respectively; P=0.03). The difference in genotype distribution was only present in men. The estimated mortality risk up to 85 years in men carrying the Val/Val genotype was 3.7 (95% confidence interval (CI), 1.3-10.9). Over the age of 85 years, mortality in men with the Val/Val genotype was increased 2.0-fold (95% CI, 1.1-3.9) and appeared to be attributable to cancer rather than cardiovascular causes of death. Among women aged 85 years and over, no deleterious effect of the *MTHFR* mutation was observed. In conclusion, the *MTHFR* mutation is associated with increased mortality in men in middle and old age, but not in women.

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Introduction

An elevated level of homocysteine in plasma is associated with the occurrence of cardiovascular disease[1] and increased mortality in patients with coronary artery disease.[2] A recent meta-analysis estimated that every 5 μmol/L increment in plasma homocysteine increases the risk of coronary heart disease by 60% for men and 80% for women.[1] Homocysteine is formed when the methyl-group of methionine is transferred to DNA, proteins or other molecules. The basal level of plasma homocysteine is mainly determined by the remethylation of homocysteine to methionine.[3,4] This reaction is regulated by the enzyme methylenetetrahydrofolate reductase (MTHFR),[3] which converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The methionine/homocysteine metabolism is disrupted by deficiencies in the essential coenzymes vitamin B6, B12 and folate, and by homozygosity for rare mutations in the genes encoding MTHFR and cystathionine β synthase.[5,6] These genetic defects give rise to greatly elevated homocysteine levels in plasma and result in mental retardation, bone malformations and premature atherosclerotic disease.[5,6]

Recently, a common C_{677}-to-T (Ala-to-Val) mutation in the *MTHFR* gene was identified,[7] which leads to a less severe disturbance of the methionine/homocysteine metabolism. About half of the general population carries at least one mutated allele and the frequency of the homozygous mutated genotype (Val/Val) varies from 8% to 18% depending on the population.[8-19] The *MTHFR* mutation was shown to render the enzyme thermolabile, and homozygotes and heterozygotes had about a 70% and 35% reduced MTHFR activity, respectively.[7] Furthermore, homozygosity for the mutation is associated with elevated levels of homocysteine in plasma.[7,9,11,15-17,19-21] This association is dependent on age[15,17] and nutrition. Plasma homocysteine levels are predominantly elevated among carriers of the Val/Val genotype who have a low level of plasma folate.[9,11,15] It was shown that especially in Val/Val carriers the level of plasma homocysteine was lowered by folic acid supplementation.[22] In various reports the *MTHFR* mutation has been implicated in the risk of cardiovascular disease[4,10,14-16,18,19,21,23-28] and cancer[12,29] but the data are equivocal.

To explore the possible detrimental effects of a disturbed methionine/homocysteine metabolism, we studied the association between the *MTHFR* mutation and mortality, nested in a population-based study of subjects aged 85 years and over (Leiden 85-plus Study). This was done (i) in a cross-sectional analysis comparing the occurrence of the *MTHFR* mutation between subjects aged 85 years and over and young subjects aged 18-40 years whose families originated from the same geographical region as the elderly subjects, and (ii), prospectively, over a 10-year follow-up period in the entire elderly cohort. The prospective study included the analysis of cause-specific mortality risks.

Materials and methods

The Leiden 85-plus Study is a population-based study in which all inhabitants of Leiden, The Netherlands, aged 85 years and over were invited to take part.[30] Of a total of 1258 eligible subjects, 221 died before enrolment which lasted from December 1, 1986, to March 1, 1988. Of the 1037 remaining subjects, 977 (94%) participated and were medically interviewed at home.[31] After the exclusion of subjects with a non-Dutch (n=29)

or unknown (n=69) place of birth, sufficient cell material was available from 666 (188 men/478 women) subjects for the present genetic study. DNA was extracted[32] and *MTHFR* genotypes were determined by the PCR-amplification of a 198 bp fragment containing the C_{677}-to-T transition, followed by digestion with *Hin*fI as previously described.[7] *MTHFR* genotypes were independently assessed by two observers. As a standard laboratory procedure a randomly chosen 10% of the samples was reamplified. No genotyping errors were observed. The study was approved by the Medical Ethics Committee of the Leiden University and informed consent was obtained from all participants.

Cross-sectional analysis

MTHFR genotype distributions were compared in elderly subjects aged 85 years and over and young controls. The subjects in the elderly population are survivors of a cohort born between 1887-1901. To avoid false associations with the *MTHFR* mutation due to differences in geographical origin rather than age, the cross-sectional comparison of elderly and young subjects accounted for local variations in *MTHFR* genotype distribution that may have existed in the past. Subjects aged 85 years and over who were born in Leiden (n=365; 56%) were compared with a control population that consisted of 250 (139 men/111 women) blood donors aged 18-40 years of Dutch descent with either one parent born in Leiden and the other within a 12-km distance from Leiden, or with two Leiden-born parents. Information regarding the birthplace of their grandparents was obtained from a written questionnaire. If a specific Leiden *MTHFR* genotype distribution had existed in the past, the genotype distribution in young controls would have been increasingly deviating the greater their number of Leiden-born grandparents. The upper age limit of the young controls was chosen since selection against genotypes contributing to population mortality was not expected to occur before the age of 40 years.

Prospective study

All participants in the Leiden 85-plus Study were followed up for mortality until October 1, 1996. Among the 666 subjects of the cohort studied, 2 were lost to follow-up. Primary causes of death were assessed by linking the death certificate numbers, obtained from civic registers, to the causes of death as recorded by the Dutch Central Bureau of Statistics. Causes of death were classified according to the ninth revision of the *International Classification of Diseases (ICD-9)*.[33] Death certificates from 1996, coded according to the tenth revision of the *International Classification of Diseases*, were recoded according to the ninth revision. *ICD-9* codes were reviewed and each code was categorised for cardiovascular disease (*ICD-9* 390-459), cancer (*ICD-9* 140-239) and all causes (*ICD-9* 000-999). Death from infection was coded as previously described.[34]

Statistical analysis

Differences in baseline characteristics were tested for significance with the χ^2 test for categorical and the Student's *t*-test for continuous variables. In the cross-sectional analysis, distributions of alleles and genotypes were compared by the χ^2 test, and

mortality risks and 95% CIs were estimated using the exposure odds ratio. Mantel's extension of the Mantel-Haenszel test was used to test for trend in stratified analyses.[35] In the prospective study, survival times for subjects were computed from the date of the home visit to the date of one of the following events: death from a specific cause, death from any cause, or October 1, 1996. Survival was estimated using the Kaplan-Meier product limit method and compared with the log-rank test. Adjusted mortality risks and 95% CIs were estimated with Cox proportional hazards models. Causes of death were assumed to be independent. P-values of less than 0.05 were considered to indicate statistical significance and all P-values were based on two-sided tests. The analyses were performed using the SPSS statistical software package.

Results

Cross-sectional analysis

Table 1 shows the baseline characteristics of the study subjects from the cohort of Leiden inhabitants aged 85 years and over (n=666). A gender difference was observed with respect to smoking habits, alcohol consumption and the prevalence of hypertension and cancer. The *MTHFR* genotype distribution in the complete cohort was 46.5% (Ala/Ala), 44.4% (Ala/Val) and 9.0% (Val/Val). For the cross-sectional analysis, *MTHFR* genotype frequencies in the elderly subjects born in Leiden (56% of the complete cohort) were compared with those in young subjects aged 18-40 years whose families originated from the Leiden area (table 2). Genotype frequencies in both groups were in agreement with the distribution predicted by the Hardy-Weinberg equilibrium. The prevalence of the Val/Val genotype in the control population (12.4%, n=250) was consistent with the 10% to 18% reported for other populations greater than 200 subjects of European, North-American and Australian origin.[8-13,15-17]

The frequency of the Val allele was significantly lower in elderly subjects than in young subjects (0.30 and 0.36, respectively; $\chi^2_{df=1}=4.74$, $P=0.030$). This observation was illustrated by the overrepresentation of the Ala/Ala genotype (48.2% versus 40.0%) and the underrepresentation of Ala/Val (43.0% versus 47.6) and Val/Val genotype (8.8%

Table 1. Baseline characteristics of the 666 study subjects aged 85 years and over.

Characteristic	All subjects	Men	Women	Gender difference
Number	666	188	478	
Born in Leiden - number (%)	365 (56%)	114 (61%)	251 (53%)	
Age - median (range)	89 (85-100)	89 (85-100)	89 (85-100)	*P*=0.48
History of myocardial infarction	7.8%	8.7%	7.5%	*P*=0.57
History of cerebrovascular disease	2.4%	2.2%	2.5%	*P*=0.75
Hypertension[a]	22.6%	8.7%	28.0%	*P*<0.0001
Diabetes	11.8%	9.1%	12.9%	*P*=0.13
Cancer	7.1%	10.5%	5.8%	*P*=0.014
Smoking	17.3%	51.4%	4.1%	*P*<0.0001
Use of alcohol	25.3%	49.4%	16.1%	*P*<0.0001

[a] Includes a self-reported history of hypertension, diastolic blood pressure >95 mmHg and/or the use of anti-hypertensive medication.

Table 2. *MTHFR* genotype distributions and estimated mortality risks in subjects aged 85 years and over and young subjects whose families originated from the same geographical region.

MTHFR Genotype	Subjects				Mortality risk 95% CI[b]		Test for trend
	Elderly (%)		Young[a] (%)				
all subjects							
Ala/Ala	176	(48.2%)	100	(40.0%)	1		
Ala/Val	157	(43.0%)	119	(47.6%)	1.3	0.9-1.9	
Val/Val	32	(8.8%)	31	(12.4%)	1.7	1.0-3.0	*P*=0.028
men only							
Ala/Ala	55	(48.2%)	50	(36.0%)	1		
Ala/Val	54	(47.4%)	72	(51.8%)	1.5	0.9-2.5	
Val/Val	5	(4.4%)	17	(12.2%)	3.7	1.3-10.9	*P*=0.011
women only							
Ala/Ala	121	(48.2%)	50	(45.0%)	1		
Ala/Val	103	(41.0%)	47	(42.3%)	1.1	0.7-1.8	
Val/Val	27	(10.8%)	14	(12.6%)	1.3	0.6-2.6	*P*=0.51

[a] Median age 31 years (range 18-40).
[b] Mortality risks and 95% CIs were estimated with the exposure odds ratio.

versus 12.4%) in elderly subjects as compared with young subjects.

Since mortality and the distribution of specific causes of death as well as the exposure to factors that potentially modulate the effects of the *MTHFR* mutation may vary between men and women, the association was explored for both genders separately. The prevalence of the Val/Val genotype in elderly men was significantly lower than in elderly women (4.4% and 10.8%, respectively; $\chi^2_{df=1}$=3.97, *P*=0.046), whereas the Val/Val frequency was virtually the same in young men and women (12.2% and 12.6%, respectively; $\chi^2_{df=1}$=0.01, *P*=0.93). Hence, the frequency of the Ala/Val and Val/Val genotypes were significantly reduced in elderly men as compared with young men ($\chi^2_{df=1}$=6.40; *P* for trend=0.011), but similar in elderly and young women ($\chi^2_{df=1}$=0.42; *P* for trend=0.51) (table 2).

Mortality risks were estimated on the basis of the *MTHFR* genotype distributions in elderly and young subjects. The mortality risks associated with the Ala/Val and the Val/Val genotype were estimated at 1.3 (95% CI, 0.9-1.9) and 1.7 (95% CI, 1.0-3.0) fold increased, respectively (table 2). The mortality risk for men carrying the Val/Val genotype compared with those carrying the Ala/Ala genotype was estimated at 3.7 (95% CI, 1.3-10.9), whereas an increased mortality risk was virtually absent in women.

The elderly subjects were the survivors of a cohort born in Leiden between 1887-1901.

Table 3. *MTHFR* genotype distribution in young subjects dependent on their number of grandparents born in Leiden.

MTHFR genotype	Number of grandparents born in Leiden			
	1 or more (N=203)	2 or more (N=178)	3 or more (N=120)	4 (N=76)
Ala/Ala	38.4%	39.3%	44.2%	40.8%
Ala/Val	49.8%	48.3%	43.3%	47.4%
Val/Val	11.8%	12.4%	12.5%	11.8%

Therefore, an investigation was made of whether the young control population was likely to represent the Leiden genotype distribution of two generations before. The *MTHFR* genotype distribution in control subjects was independent of the number of grandparents born in Leiden (table 3). This indicates that the selection criterion for the control population (i.e. either two Leiden-born parents or one Leiden-born parent and the other born within a 12-km distance of Leiden) had been strict enough to obtain a population representing past Leiden genotype frequencies.

Prospective study

During the 10-year follow-up period, 591 (89%) deaths occurred in the complete 85-plus cohort investigated in this study (n=666; 2 subjects lost to follow up). The cumulative survival of men and women stratified according to *MTHFR* genotype is shown in figure 1. Men carrying the Val/Val genotype survived for a shorter time (*P* log-rank=0.020). The median survival time of this group was 11 months compared with 38 months and 36 months for men with Ala/Ala and Ala/Val genotype, respectively. Among women, the *MTHFR* mutation was not associated with a difference in life expectancy (*P* log-rank=0.16).

Overall, the Ala/Val and Val/Val genotypes were not associated with an increased mortality risk (gender and age-adjusted relative risk, 0.9 [95% CI, 0.7-1.0] and 0.9 [95% CI, 0.6-1.2], respectively). However, men homozygous for the mutation, but not heterozygous men, had a significantly increased mortality risk as compared with men carrying the Ala/Ala genotype (age-adjusted relative risk, 2.0 [95% CI, 1.1-3.9]) (table 4). This mortality risk did not appreciably change after adjusting for smoking, alcohol consumption, hypertension and diabetes (relative risk, 2.0 [95% CI, 0.9-4.4]). Among women, the *MTHFR* mutation was associated with marginally lower mortality risks, which bordered on the significant.

In the elderly cohort, the *MTHFR* mutation was not associated with a self-reported

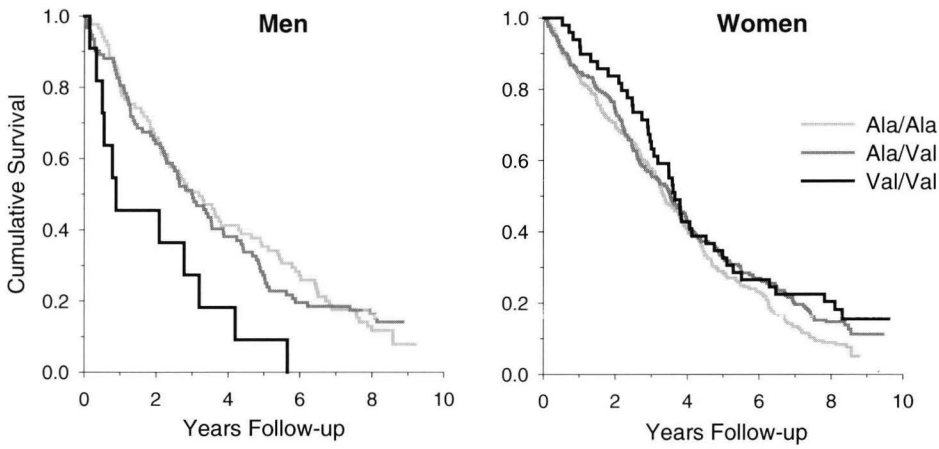

Figure 1. Kaplan-Meier estimate of 10-year cumulative survival according to *MTHFR* genotype for men and women aged 85 years and over.

history of myocardial infarction or cerebrovascular disease (data not shown). The mutation was also not associated with mortality from cardiovascular disease either in men or women (table 4). The mortality risk from cancer, however, was significantly higher among men with the Val/Val genotype (age-adjusted relative risk, 4.2 [95% CI, 1.3-13.5]), whereas among women carrying the Val/Val genotype the risk was not significantly different.

Discussion

In this study we have explored the possible detrimental effects of a disturbed methionine/homocysteine metabolism. This was done by investigating the association of the common *MTHFR* Ala-to-Val mutation with mortality in a cohort of men and women born between 1887-1901. It can be assumed that carriers of the *MTHFR* mutation, in general, have a mildly disturbed methionine/homocysteine metabolism during their whole life. The effect of the *MTHFR* mutation on mortality before the age of 85 years was studied in a cross-sectional design that accounted for possible geographical differences in the *MTHFR* genotype distribution. The *MTHFR* mutation was associated with an increased mortality risk as indicated by an underrepresentation of the mutation in elderly as compared with young subjects. This association was predominantly present in men. Men homozygous for the mutation had about a 4-fold increased mortality risk. Our findings are supported by the reduction of the Val/Val genotype observed in French centenarians[17] and the gradual decline in prevalence of the Val/Val genotype with increasing age found in Japanese subjects,[36] whereas two other cross-sectional studies[37,38] did not observe a decreased prevalence of the *MTHFR* mutation in old age. The design of these studies did, however, not extensively control for geographical variations in genotype distribution or gene-pool effects. Also, population differences in factors modulating the effect of the *MTHFR* mutation may have contributed to these variable results.

Mortality after the age of 85 years was studied prospectively over a 10-year follow-up

Table 4. All-cause and cause-specific 10-year mortality risks according to *MTHFR* genotype in subjects aged 85 years and over.

***MTHFR* genotype**	**N**	**all cause**		**cardiovascular disease**		**cancer**		**infectious disease**	
		RR	95% CI	RR	95% CI	RR	95% CI	RR	95% CI
men									
Ala/Ala	85	1		1		1		1	
Ala/Val	92	1.0	0.7-1.4	0.7	0.4-1.2	1.2	0.6-2.6	1.1	0.4-3.1
Val/Val	11	2.0	1.1-3.9	0.7	0.2-3.1	4.2	1.3-13.5	1.4	0.1-14.6
women									
Ala/Ala	223	1		1		1		1	
Ala/Val	204	0.8	0.7-1.0	0.9	0.7-1.3	0.9	0.6-1.5	0.6	0.3-1.2
Val/Val	49	0.7	0.5-1.0	0.9	0.5-1.5	0.5	0.2-1.4	0.3	0.1-1.3

RR indicates the mortality risk as estimated with a Cox proportional hazard model adjusted for age at baseline.

period. The mutation conferred a 2-fold increased mortality risk in men aged 85 years and over homozygous for the mutation, but not in women. The prospective study thus confirms the increased mortality risk associated with the *MTHFR* mutation observed in our cross-sectional analysis.

The relation of the *MTHFR* mutation with cardiovascular disease risk is controversial. Some studies reported an association of the Val/Val genotype with an increased risk of cardiovascular disease,[4,14,18,23] whereas in other studies evidence for this relation was absent.[10,15,16,19,21,24-28] Here, we show that the *MTHFR* mutation was associated with an increased mortality in men before and after the age of 85 years but, by design, no data were available concerning the causes of death in middle age. Over the age of 85 years, cardiovascular diseases did not contribute to the increased mortality of men with the Val/Val genotype. Although half of the elderly men and women died from cardiovascular disease (data not shown), the mutation was not associated with increased cardiovascular disease mortality. Two interpretations are compatible with these findings. The *MTHFR* mutation is not related to mortality due to cardiovascular diseases in middle and old age. Alternatively, carriers of the mutation are subject to an increased cardiovascular disease mortality before the age of 85 years, which has led to the selective survival of carriers who are less susceptible to cardiovascular diseases.

Although the numbers were small, we found that the increased mortality risk of men aged 85 years and over with the Val/Val genotype was largely explained by an increased risk of death from cancer. An association between the *MTHFR* mutation and the risk of colorectal cancer was reported in two other prospective studies. United States health professionals[12] and physicians[29] carrying the Val/Val genotype were found to have a reduced risk of colorectal cancer. This protective effect was abolished by moderate alcohol consumption, probably because alcohol depletes folate.[39] It is not clear how the opposite effects of the *MTHFR* mutation on cancer risk in our population-based study and the previous studies can be explained. The *MTHFR* mutation may increase the risk of cancer especially in groups with a low folate intake, such as the elderly, rather than in well-nourished health professionals and physicians.[12,29]

From the present data it remains unclear why men carrying the *MTHFR* mutation were at an increased risk of mortality whereas the mortality in women carrying the mutation was not affected. Hormonal differences with respect to estrogens are a less likely explanation for the association observed, since the gender-dependent association with mortality persisted after the age of 85 years when women are well beyond the menopause. An influence of estrogens on the methionine/homocysteine metabolism is further refuted by the absence of a long-term effect of hormone replacement therapy on the level of plasma homocysteine.[40] Since the level of plasma folate is a critical modulator of the *MTHFR* mutation, differences in the level of plasma folate between men and women may also have contributed to the gender-dependent association. A previous study in the elderly, however, reported no difference in the level of plasma folate between men and women despite the higher folate intake of women.[41]

A clear gender-difference was present with respect to smoking habits and alcohol consumption; 51% of the men and only 4% of the women reported to smoke, and 49% of the men reported to consume alcohol versus 16% of the women. Smoking is associated

with elevated plasma homocysteine[15,42] whereas alcohol is a methyl group antagonist[43] and depletes folate.[39] It may be hypothesised that the combined effects of smoking, alcohol consumption and the *MTHFR* mutation on the methionine/homocysteine metabolism might have led to an increased mortality risk in men but not in women. Especially among individuals from the 1887-1901 birth-cohort, who were middle aged during the 1930s to 1960s, the majority of men smoked whereas among women smoking was uncommon.

In conclusion, our data suggest that homozygosity for the *MTHFR* Ala-to-Val mutation increases the mortality risk in men both in middle and old age. Our study does not reveal the causes of death contributing to the increased mortality risk in middle age, but suggests that cancer rather than cardiovascular disease may be the primary cause of death of elderly men carrying the Val/Val genotype. Larger prospective population-based studies are needed to confirm the effect of the *MTHFR* mutation on all-cause and cancer mortality. Interventions starting at a young age to restore a balanced methionine/homocysteine metabolism may prove to be beneficial to carriers of the Val/Val genotype.

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Chapter 3

Association of *APOE* ε2/ε3/ε4 and promoter gene variants with dementia but not cardiovascular mortality in old age

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Abstract

The common apolipoprotein E (*APOE*) alleles ε2, ε3 and ε4 are associated with the risk of dementia and cardiovascular disease. Recently, two functional variants (-219G/T and -491A/T) have been identified in the promoter of the *APOE* gene that enable a further characterisation of the role of the *APOE* locus in disease. We investigated the contribution of these *APOE* gene variants to dementia and cardiovascular mortality in old age using a population-based cohort of 648 subjects aged 85 years and over (Leiden 85-plus Study).

Genotypes containing an *APOE* ε4 allele were associated with a 4.1-fold (95% CI, 2.2-7.7) increased risk of dementia as compared to the ε3/ε3 genotype in old subjects. Moreover, homozygosity for the -219T allele was found to be associated with a 2.4-fold (95% CI, 1.0-5.8) increased risk independently of ε2 and ε4; the -491A/T variant was not associated with dementia. Over a 10-year follow-up period the risk of cardiovascular mortality was not increased among ε4 carriers (RR, 0.8 [95% CI 0.6-1.1]) or -219T homozygous subjects (RR, 1.1 [95% CI, 0.8-1.5]) nor decreased among -491T homozygous subjects (RR, 1.0 [95% CI, 0.5-2.3]).

In conclusion, both the *APOE* ε2/ε3/ε4 and the -219G/T variant were identified as risk factors for dementia but not cardiovascular mortality in old age. Our results support the hypothesis that both the isoform and the amount of brain APOE may influence the risk of dementia. Furthermore, they emphasise the higher impact of variation at the *APOE* locus on the risk of dementia than on the risk of cardiovascular disease in old age.

Submitted for publication

Introduction

The common apolipoprotein E (*APOE*) alleles ε2, ε3 and ε4 are strongly associated with age of onset and risk of dementia.[1] The biological mechanism underlying the association with dementia is not known. Hypotheses include a differential effect of *APOE* alleles on amyloid deposition,[2] tangle formation,[3] neuronal plasticity[4] and cholinergic function.[5] In addition, the ε4 allele is associated with an increased risk of cardiovascular disease,[6-8] which may be the consequence of higher LDL cholesterol and triglyceride levels in plasma among individuals carrying an ε4 allele.[9]

Two variants in the promoter of the *APOE* gene, -219G/T and -491A/T, were found to influence transcriptional activity *in vitro*[10] and thereby enable further characterisation of the role of the *APOE* locus in disease. The contribution of these promoter variants to dementia has been investigated in several studies. Some studies observed an association of the -491A/T variant with Alzheimer's type dementia,[11,12] but this finding was refuted by others[13-15] and the association of the -219G/T variant with Alzheimer's type dementia in hospital patients[13] could not be replicated.[16] Hence, the influence of the promoter variants on the risk of dementia in addition to the *APOE* ε2/ε3/ε4 variant is still largely unclear. Moreover, data on the impact of the promoter variants on cardiovascular disease risk are still lacking.

The implication of common variants at the *APOE* locus in both dementia and cardiovascular disease makes this locus a prime candidate for affecting mortality in the population at large. This has been tested previously for the APOE ε2/ε3/ε4 variants. Most[17-23] but not all[24] cross-sectional studies reported lower frequencies of ε4-containing genotypes and higher frequencies of ε2-containing genotypes in old versus young populations. The results of prospective studies are, however, not consistent. An association between the ε4 allele and mortality appears to be present before[25-27] but not after[25-29] the age of ±75 years. The exclusion of less healthy subjects and geographical differences in genotype distribution might have contributed to the discrepancy between the results of cross-sectional and prospective studies.

We further explored the relevance of variation at the *APOE* locus to dementia, cardiovascular diseases and mortality in old age, as in this age group dementia is common and cardiovascular diseases are the primary cause of death. To this end subjects aged 85 years and over from the Leiden 85-plus Study[30] were investigated using both a cross-sectional and a prospective design.[31] The study had a high response rate (94%) and permitted the comparison of subjects with a homogeneous geographic background.

Methods

Subjects

The Leiden 85-plus Study is a population-based study in which all subjects aged 85 years and over living in Leiden, a city with 108,000 inhabitants in the western part of The Netherlands, were invited to take part [30]. Out of a total of 1258 eligible subjects, 221 died before enrolment. Of the 1037 remaining subjects, 977 (94%) participated and were medically interviewed at home. After exclusion of subjects with a non-Dutch (n=29) or unknown (n=69) place of birth, sufficient cell material was available from 666 (188

men/478 women) subjects for the present genetic study.

To identify patients with dementia, participants with a Mini-Mental State Examination (MMSE) score lower than 24 out of 30 (n=226) were evaluated by a psychiatrist using the Dutch version of the Geriatric Mental State Schedule. 149 subjects were found to be demented according to DMS-III criteria[32]; cognitive status could not be established in 74 subjects.

All participants were followed for mortality until October 1, 1996 (2 lost to follow-up). Primary causes of death were obtained from the Dutch Central Bureau of Statistics. Cardiovascular causes were coded according to the ninth revision of the International Classification of Diseases (codes 390-459).[33]

As a young control group, a population consisting of 250 (139 men/111 women) blood donors aged 18-40 years of Dutch descent was recruited. The young subjects had either two Leiden-born parents or had one Leiden-born parent and one born within a 12-km radius of Leiden. Information regarding the birthplace of their parents and grandparents was obtained from a written questionnaire. Of the 250 young subjects, 78 (38%) had four Leiden-born grandparents and 72 (29%) ≤1 definite Leiden-born grandparents. The study was approved by the Medical Ethics Committee of the Leiden University and informed consent was obtained from all participants.

APOE genotyping

DNA was extracted by protein precipitation using potassium acetate and chloroform extraction. *APOE* ε2/ε3/ε4,[34] -219G/T[35] and -491A/T[36] genotypes were determined as previously described. Digestion products were separated on 7.5% (-491A/T and ε2/ε3/ε4) and 10% (-219G/T) polyacrylamide MADGE-gels (microtitre array diagonal gel electrophoresis).[37] In 97% (648/666) of the subjects for whom DNA was available, all three *APOE* genotypes were successfully determined. Two observers independently assessed the *APOE* genotypes. As a standard laboratory procedure a randomly chosen 10% of the samples was reamplified for each *APOE* variant. In all cases the previously assigned genotype was confirmed.

Statistical analyses

In the cross-sectional analyses, distributions of genotypes were compared by the χ^2-test. Risks of dementia and mortality and 95% confidence intervals (CIs) were estimated using logistic regression. Pairwise linkage disequilibria and maximum likelihood haplotype frequencies were estimated using Arlequin software version 1.1.[38] In the prospective study, survival times for subjects were computed from the date of the home visit to the date of one of the following events: death from a specific cause, death from any cause, or October 1, 1996. Mortality risks and 95% CIs were estimated with Cox' proportional hazards models. All P-values were based on two-sided tests. The analyses were performed using the SPSS statistical software package version 8.0.

Results

Population characteristics

APOE genotypes were determined in 66% (648/977) of the complete cohort of subjects age 85 years and over. 10-year survival was very similar for subjects with and without data on *APOE* genotypes (P=0.92) and both groups were also very similar with respect to cardiovascular risk factors and health status, including cognitive function (table 1). Subjects with data on *APOE* genotypes were on average 5 months older.

Since the 85-plus population comprises subjects born in different regions in The Netherlands, it was investigated whether *APOE* allele frequencies were dependent on geographical origin of the subjects. The ε2 frequency was higher and the ε4 frequency was lower among the 352 subjects born in Leiden as compared with the 296 subjects born elsewhere in The Netherlands (ε2: 0.121 versus 0.084, ε4: 0.095 versus 0.115; P=0.068).

Higher ε2 and lower ε4 frequencies were also observed in 76 young subjects with 4 Leiden-born grandparents as compared with in 72 subjects with 1 or less definite Leiden-born grandparents (ε2: 0.092 versus 0.034 and ε4: 0.145 versus 0.208, respectively; P=0.064). Such geographical differences in allele frequency were not observed for the -219G/T and -491A/T *APOE* promoter variants. To eliminate confounding effects of geographical origin, all comparisons were based on old subjects born in Leiden and young subjects with 4 Leiden-born grandparents. Genotype distributions were in Hardy-Weinberg equilibrium.

Dementia

APOE ε2/ε3/ε4 genotypes were strongly associated with the risk of dementia in the cohort aged 85 years and over (table 2; P<0.0005). Genotypes with an ε4 allele were

Table 1. Characteristics of subjects aged 85 years and over according to the availability of *APOE* genotypes.

Characteristic	*APOE* genotype	
	Yes (n=648)	No (n=329)
Age (median, IQR)[a]	89.2 (87.5-91.6)	88.7 (87.2-90.7)
Women	71.8%	74.5%
Born in Leiden	54.3%	59.5%
Smoking[b]	17.2%	22.9%
Use of Alcohol	25.3%	25.3%
Prevalence of dementia	24.6%	22.9%
History of hypertension[c]	22.9%	21.5%
History of diabetes[d]	11.8%	12.5%
Total Cholesterol (mean, SD)	5.7 (1.3)	5.7 (1.3)
Mini Mental State Examination (median, IQR)	26 (21-29)	26 (20-28)

[a] P=0.004

[b] Smoking includes former smokers who had stopped for less than 10 years.

[c] Includes self-reported hypertension, diastolic blood pressure >95 mmHg and/or the use of anti-hypertensive medication.

[d] Includes self-reported diabetes, serum glucose >11 mmol/L in a non-fasting blood sample or the use of medication for treatment of diabetes.

associated with a 4.1-fold (95% CI, 2.2-7.7) increased risk, while the ε2/ε2 and ε2/ε3 genotypes were associated with a 0.7 (95% CI, 0.3-1.5) reduced risk as compared to the ε3/ε3 genotype. A significant association with dementia was also observed for the -219G/T variant (P=0.019), but not for the -491A/T variant (P=0.54; table 2). Both promoter variants were in partial linkage disequilibrium with the *APOE* ε2/ε3/ε4 variant (P<0.0001), but not with each other (P=0.92). On the basis of haplotype frequencies estimated for maximum likelihood (data not shown), none of the -219T alleles occurred on a haplotype with the ε2 allele and 18.4% occurred in combination with the ε4 allele. For the -491T allele these frequencies were 41.2% and 5.5%, respectively. When restricting the analysis to ε3 homozygous subjects (n=198) to circumvent the effect of linkage disequilibrium, the -219T/T genotype was associated with a significantly increased risk of dementia (OR, 2.4 [95% CI, 1.0-5.8]). A similar association was observed when was controlled for ε2 and ε4 effects using logistic regression (OR, 1.9 [95% CI, 0.9-4.0]). The low frequency of the -491T allele precluded an assessment of its effect independent of the ε2 and ε4 alleles.

Mortality

Association of *APOE* genotypes with mortality prior to the age of 85 years was assessed by comparing genotype frequencies in old and young subjects of Leiden origin (table 3). The young population consisted of subjects with 4 Leiden-born grandparents and is, thus, likely to represent the Leiden gene pool of two generations before. No overall significant difference in genotype frequency was observed for the ε2/ε3/ε4 variant (P=0.29; table 3). The ε4/ε4 genotype, however, was associated with a 4.8-fold (95% CI, 0.9-24.4) increased mortality risk. Although a trend was observed towards different

Table 2. *APOE* genotypes in subjects with and without dementia aged 85 years and over born in Leiden.

APOE **genotype**	**Dementia (n=78)**	**No dementia (n=242)**	**Risk of dementia (95% CI)**[a]	
ε2/ε3/ε4				
ε3/ε3	51.3%	65.3%	1	(referent)
ε2/ε2	0.0%	1.7%	-	(-)
ε2/ε3	12.8%	21.9%	0.7	(0.3-1.6)
ε2/ε4	7.7%	2.1%	4.7	(1.4-16.3)
ε3/ε4	26.9%	8.3%	4.1	(2.1-8.4)
ε4/ε4	1.3%	0.8%	2.0	(0.2-22.3)
-219G/T				
G/G	25.6%	33.1%	1	(referent)
G/T	50.0%	55.4%		
T/T	24.4%	11.6%	2.5	(1.3-4.7)
-491A/T				
A/A	71.8%	71.5%	1	(referent)
A/T	26.9%	24.8%		
T/T	1.3%	3.7%	0.3	(0.0-2.7)

[a] Risks of dementia were estimated using logistic regression.

-219G/T genotype distributions in the young and old population (P=0.11; table 3), the estimated risk of mortality associated with the -219T/T genotype approximated unity when the analysis was restricted to ε3 homozygous subjects (n=266; mortality risk, 1.0 [95% CI, 0.4-2.5]) or when adjusting for ε2 and ε4 effects using logistic regression (mortality risk, 1.5 [95% CI, 0.7-2.9]). Genotype frequencies for the -491A/T variant were similar (P=0.32; table 3). An assessment of its effects independent of the ε2/ε3/ε4 variant was precluded by the absence of the –491T/T genotype among the young subjects.

The association between *APOE* genotypes and the risk of mortality after the age of 85 years was investigated over a follow-up period of 10 years. All old subjects (n=648) were now included in the analyses since this design is not distorted by geographical variation in genotype distributions. During follow-up, 574 (89%) died from any cause and 245 (38%) died from cardiovascular causes. Old subjects that were either heterozygous or homozygous for the ε4 allele did not have an increased risk of all-cause (RR, 1.0 [95% CI, 0.8-1.2]) or cardiovascular (RR, 0.8 [95% CI, 0.6-1.1]) mortality as compared to ε3 homozygous subjects. No associations of the promoter variants with either end-point were detected (table 4); this result was not affected by controlling for ε2 and ε4 alleles (data not shown).

Discussion

The main findings of the present study are: first, the *APOE* ε2/ε3/ε4 variant was strongly associated with the risk of dementia in subjects aged 85 years and over; second,

Table 3. Comparison of *APOE* genotypes in young and old persons reflecting *APOE*-associated mortality before the age of 85 years.

APOE **genotype**	**Old**[a] **(n=352)**	**Young**[a] **(n=76)**	**Mortality risk**[b] **(95% CI)**	
ε2/ε3/ε4				
ε3/ε3	62.5%	60.5%	1	(referent)
ε2/ε2	1.1%	0.0%	-	(-)
ε2/ε3	18.2%	14.5%	0.8	(0.4-1.7)
ε2/ε4	3.7%	3.9%	1.1	(0.3-4.0)
ε3/ε4	13.6%	17.1%	1.3	(0.6-2.6)
ε4/ε4	0.9%	3.9%	4.8	(0.9-24.4)
-219G/T				
G/G	31.5%	34.2%	1	(referent)
G/T	53.4%	42.1%		
T/T	15.1%	23.7%	1.8	(1.0-3.2)
-491A/T				
A/A	73.0%	76.3%	1	(referent)
A/T	24.1%	23.7%		
T/T	2.8%	0.0%	0	(-)

All subjects have the same geographical origin, i.e. old subjects were born in Leiden and young subjects had 4 Leiden-born grandparents.

[a] Mean age old and young subjects 89 years (range 85-100 years) and 31 years (range 18-40 years), respectively.

[b] Mortality risks were estimated using logistic regression.

the -219G/T but not the -491A/T *APOE* promoter variant was indicated to be a risk factor for dementia independent of the ε2 and ε4 alleles; third, the ε4/ε4 genotype, but not any of the other *APOE* genotypes, was associated with increased mortality prior to the age of 85 years; fourth, *APOE* genotypes were not related to all-cause or cardiovascular mortality after the age of 85 years.

The potential influence of the ε4 allele on mortality has been explored in numerous studies. Our prospective study adds to the evidence from previous ones indicating that the ε4 allele does not affect mortality in old age (>±75 years).[25-29] The ε4 allele is, however, associated with a moderately increased mortality rate at less old ages (<±75 years).[25-27] This association is generally attributed to an increased risk of cardiovascular disease[6-8] among ε4 carriers. Since cardiovascular diseases are the primary cause of death both before and after the age of 75 years and dementia becomes a common disease only after this age, the ε4 allele may have been expected to affect mortality in old age as well. The absence of this relation may be explained by attenuated effects of the ε4 allele on the risk of coronary atherosclerosis[39] and dementia[1] at older ages. Unknown age-dependent differences in the pathogenesis of cardiovascular diseases and dementia might underlie these observations.

Our finding that ε3/ε4 genotype frequency was not markedly lower in old age contrasts to the majority of previous cross-sectional studies.[17-23] The selection of older groups in positive studies can be rejected as an explanation for these discrepancies since the ε3/ε4 genotype does not contribute to mortality in old age. The discrepancies may, however, be related to other factors than mortality that can give rise to decreased ε3/ε4

Table 4. Mortality risks in subjects aged 85 years and over dependent on *APOE* genotype.

***APOE* genotype**	**N**	**Mortality risk (95% CI)**			
		All causes		**Cardiovascular causes**	
ε2/ε3/ε4					
ε3/ε3	408	1	(referent)	1	(referent)
ε2/ε2	5	1.9	(0.8-4.6)	0.8	(0.1-5.5)
ε2/ε3	104	1.0	(0.8-1.2)	0.9	(0.6-1.3)
ε2/ε4	21	1.1	(0.7-1.8)	0.7	(0.3-1.7)
ε3/ε4	102	1.0	(0.8-1.2)	0.8	(0.6-1.2)
ε4/ε4	6	0.9	(0.4-2.1)	0.3	(0.0-2.4)
-219G/T					
G/G	221	1	(referent)	1	(referent)
G/T	331				
T/T	104	1.0	(0.8-1.3)	1.1	(0.8-1.5)
-491A/T					
A/A	471	1	(referent)	1	(referent)
A/T	162				
T/T	13	1.0	(0.6-1.8)	1.0	(0.5-2.3)

All subjects were followed for 10 years; 574 (89%) died of any cause and 245 (38%) died of cardiovascular disease.
Mortality risks were estimated using a Cox proportional hazards model.

frequencies in old populations. Since the ε3/ε4 genotype is linked to impaired cognition, decreased ε3/ε4 frequencies may be the result of a bias towards an overrepresentation of cognitively healthy elderly. For example, previous cross-sectional studies included only ambulatory, autonomous octogenarians without mental illnesses,[17] healthy normal individuals[18] or elderly subjects who were ambulatory and not institutionalised.[19] Studies with lower response rates are prone to this bias as well since unhealthy individuals are less inclined to participate in scientific studies. Non-mortality related differences in genotype distribution may also arise when the old and young populations do not originate from the same gene pool. Our population-based study among old subjects had a relatively high response rate and the old and young populations were likely to be descended from the same gene pool since the old subjects were born in the same municipality as the four grandparents of the young subjects. According to our cross-sectional analysis, the increase in mortality before the age of 85 years associated with the ε3/ε4 genotype was modest and non-significant. Some of the previous cross-sectional studies may have overestimated the effect of the genotype on mortality.

Our study once more illustrates the importance of comparing groups with a homogeneous geographic background in genetic association studies. Previously, a north-south gradient in the frequency of *APOE* ε2/ε3/ε4 alleles was found to exist over Europe.[40] Here, we present data suggesting that frequencies of the ε2 and ε4 alleles vary between Leiden and non-Leiden subjects indicating the presence of geographical differences on a much smaller scale. These differences would not have been detected in smaller studies and might have led to spurious associations. Remarkably, the genotype distributions of polymorphisms in four other genes were not dependent on geographical region.[31,41,42] The frequencies of the *APOE* promoter variants were also not dependent on geographical region owing to the fact that the rare promoter alleles mainly occurred on the same haplotype as the ε3 allele, which dilutes the ε2 and ε4 effects. Our findings indicate that equal distributions of one or several gene variants do not guarantee that future differences are due to an association with disease risk rather than gene pool effects

In conclusion, the ε2/ε3/ε4 and -219G/T *APOE* variants were associated with an increased risk of dementia (prevalence 25%), but did not predict cardiovascular mortality (38% of deaths) in old age. Our results support the hypothesis that both the amount and the isoform of brain APOE may influence the risk of dementia.[13] Furthermore, they emphasise the higher impact of variation at the *APOE* locus on the risk of dementia[1] than on the risk of cardiovascular disease in old age.[6-8]

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Chapter 4

Common paraoxonase gene variants, mortality risk and fatal cardiovascular events in elderly subjects

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Abstract

Recent studies indicate that the enzyme paraoxonase may be an important modulator of cardiovascular disease risk because of its ability to protect LDL from oxidation. We tested for association between two functional variants of the paraoxonase gene (Met55/Leu and Gln192/Arg) and both all-cause mortality and fatal cardiovascular disease. This was done within a population-based study among subjects aged 85 years and over in a cross-sectional and a prospective design.

In the cross-sectional analysis, the distribution of both paraoxonase genotypes was found to be similar in the subset of 364 elderly subjects who were born in Leiden, The Netherlands, as compared to 250 young subjects whose families originated from the same geographical region. The polymorphisms were in strong linkage disequilibrium ($P<0.00001$) and the frequency of the haplotype carrying both risk alleles was not lower in the elderly than in the young (0.313 versus 0.284).

The complete cohort of 666 elderly subjects was followed over 10 years. The risk of all-cause and cardiovascular mortality was not increased in elderly subjects with the paraoxonase Leu/Leu (RR, 1.1; 95% CI, 0.9-1.5, and 1.3; 95% CI, 0.8-2.0, respectively) or the Arg/Arg genotype (RR, 0.9; 95% CI, 0.7-1.2, and 0.7; 95% CI, 0.4-1.3, respectively). In a subset of patients with diabetes, the all-cause mortality risk was elevated in Arg/Arg carriers (RR, 2.1; 95% CI, 0.8-5.8) but this did not reach statistical significance. Analyses of genotype combinations did not yield significant associations with mortality.

The paraoxonase gene variants, previously associated with coronary artery disease, are not likely to have a major effect on the risk of fatal cardiovascular disease in the population at large. Adverse effects of the gene variants might be observed in subjects exposed to factors that enhance oxidative stress such as diabetes.

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Introduction

Oxidised low density lipoprotein (oxLDL) is thought to play a central role in atherogenesis.[1,2] Evidence is accumulating that the enzyme paraoxonase protects LDL from oxidation. Paraoxonase, which is physically associated with apolipoprotein A-I in HDL, inhibits Cu^{2+}-induced oxidation of LDL *in vitro*[3] by destroying proinflammatory lipid peroxides.[4] Subsequent studies using a cell co-culture model showed that HDL from wild-type mice but not from paraoxonase deficient mice inhibits the monocyte chemotactic activity of LDL, which becomes oxidised in the subendothelial matrix if HDL is absent.[5] Moreover, paraoxonase deficient mice are more susceptible to atherosclerosis than wild-type mice when fed a high-fat/high-cholesterol diet.[5]

The contribution of oxLDL to cardiovascular disease in humans may thus be evaluated by studying functional variants of the paraoxonase gene (*PON1*). Two variants in the coding region have been identified leading to a methionine-55 to leucine and a glutamine-192 to arginine amino acid substitution.[6] The inter-individual variation in the ability of paraoxonase to hydrolyse organophosphorous compounds is determined by the Gln192/Arg polymorphism. The effect, however, is substrate-dependent.[7,8] *In vitro*, the Arg-isoform was found to be less effective in preventing LDL from oxidation by Cu^{2+} than the Gln-isoform.[9] Hence, the Arg-allele may be a risk factor for cardiovascular disease. Evidence for this hypothesis was obtained in three studies in Caucasian subjects, which found the Arg-allele to be more common in type 2 diabetic patients with coronary heart disease[10,11] and in patients with more than 75% luminal stenosis in a coronary artery[12] than in controls. Two small studies suggested that the Arg-allele was associated with coronary heart disease in the Japanese.[13,14] In contrast, no increased frequency of the Arg-allele was observed in myocardial infarct patients in two studies,[11,15] in Finnish patients who underwent coronary bypass surgery[16] and in Italian patients with more than 50% stenosis.[17]

The Met55/Leu polymorphism has been associated with the level of paraoxonase in serum[8] and mRNA in liver biopsies.[18] Surprisingly, it was the high-level associated Leu-allele that was found to represent an increased risk of coronary heart disease in Caucasian patients with type 2 diabetes.[8] This might suggest that the Leu-allele is not only associated with paraoxonase serum level, but also has a detrimental effect on enzyme function. In a study among Asian Indians and Chinese, the Met55/Leu polymorphism was not associated with coronary heart disease.[19]

Until now, none of the studies that assessed the possible disease association of the paraoxonase polymorphisms were prospective, nor did they include fatal cases. Therefore, we explored whether both polymorphisms, either separately or in combination, are associated with all-cause and cardiovascular mortality in the general population. This was done within a population-based study among subjects aged 85 years and over (Leiden 85-plus Study[20]) using two designs.[21] The impact of the gene variants on mortality before the age of 85 years was studied cross-sectionally, by comparing the paraoxonase genotype distribution between subjects aged 85 years and over and young subjects with families from the same geographical region. In a prospective study with a 10-year follow-up period, the relation of the gene variants to all-cause and cardiovascular mortality above the age of 85 years was investigated.

During follow-up, the all-cause mortality was 89% and the cardiovascular mortality was 38%.

Methods

The Leiden 85-plus Study is a population-based study in which all inhabitants of Leiden, The Netherlands, aged 85 years and over were invited to take part.[20] Out of a total of 1258 eligible subjects, 221 died before enrolment. Of the 1037 remaining subjects, 977 (94%) participated and were medically interviewed at home. Diabetes was diagnosed on the basis of the medical interview, use of medication for treatment of diabetes and/or a serum glucose level over 11.0 mmol/l in a non-fasting blood sample. After exclusion of subjects with a non-Dutch (n=29) or unknown (n=69) place of birth, sufficient cell material was available from 666 (188 men/478 women) subjects for the present genetic study. Subjects of who a DNA sample was available did not significantly differ from subjects of who was not with respect to age (P=0.2), gender (P=0.2), smoking (P=0.8), the prevalence of diabetes (P=0.8) or the prevalence of hypertension (P=0.8). DNA was extracted by protein precipitation using potassium acetate and chloroform extraction.[22] The paraoxonase Met55/Leu and Gln192/Arg genotypes were determined by PCR-amplification followed by digestion with *Nla*III and *Alw*I, respectively.[6] For genotyping of the Gln192/Arg polymorphism an alternative downstream primer was used (5'-GAGAATCTGAGTAAATCCACTACATTTCAG) which results in a 64 bp and a 172 bp DNA fragment after digestion if the Arg-allele is present. Digestion products were separated on 7.5% polyacrylamide MADGE-gels (microtitre array diagonal gel electrophoresis).[23] Paraoxonase genotypes were independently assessed by two observers. As a standard laboratory procedure a randomly chosen 10% of the samples was reamplified. In all cases the previously assigned genotype was confirmed. The study was approved by the Medical Ethics Committee of the Leiden University and informed consent was obtained from all participants.

Cross-sectional analysis

Paraoxonase genotype distributions were compared among elderly subjects aged 85 years and over and young controls. To avoid false associations with the paraoxonase polymorphisms due to differences in geographical origin rather than age, only those subjects aged 85 years and over who were born in Leiden (n=364; 55%) were compared with a control population which consisted of 250 (139 men/111 women) blood donors aged 18-40 years of Dutch descent with either two Leiden-born parents or one Leiden-born parent and the other born within a 12-km distance of Leiden. Information regarding the birthplace of their grandparents was obtained from a written questionnaire.

The elderly subjects were survivors of a cohort born in Leiden between 1887-1901. An investigation was made of whether the young control population was likely to represent the Leiden genotype distribution of two generations before. The frequencies of the Leu/Leu genotype were 41.4%, 41.0%, 42.5% and 42.1% among young subjects with either 1 or more (n=203), 2 or more (n=178), 3 or more (n=120) and 4 (n=76) Leiden-born grandparents, respectively. For the Arg/Arg genotype these frequencies were 9.4%,

9.6%, 11.7% and 9.2%, respectively. Thus, the paraoxonase genotype frequencies in control subjects were independent of the number of grandparents born in Leiden. This indicates that no specific Leiden paraoxonase genotype distribution existed around the year 1900.

Prospective study

All participants in the Leiden 85-plus Study were followed for mortality until October 1, 1996. Among the 666 subjects of the cohort studied, 2 were lost to follow-up. Primary causes of death were obtained from the Dutch Central Bureau of Statistics and categorised for cardiovascular disease (*ICD-9* codes[24] 390-459), ischaemic heart disease (410-414), cerebrovascular disease (430-438).

Statistical analysis

When analysing the paraoxonase polymorphisms separately, the genotypes were not grouped because previous studies reported a co-dominant association with the risk of coronary heart disease.[10-12] In the cross-sectional analysis, distributions of genotypes and haplotypes were compared by the χ^2-test. Mortality risks up to the age of 85 years and 95% confidence intervals (CIs) were estimated using the exposure odds ratio. Linkage disequilibrium between the two paraoxonase polymorphisms was tested using a likelihood-ratio test and maximum-likelihood haplotype frequencies were computed using an expectation-maximisation algorithm. Both procedures were performed using Arlequin software version 1.1.[25] The latter procedure is an iterative process aiming at obtaining maximum-likelihood estimates of haplotype frequencies from multi-locus genotypic data when the gametic phase is unknown. In this case, simple gene counting is not possible because several haplotypes are possible for individuals heterozygous at both paraoxonase loci. The expectation-maximisation algorithm starts with arbitrary (random) estimates of haplotype frequencies. These estimates are used to compute expected genotype frequencies assuming Hardy-Weinberg equilibrium (expectation step). The relative genotype frequencies obtained are used as weightings for their two constituting haplotypes in a gene counting procedure leading to new estimates of haplotype frequencies (maximisation step). The expectation and maximisation steps are repeated until the haplotype frequencies reach equilibrium. The maximum likelihood estimate of the population linkage-disequilibrium parameter D and the percent of its maximum possible value were calculated as described by Thompson *et al.*[26]

In the prospective follow-up study, survival times for subjects were computed from the date of the home visit to the date of one of the following events: death from a specific cause, death from any cause, or October 1, 1996. Survival was estimated using the Kaplan-Meier product limit method. Age and gender adjusted mortality risks and 95% CIs were estimated with Cox proportional hazards models. Causes of death were assumed to be independent. P-values of less than 0.05 were considered to indicate statistical significance and all P-values were based on two-sided tests.

Results

Cross-sectional analysis

The genotype distributions of the paraoxonase polymorphisms were determined in a cohort of 666 subjects aged 85 years and over. The frequencies for the Met55/Leu polymorphism were 10.8% (Met/Met), 48.3% (Met/Leu) and 40.8% (Leu/Leu); for the Gln192/Arg polymorphism the frequencies were 47.0% (Gln/Gln), 44.9% (Gln/Arg) and 8.1% (Arg/Arg). Excess mortality before the age of 85 years among carriers of the putative risk-genotypes would be reflected in a reduced frequency of these genotypes in the elderly population. Therefore, paraoxonase genotype frequencies in the elderly subjects born in Leiden (n=364; 55% of the cohort studied) were compared with those in young subjects aged 18 to 40 years whose families originated from the Leiden area (n=250) (table 1). The genotype distributions of both polymorphisms were in Hardy-Weinberg equilibrium. The Met55/Leu genotype distribution was virtually identical among elderly and young subjects (P=0.73). The estimated mortality risk up to the age of 85 years associated with the Leu/Leu genotype was not increased compared with the Met/Met genotype (OR, 0.8; 95% CI, 0.5-1.4). The Gln192/Arg genotype distribution was also similar in the elderly and the young (P=0.26). The mortality risk associated with the Arg/Arg genotype was not increased (OR, 0.9; 95% CI, 0.5-1.6) as compared to that with the Gln/Gln genotype. Separate analysis of men and women yielded similar results. The frequencies of the Leu/Leu and Arg/Arg genotypes observed for elderly men (n=115; 33.9% and 8.7%, respectively) and for elderly women (n=249; 43.4% and 8.4%, respectively) were not significantly different from those in young subjects (39.6% and 8.4%, respectively).

The Met55/Leu and Gln192/Arg polymorphisms were in strong negative linkage disequilibrium (P<0.00001; D=100% in young and D=96% in elderly subjects). Thus, the rare Arg-allele almost always occurred in combination with the frequent Leu-allele,

Table 1. Paraoxonase genotype distributions in subjects aged 85 years and over and young subjects whose families originated from the same geographical region.

Paraoxonase genotype	Subjects			
	Elderly[a] (n=364)		**Young**[b] (n=250)	
Met55/Leu				
Met/Met	39	(10.7%)	32	(12.8%)
Met/Leu	178	(48.9%)	119	(47.6%)
Leu/Leu	147	(40.4%)	99	(39.6%)
Gln192/Arg				
Gln/Gln	164	(45.1%)	129	(51.6%)
Gln/Arg	169	(46.4%)	100	(40.0%)
Arg/Arg	31	(8.5%)	21	(8.4%)
Met55/Leu + Gln192/Arg				
Met/Met-Gln/Gln	39	(10.7%)	32	(12.8%)
Met/Leu-Arg/Arg + Leu/Leu-Gln/Arg	83	(22.8%)	56	(22.4%)
Leu/Leu-Arg/Arg	29	(8.0%)	21	(8.4%)

[a] Median age: 89 years (range 85-100).
[b] Median age: 31 years (range 18-40).

Table 2. Estimated paraoxonase haplotypes frequencies in subjects aged 85 years and over and young subjects whose families originated from the same geographical region.

Paraoxonase Haplotype	Elderly (728 chromosomes)	Young (500 chromosomes)
Met-Gln	0.348	0.366
Leu-Gln	0.335	0.350
Met-Arg	0.004	0.000
Leu-Arg	0.313	0.284

which gives rise to a common haplotype carrying both putative risk-alleles. The estimated haplotype frequencies were similar in elderly and young subjects (P=0.34; table 2). The analysis of combinations of genotypes did also not reveal any differences between the two groups (table 1). The estimated mortality risk up to the age of 85 years associated with homozygosity for both putative risk alleles was 1.1 (95% CI, 0.5-2.4) as compared with subjects homozygous for the Met and Gln-allele. It should be noted that because of the negative linkage disequilibrium, in the young all of the 21 Arg/Arg carriers and in the elderly 29 of the 31 Arg/Arg carriers were also homozygous for the Leu-allele.

Prospective study

During the 10-year follow-up period 89% of the 666 subjects died of any cause, 38% of the 666 of cardiovascular disease, 9% of ischaemic heart disease and 13% of cerebrovascular disease. The 10-year survival of 666 elderly subjects according to paraoxonase Met55/Leu and Gln192/Arg genotypes is shown in figure 1. The all-cause mortality risk was neither increased among carriers of the Leu/Leu genotype, nor among carriers of the Arg/Arg genotype, nor in carriers homozygous for both putative

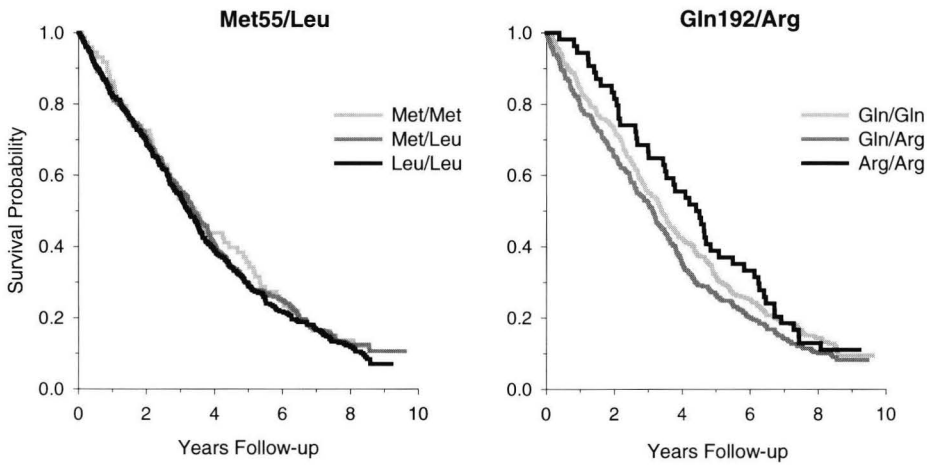

Figure 1. Kaplan-Meier estimate of 10-year cumulative survival according to paraoxonase Met55/Leu and Gln192/Arg genotype for subjects aged 85 years and over.

risk alleles (RR, 1.1; 95% CI, 0.9-1.5, 0.9; 95% CI, 0.7-1.2, and 0.9; 95% CI, 0.6-1.4, respectively; table 3). No significantly increased risks of cardiovascular death causes were observed for (combinations of) paraoxonase genotypes, except for the heterozygous Gln/Arg genotype that was associated with an increased risk of ischaemic heart disease. This finding is, however, not compatible with a recessive or (co-)dominant effect. Analysis of men and women separately yielded similar results. The Leu/Leu genotype was associated with an all-cause mortality of 1.1 (95% CI, 0.7-1.8) in men (n=189) and 1.1 (95% CI, 0.8-1.6) in women (n=475) as compared with the Met/Met genotype. For cardiovascular mortality, these risks were 1.5 (95% CI, 0.7-3.6) and 1.1 (95% CI, 0.7-1.9), respectively. The Arg/Arg genotype was associated with an all-cause mortality of 0.7 (95% CI, 0.4-1.3) in men and 1.0 (95% CI, 0.7-1.5) in women as compared with the Gln/Gln genotype. For cardiovascular mortality these risks were 0.7 (95% CI, 0.3-2.1) and 0.7 (95% CI, 0.4-1.4), respectively.

It has been hypothesised that the effects of the putative paraoxonase risk-alleles may be enhanced by factors which increase oxidative stress such as diabetes and smoking.[4] Therefore, we repeated our analyses for elderly subjects with diabetes (n=72) and for those who smoked (n=109). In the subset of patients with diabetes, the all-cause mortality risk was elevated in Arg/Arg carriers (RR, 2.1; 95% CI, 0.8-5.8) but this did not reach statistical significance. The relative risk of cardiovascular disease mortality was 1.8 (95% CI, 0.4-8.5) in carriers of the Arg/Arg genotype and 1.9 (95% CI, 0.5-6.6) in carriers of the Leu/Leu genotype. Among the subset of smoking elderly no elevated all-cause or

Table 3. 10-Year all-cause and cardiovascular disease mortality risks according tot paraoxonase genotype in subjects aged 85 years and over.

Paraoxonase genotype	N	All causes (n=593)		Cardio-vascular disease (n=250)		Ischaemic heart disease (n=62)		Cerebro-vascular disease (n=84)	
		RR	95% CI	RR	95% CI	RR	95% CI	RR	95% CI
Met55/Leu									
Met/Met	72	1		1		1		1	
Met/Leu	320	1.0	0.8-1.4	1.2	0.8-1.9	1.0	0.5-2.4	1.0	0.5-2.1
Leu/Leu	272	1.1	0.9-1.5	1.3	0.8-2.0	1.1	0.5-2.5	1.1	0.5-2.3
Gln192/Arg									
Gln/Gln	312	1		1		1		1	
Gln/Arg	298	1.2	1.0-1.4	1.3	1.0-1.7	1.9	1.1-3.3	1.3	0.8-2.0
Arg/Arg	54	0.9	0.7-1.2	0.7	0.4-1.3	0.9	0.3-2.6	0.4	0.1-1.3
Met55/Leu + Gln192/Arg									
Met/Met-Gln/Gln	71	1		1		1		1	
Met/Leu-Arg/Arg + Leu/Leu-Gln/Arg	147	1.3	0.9-1.7	1.5	0.9-2.4	1.4	0.6-3.4	1.3	0.6-2.8
Leu/Leu-Arg/Arg	51	0.9	0.6-1.4	0.9	0.4-1.6	0.7	0.2-2.5	0.4	0.1-1.5

RR indicates the mortality risk as estimated with a Cox proportional hazards model adjusted for gender and age at baseline.

cardiovascular mortality risks were observed (data not shown).

Discussion

Polymorphisms in the paraoxonase gene are associated with paraoxonase levels in serum (Met55/Leu)[8] and differential susceptibility of LDL to oxidation *in vitro* (Gln192/Arg).[9] In this study we investigated the contribution of these paraoxonase polymorphisms to mortality in a cohort born between 1887-1901. We found that paraoxonase genotypes previously associated with an increased risk of cardiovascular disease (i.e. containing a Leu[8] or an Arg-allele[10-12]) were not associated with mortality in middle or old age. The prevalence of the putative risk genotypes was not less among elderly subjects (>85 years) than among young subjects (18-40 years) nor were the genotypes associated with all-cause mortality or fatal cardiovascular events in elderly subjects followed over a 10-year period.

It has been hypothesised that the effects of the paraoxonase polymorphisms are enhanced in subjects with type 2 diabetes, because these patients are exposed to higher levels of oxidative stress.[4] There are additional findings that suggest such enhanced effects. It has been reported that type 2 diabetes is associated with elevated levels of acute-phase proteins[27,28] and that the acute-phase response results in a decline in HDL-associated paraoxonase activity in humans, rabbits and experiments *in vitro*.[29] Indications for increased deleterious effects of the paraoxonase gene variants were found in the two-fold higher cardiovascular mortality among diabetes patients with the Arg/Arg or the Leu/Leu genotype. These associations did, however, not reach statistical significance and should be further explored in more extensive studies.

The two paraoxonase polymorphisms were found to be in a strong negative linkage disequilibrium in the Dutch population: almost all of the rare Arg-alleles occur on a haplotype carrying the common Leu-allele and, vice versa, about half of the Leu-alleles are found on a haplotype carrying the Arg-allele. This should be taken into account when studying the effects of the polymorphisms on enzyme function or disease risk. In fact, it is impossible to study the influence of the Arg-allele independently of the effect of the Leu-allele. If the linkage disequilibrium between the two polymorphism is weaker in non-Caucasian populations, these may be used to study the effects independently.

The absence of an association with all-cause mortality in middle and old age does not exclude the possibility that the polymorphisms are associated with an increased risk of fatal cardiovascular disease. It indicates, however, that this potential increase in risk is limited. The finding that the paraoxonase gene variants were not a risk factor for fatal cardiovascular disease in old age substantiates this interpretation. It may be hypothesised that paraoxonase gene variants do contribute to coronary artery disease as suggested by several other studies,[8,10-14] but not to the acute complications of atherosclerosis leading to fatal cardiovascular events. The absence of an association with myocardial infarction would be in agreement with this view.[11,15] Assuming that the paraoxonase gene variants are a causal factor in atherogenesis because they affect the degree of LDL oxidation and oxidised LDL is central to the pathogenesis of atherosclerosis, our results may imply that not atherosclerosis as such, but other factors

(for example those influencing plaque stability) are critical in determining cardiovascular mortality.

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Chapter 5

Association of the TNFα -308G/A polymorphism with the risk of diabetes in an elderly population-based cohort

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Abstract

Ample evidence supports a role for tumour necrosis factor α (TNFα) in the development of type 2 diabetes and cardiovascular disease. TNFα expression was found to be influenced by a –308G/A polymorphism in the promoter of the TNFα-gene. We investigated the contribution of this polymorphism to diabetes and cardiovascular mortality in a population-based cohort of 664 subjects aged 85 years and over (Leiden 85-plus Study).

The –308G/A TNFα promoter polymorphism was associated with the prevalence of diabetes in old age (P=0.004). The risk of diabetes among subjects homozygous for the A-allele was estimated to be 5.5-fold (95% CI, 1.9-16.0) higher than among subjects homozygous for the common G-allele. The promoter polymorphism did, however, not predict mortality from all causes, cardiovascular diseases, cancer or infectious diseases during a 10-year follow-up period. In addition to the promoter polymorphism, TNFa and TNFc short tandem repeat genotypes were determined but these polymorphisms were not associated with morbidity or mortality.

In conclusion, the -308G/A polymorphism in the TNFα promoter is strongly associated with the risk of diabetes but not cardiovascular mortality in old age.

Submitted for publication

Introduction

Ample evidence supports a role of tumour necrosis factor α (TNFα) in the development of cardiovascular disease. TNFα is expressed in atherosclerotic plaques but not in healthy vessels.[1] In atherosclerotic plaques, TNFα may contribute to foam cell formation, to T-lymphocyte activation and to the expression of matrix metalloproteinases that may destabilise the plaque by degrading the extra-cellular matrix.[2,3] Detailed studies also implicated TNFα in the aetiology of insulin resistance, a key feature of type 2 diabetes and a major risk factor for cardiovascular disease in the elderly. TNFα mRNA expression is increased in adipose tissue of severely obese and insulin resistant *fa/fa* rats,[4] while TNFα-deficiency results in an increased peripheral insulin sensitivity in obese mice.[5] In humans, there is a strong positive association between levels of TNFα mRNA in adipose tissue and the extent of hyperinsulinaemia,[6] and TNFα plasma levels are increased in patients with type 2 diabetes.[7] The proposed molecular mechanism underlying these correlations is that TNFα inhibits the insulin induced tyrosine kinase activity of the insulin receptor.[5,8,9]

The A allele of a common -308G/A polymorphism in the promoter region of the TNFα gene is associated with higher reporter gene activity[10,11] and TNFα production in whole blood cell cultures.[12] In addition, several short tandem repeat polymorphisms have been identified at the TNF locus, of which TNFa and TNFc were suggested to be associated with differences in TNFα secretion by human monocytes.[13] We have assessed the contribution these polymorphisms to diabetes and all-cause and cause-specific mortality in a population-based cohort of 664 subjects aged 85 years and over.

Methods

Subjects

The Leiden 85-plus Study is a population-based study in which all inhabitants of Leiden, The Netherlands, aged 85 years and over were invited to take part.[14] Out of a total of 1258 eligible subjects, 221 died before enrolment. Of the 1037 remaining subjects, 977 (94%) participated and were medically interviewed at home. Diabetes was diagnosed on the basis of a previous history, use of medication against diabetes and/or a glucose level over 11.0 mmol/l in a non-fasting blood sample. After the exclusion of subjects with a non-Dutch (n=29) or unknown (n=69) place of birth, sufficient cell material was available from 666 (188 men/478 women) subjects for the present genetic study. The study was approved by the Medical Ethics Committee of the Leiden University and informed consent was obtained from all participants.

Genotyping and HLA typing

The TNFα –308G/A genotypes were determined by PCR-amplification followed by *Nco*I digestion.[15] Digestion products were separated on a 7.5% polyacrylamide MADGE-gel (microtitre array diagonal gel electrophoresis).[16] Genomic regions containing the TNFa and TNFc short tandem repeats were amplified in a multiplex PCR [17] and alleles were separated with an ALF-express automated sequencer (Amersham Pharmacia Biotech).

Because of technical reasons 2, 4, and 5 subjects could not be typed for the -308G/A, TNFa, and TNFc polymorphism, respectively. The TNF genotypes were independently assessed by two observers. As a standard laboratory procedure a randomly chosen 10% of the samples was reamplified.

Typing for HLA-DR antigens were performed with a two colour fluorescence test using a set of highly selected alloantisera to class II antigens.

Prospective study

All participants in the Leiden 85-plus Study were followed up for mortality until October 1, 1996. Among the 666 subjects of the cohort studied, 2 were lost to follow-up. Primary causes of death were obtained from the Dutch Central Bureau of Statistics and categorised for cardiovascular disease (*ICD-9* codes[18] 390-459), ischaemic heart disease (410-414), cerebrovascular disease (430-438) and cancer (140-239). Death from infection was coded as previously described.[14]

Statistical analyses

Distributions of genotypes, alleles and HLA phenotypes were compared by the χ^2-test. Age and gender adjusted odds ratios for diabetes and 95% confidence intervals (CIs) were calculated using logistic regression analysis. The Mantel-Haenszel method was used to test for heterogeneity of associations for strata of geographical origin. Pairwise linkage disequilibria between the three TNF polymorphisms and maximum likelihood haplotype frequencies were estimated using Arlequin software version 2.000.[19]

In the prospective study, survival times for subjects were computed from the date of the home visit to the date of one of the following events: death from a specific cause, death from any cause, or October 1, 1996. Age and gender adjusted mortality risks and 95% CIs were estimated with Cox proportional hazards models. Causes of death were assumed to be independent. P-values of less than 0.05 were considered to indicate statistical significance and all P-values were based on two-sided tests.

Results

The genotype distribution of TNFα -308G/A polymorphism was 65.4% (G/G), 32.2% (G/A) and 2.4% (A/A) in the complete cohort of 664 subjects aged 85 years and over and was in Hardy-Weinberg equilibrium. The polymorphism was significantly associated with the risk of diabetes (P=0.004; table 1). Adjusted for age and gender, the risk of diabetes associated with the G/A and the A/A genotypes were estimated at 1.3-fold (95% CI, 0.7-2.1) and 5.5-fold (95% CI, 1.9-16.0) increased as compared with the common G/G genotype, respectively. The risk estimate for the A/A genotype was 3.3 (95% CI, 0.6-19.4) among men and 6.4 (95% CI, 1.6-25.5) among women. There was no indication for heterogeneity of the association among subjects born in Leiden (55%) and elsewhere in The Netherlands (test for heterogeneity: P=0.40 and P=0.64 for the G/A and A/A genotypes, respectively).

The cohort was followed for mortality over a 10-year period. TNFα -308G/A genotypes were not associated with all-cause mortality (89%), cardiovascular mortality

Table 1. TNFα -308G/A genotype distributions in subjects aged 85 years and over with and without diabetes.

TNFα -308G/A genotype	Diabetes (n=72)		Controls (n=529)	
G/G	41	(56.9%)	350	(66.2%)
G/A	25	(34.7%)	169	(31.9%)
A/A	6	(8.3%)	10	(1.9%)

Test for difference genotype distributions P=0.004

(37%), or death from cancer (15%) and infectious diseases (9%) (table 2). Similar risk estimates were obtained when men and women were analysed separately.

In addition to the -308G/A polymorphism, the TNFa (14 alleles) and TNFc (2 alleles) short tandem repeat polymorphisms were measured. The three polymorphisms were in linkage disequilibrium (all pairwise linkage disequilibria P<0.00001). However, the TNFa and TNFc polymorphisms were not associated with diabetes (P=0.95 and P=0.72, respectively) nor were they associated with mortality from any cause or from a specific cause (data not shown). The absence of an association between the short tandem repeats and diabetes despite their strong linkage disequilibrium with the -308G/A polymorphism is explained by the fact that the -308A allele is distributed over several haplotypes (table 3). For example, all the -308A alleles occur in combination with a TNFc1 allele, but about 70% of the TNFc1 alleles do not occur in combination this allele; about 65% of the -308A alleles occur in combination with a TNFa2 allele, but about 56% of the TNFa2 alleles do not occur in combination with this allele.

The TNFα gene is located in the HLA region, which is characterised by strong linkage disequilibrium. The association with diabetes found here might therefore have been the result of linkage disequilibrium between the -308G/A polymorphism and variation elsewhere in the HLA region influencing diabetes risk. The occurrence of diabetes was, however, independent of HLA-DR3 and DR4 phenotypes (P=0.67 and P=0.86, respectively), indicating that this was unlikely to be the case.

Discussion

The -308G/A polymorphism in the promoter of the TNFα gene strongly contributed to the risk of diabetes in a population-based cohort of elderly subjects aged 85 years and over. Homozygosity for A-allele conferred a more than 5-fold increased risk of diabetes. This is in agreement with the extensive evidence for a direct role of TNFα in the

Table 2. 10-Year all-cause and cause-specific mortality risks according to TNFα -308G/A genotype in subjects aged 85 years and over.

TNFα -308G/A Genotype	N	All causes (n=591)		Cardiovascular diseases (n=248)		Cancer (n=99)		Infectious disease (n=62)	
		RR	(95% CI)	RR	(95% CI)	RR	(95% CI)	RR	(95% CI)
G/G	433	1		1		1		1	
G/A	213	0.9	(0.8-1.1)	0.8	(0.6-1.0)	0.9	(0.6-1.4)	1.4	(0.9-2.4)
A/A	16	0.9	(0.5-1.5)	0.9	(0.4-2.1)	0.8	(0.2-3.5)	1.4	(0.3-6.0)

Tabel 3. Frequent TNF haplotypes in subjects aged 85 years and over.

Haplotype			
-308G/A	**TNFc**	**TNFa**	**Frequency[b]**
G	c1	a6	0.129
G	c1	a10	0.090
G	c1	a11	0.188
G	c2	a2	0.150
G	c2	a5	0.054
A	c1	a2	0.116
A	c1	a4	0.063

[a] Haplotypes frequencies were estimated on the basis of 1236 chromosomes of subjects with a diagnosis of diabetes.

[b] Only those haplotypes with a frequency higher than 0.05 are included (7 of 24 occurring haplotypes).

aetiology of insulin resistance and type 2 diabetes.[5,8,9]

It is likely that the -308G/A polymorphism itself is the functional variation underlying the association with diabetes. The A-allele was associated with higher gene expression levels in several studies.[10-12] The absence of this effect in another study[20] may be related to the use of different reporter gene constructs or cell culture conditions. Furthermore, the association with diabetes in our study was independent of other genetic variation at the TNF locus as measured by two short tandem repeat polymorphisms and the -308A allele does not occur on the same haplotype as the rare alleles of other promoter polymorphisms (-238G/A, -851C/T and -857C/A) in Caucasians.[21] Finally, in view of the suggested shared genetic susceptibility of type 1 and type 2 diabetes[22,23] it is notable that linkage disequilibrium of the promoter polymorphism and HLA-DR3 and DR4 could be excluded as the underlying cause of the association.

It is interesting to note that the TNFa (14 alleles) and the TNFc (2 alleles) short tandem repeats were not associated with the risk of diabetes despite their strong linkage disequilibrium with the -308G/A promoter polymorphism. This illustrates that the complexity of haplotype structures may severely hamper the usefulness of linkage disequilibrium mapping as a tool in unravelling the genetic component of complex diseases.

The –308G/A polymorphism was not associated with type 2 diabetes in a previous study among 138 patients and 57 controls with a mean age of 57 years.[24] Apart from differences in study design and environmental and genetic background of the subjects studied, this may suggest that other genetic factors contribute to the disease at younger ages. Possibly, the adverse effects of mild alterations in TNFα expression become apparent only in old age. The association of the promoter polymorphism with insulin sensitivity among subjects with a mean age of 36 years[25] but not among subjects with a mean age of 25 years[26] would be consistent with this hypothesis. More extensive studies are warranted to more precisely characterise the role of the TNFα promoter polymorphism in type 2 diabetes.

The -308G/A polymorphism was not related to all-cause and cardiovascular mortality during a 10-year follow-up period. In previous studies, the polymorphism was not

associated with the risk of myocardial infarction[21] or coronary artery disease.[27,28] This may reflect that the aetiology of cardiovascular diseases is much more heterogeneous than type 2 diabetes.

In conclusion, our study indicates that the TNFα -308G/A polymorphism may be a potent risk factor for diabetes in old age. We did not find evidence that the polymorphism contributes to the risk of cardiovascular mortality.

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Chapter 6

The risk of mortality and the factor V Leiden mutation in a population-based cohort

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Abstract

The factor V Leiden mutation (conferring resistance to activated protein C) has been implicated in the risk of arterial thrombosis and is a well-established risk factor for venous thrombosis especially in the elderly. We studied whether the disease association of the factor V mutation is reflected in an increased all-cause and cause-specific mortality.

First, the prevalence of the factor V Leiden mutation was determined in a population-based study among subjects aged 85 years and over (4.7%, n=660) and was found to correspond to the prevalence in young subjects aged 18 to 40 years (5.0%, n=321). Secondly, we studied the association of factor V Leiden with the risk of all-cause mortality and specific causes of death in the elderly cohort during a 10-year follow-up period. Neither the all-cause mortality risk (RR 1.0; 95% CI, 0.7-1.5), nor the risk of death from cardiovascular disease (RR 0.9; 95% CI, 0.5-1.7) were increased in elderly subjects heterozygous for factor V Leiden. Our study thus indicates that heterozygosity for the factor V Leiden mutation does not affect mortality in the general population.

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Introduction

The factor V Leiden mutation is the most common genetic predisposition to venous thrombosis with a prevalence of heterozygous carriers of 3% to 6%.[1,2] The mutation leads to an arginine-506 to glutamine amino-acid substitution at the cleavage site for activated protein C (APC), which results in a 10-fold decreased inactivation rate of the variant factor V.[3] As a consequence, individuals with the mutation have a poor anticoagulant response (APC-resistance). Heterozygous carriers have a 3.5- to 7-fold increased risk of venous thrombosis.[1,2] The majority of studies did not demonstrate associations of factor V Leiden with arterial thrombosis[2,4,5] although the factor V mutation was found to be associated with myocardial infarction in smoking women younger than 45 years.[6]

The disease association with factor V Leiden raises the concern that the mutation confers excess mortality among carriers due to an increased risk of thromboembolic complications. If the factor V mutation were to have a deleterious effect on mortality risk, this would probably be observed in the elderly since the relative contribution of arterial and venous thrombosis to total mortality is greater with advancing age. Moreover, the incidence of venous thrombosis among factor V Leiden carriers has been found to increase with age at a significantly greater rate than among subjects without the mutation and increases especially after the age of 70 years.[7]

We studied the association of the factor V Leiden mutation with mortality in a population-based study among subjects aged 85 years and over (Leiden 85-plus Study). First, the association with all-cause mortality was assessed by comparing the prevalence of the factor V mutation in the elderly cohort with that in subjects aged 18 to 40 years, since excess mortality would be reflected in a reduced frequency of the mutation in older age-groups. Secondly, the association of factor V Leiden with all-cause mortality and specific causes of death in old age was studied in the elderly cohort over a 10-year follow-up period.

Methods

The Leiden 85-plus Study is a population-based study in which all the inhabitants of Leiden, The Netherlands, aged 85 years and over were invited to take part.[8] Out of a total of 1258 eligible subjects, 221 died before the enrolment which lasted from December 1, 1986, to March 1, 1988. Of the 1037 remaining subjects, 977 (94%) participated and were medically interviewed at home. After the exclusion of subjects with a non-Dutch (n=29) or unknown (n=69) place of birth, sufficient cell material was available from 660 (186 men/474 women) subjects for the present genetic study. DNA was extracted and the presence of factor V Leiden detected by the PCR-amplification of a 220 bp fragment containing the G_{1691}- to-A transition, followed by digestion with *Mnl*I as previously described.[1] The factor V genotype was independently assessed by two observers and the sample was reamplified if their observations did not match. The study was approved by the Medical Ethics Committee of Leiden University and informed consent was obtained from all participants.

In the cross-sectional analyses, subjects aged 85 years and over were compared with a control population that consisted of 320 (191 men/129 women) blood donors of Dutch

descent aged 18-40 years.

All participants in the Leiden 85-plus Study were followed up for mortality until October 1, 1996. Among the 660 subjects of the cohort studied, 2 were lost to follow-up. Primary causes of death were obtained from the Dutch Central Bureau of Statistics and categorised for cardiovascular disease (*ICD-9* 390-459), ischaemic heart disease (410-414), cerebrovascular disease (430-438), cancer (*ICD-9* 140-239) and all causes (*ICD-9* 000-999). Death from infection was coded as previously described.[9]

In the cross-sectional analysis, distributions of genotypes were compared by the χ^2 test, and mortality risks and 95% confidence intervals (CI) were estimated using the exposure odds ratio. In the prospective follow-up study, survival times for subjects were computed from the date of the home visit to the date of one of the following events: death from a specific cause, death from any cause, or October 1, 1996. Survival was estimated using the Kaplan-Meier product limit method and compared with the log-rank test. Adjusted mortality risks and 95% CIs were estimated with Cox proportional hazards models.

Results

The prevalence of the factor V Leiden mutation among subjects aged 85 years and over (31/660, 4.7%) corresponded with that among young controls aged 18-40 years (16/321, 5.0%; $\chi^2_{df=1}=0.04$; P=0.84). Both populations were in Hardy-Weinberg equilibrium and no homozygotes for the factor V mutation were identified. The all-cause mortality risk up to the age of 85 years, as estimated with the exposure odds ratio, associated with carrying factor V Leiden was estimated at 1.1 (95% CI, 0.6-2.0). For men the risk was estimated at 0.9 (95% CI, 0.4-2.1) and for women at 1.1 (95% CI, 0.7-1.8).

During the 10 years follow-up period, 587 (89.2%) deaths occurred in the 85-plus cohort investigated in this study (n=660; 2 subjects lost to follow up). The mortality of carriers of the factor V Leiden mutation was similar to that of non-carriers (RR 1.0; 95% CI, 0.67-1.46; adjusted for age and gender; figure 1). The estimates were similar for men (RR 0.9; 95% CI, 0.5-1.7; adjusted for age) and women (RR 1.1; 95% CI, 0.7-1.8; adjusted for age). The mortality risk associated with heterozygosity for factor V Leiden was 0.7 (95% CI, 0.3-1.6) in smokers (n=109) and 1.1 (95% CI, 0.7-1.8) in non-smokers (n=524).

The mortality risks for specific causes of death were not significantly different for factor V Leiden carriers as compared to non-carriers (table 1). The risk of death from

Table 1. All-cause and cause-specific 10-year mortality risks of factor V Leiden carriers as compared with non-carriers in subjects aged 85 years and over.

Cause of death	N	Mortality risk (95% CI)	
Cardiovascular disease	250	0.9	(0.5-1.7)
Ischaemic heart disease	63	0.3	(0.0-2.4)
Cerebrovascular disease	83	1.7	(0.7-4.0)
Cancer	97	1.7	(0.8-3.7)
Infectious disease	62	2.2	(0.9-5.5)
All-cause	587	1.0	(0.7-1.5)

Mortality risks were estimated with a Cox proportional hazard model and adjusted for gender and age at baseline.

cardiovascular disease in factor V Leiden carriers who smoked (RR 0.7; 95% CI, 0.2-3.1) was approximately similar to that in non-smoking carriers (RR 0.9; 95% CI, 0.4-2.0). One subject, a 94-year old woman, died from the complications of venous thrombotic disease at the age of 97 and did not carry the factor V mutation.

Discussion

We have assessed the impact of the factor V Leiden mutation, the most common genetic risk factor for venous thrombosis, on mortality in two study designs. A cross-sectional comparison of the prevalence of factor V Leiden in young and elderly subjects did not indicate a major effect of the factor V Leiden mutation on population mortality. A 10-year follow-up study among elderly subjects confirmed these results. Furthermore, heterozygosity for factor V Leiden was not associated with an increased risk of common specific causes of death in old age.

Previous studies showed that the deleterious effect of the factor V Leiden mutation is enhanced by non-genetic factors. The deleterious effect of the mutation on the risk of venous thrombosis was found to be more pronounced among elderly individuals.[7] Nevertheless, the mortality of elderly subjects in our study was not influenced by the factor V mutation. Another previously suggested modulating factor is smoking. Factor V Leiden was reported to be associated with the risk of myocardial infarction in women younger than 45 years of age who smoked, whereas among non-smoking carriers the risk was not increased.[6] This association was attributed to the low prevalence of atherosclerosis in this specific group and assumed to be absent in the general population. Indeed, we did not detect any increased all-cause or cardiovascular disease mortality among elderly factor V Leiden carriers who smoked.

The women in the cohort studied were born between 1887 and 1901 and oral contraceptives were not available during their reproductive period. Should the reported

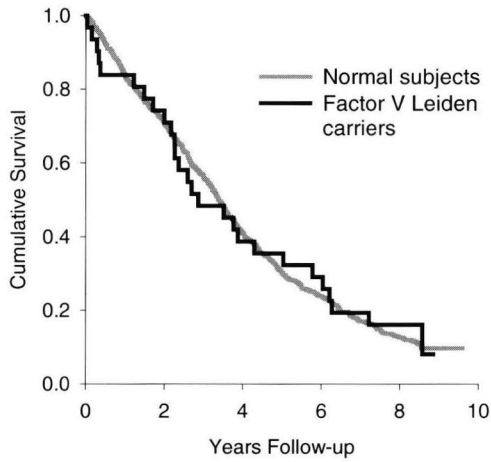

Figure 1. Kaplan-Meier estimate of 10-year cumulative survival for factor V Leiden carriers and subjects without the mutation age 85 years and over.

interaction between the factor V mutation and the use of oral contraceptives[10] confer an increased mortality, this would not have affected the mortality in the generation studied. By similar reasoning, it cannot be excluded that the mortality of women with the factor V Leiden mutation from subsequent generations might be higher.

It may be argued that our study did not have sufficient power to detect small increases in mortality risk among factor V Leiden carriers. However, combined with data from previous studies it seems unlikely that heterozygosity for factor V Leiden contributes to mortality in the general population. First, factor V Leiden is not an important risk factor for arterial thrombosis in the general population.[2,4,5] Second, although the mutation is associated with a considerable risk of deep vein thrombosis,[1,2] the increase in risk of its lethal complication, i.e. pulmonary embolism, is limited.[11-14] Third, parents of subjects with factor V Leiden did not suffer increased all-cause or cause-specific mortality.[15] Finally, heterozygosity for factor V Leiden is compatible with extreme longevity.[16,17]

Since our study indicates that factor V Leiden carriers in general are not subject to increased mortality, long-term prophylactic anti-coagulant therapy, which induces the risk of fatal haemorrhage,[18] should not be considered solely on the basis of factor V Leiden status.

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Chapter 7

Angiotensin I-converting enzyme and plasminogen activator inhibitor-1 gene variants: risk of mortality and fatal cardiovascular disease in an elderly population-based cohort

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Abstract

The angiotensin I-converting enzyme deletion/deletion (*ACE* D/D) and plasminogen activator inhibitor-1 (*PAI1*) 4G/4G genotypes have been consistently associated with elevated plasma activities of the gene products. Their role in cardiovascular disease, although explored intensively, is still equivocal. We studied the contribution of the putative *ACE* and *PAI1* risk genotypes to all-cause and cardiovascular mortality in a population-based cohort.

ACE and *PAI1* genotypes were determined in 648 subjects aged 85 years and over. In a cross-sectional analysis, the genotype distributions in a subset of 356 elderly subjects who were born in Leiden, The Netherlands, were compared with those in 250 young subjects whose families originated from the same geographical region. In addition, the complete cohort of elderly subjects was followed over 10 years for all-cause and cardiovascular mortality and stratified according to genotype.

In the cross-sectional analysis, the *ACE* and *PAI1* genotype distributions were similar in elderly and young subjects. In the prospective follow-up study, however, the age-adjusted risk of fatal ischaemic heart disease was increased 3-fold (95% CI, 1.2-7.6) in elderly men carrying the *PAI1* 4G/4G genotype. The risk of all-cause mortality was not increased among elderly subjects carrying the *PAI1* 4G/4G (RR, 0.9; 95% CI, 0.7-1.1) or the *ACE* D/D genotype (RR, 0.9; 95% CI, 0.7-1.1), nor did we observe elevated risks of death from all cardiovascular diseases combined. There was no interaction between the genotypes. The PAI 4G/4G genotype may be a risk factor for fatal ischaemic heart disease in elderly men. However, the impact of moderately increased ACE and PAI-1 activities associated with the *ACE* D/D and *PAI1* 4G/4G genotypes is too small to affect mortality in the general population.

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Introduction

Over the last years a number of common gene variants have been identified that are associated with plasma levels of the gene products and the risk of cardiovascular disease. Most studies performed thus far have not addressed the association of these variants with fatal cardiovascular disease or such studies included only a small number of fatal cases. Also, very few studies have yet explored how combinations of possible risk genotypes affect disease risk. Establishing such genetic risk profiles is only feasible when relatively large populations are available and the putative risk alleles have a high frequency in the general population. Two highly frequent genetic variants that may be linked to cardiovascular disease risk have been identified in the genes encoding angiotensin I-converting enzyme (ACE) and plasminogen activator inhibitor 1 (PAI-1).

ACE catalyses the conversion of angiotensin I to angiotensin II, which is a potent vasoconstrictor and promotes the growth of vascular smooth muscle cells, and also degrades bradykinin, which is a vasodilator. The *ACE* insertion/deletion (I/D) polymorphism arises from the presence or absence of an *alu* repeat located in intron 16 of the *ACE* gene (frequency *ACE* D-allele in Caucasians ~0.54).[1] The *ACE* D-allele is associated with increased ACE activity in plasma[1,2] and tissue.[3] Several studies have reported an increased frequency of the *ACE* D/D genotype in survivors of a myocardial infarction as compared with healthy subjects.[4-7] However, these results have been challenged by the negative findings from other studies.[8-15] In addition to the ongoing debate on the deleteriousness of the *ACE* D/D genotype, a beneficial influence on survival in very old age was suggested by its increased frequency in French centenarians.[16]

PAI-1 is the primary inhibitor of fibrinolysis. A 4/5-guanine-tract (4G/5G) polymorphism was identified in the promoter of the *PAI1* gene 675 basepairs upstream from the start of transcription.[17] The *PAI1* 4G allele is associated with elevated PAI-1 levels in plasma (frequency *PAI1* 4G-allele in Caucasians ~0.52).[17-19] This association is especially strong among subjects with relatively high plasma levels of triglycerides, insulin or glucose.[20,21] Studies *in vitro* indicate that the 4G-allele is unable to bind a repressor and is associated with increased transcription of the *PAI1* gene.[17,18] In addition, a VLDL-responsive element was found to partly overlap the guanine tract, which may provide a molecular explanation for the effect modification by triglycerides.[22] The elevated plasma PAI-1 level in 4G/4G homozygotes may result in an increased risk of coronary heart disease as a consequence of a diminished fibrinolytic capacity. Studies investigating this hypothesis, however, have produced conflicting results.[18,19,23-26]

If a deleterious effect of the *ACE* D and the *PAI1* 4G-allele is present, carriers of both risk alleles may be especially susceptible to disease due to the link that exist between the renin-angiotensin system and fibrinolytic function. Infusion of angiotensin II increases plasma PAI-1 activity in humans[27] and, recently, two studies showed that the *ACE* D/D genotype was associated with elevated PAI-1 levels in plasma.[28,29] Hence, the *ACE* and *PAI1* polymorphisms may be involved in cardiovascular disease by the same etiologic pathway.

The aim of the present study was to assess whether the *ACE* and *PAI1* polymorphisms, either separately or in combination, are associated with mortality in the

general population. This was done using two designs within a population-based study among subjects aged 85 years and over (Leiden 85-plus Study[30]). The influence of the gene variants on mortality before the age of 85 years was studied in a cross-sectional design by comparing the genotype distributions in Leiden-born subjects aged 85 years and over with those in young subjects whose families originated from the same geographical region.[31] The relation of the gene variants to all-cause and cardiovascular mortality above the age of 85 years was investigated in a prospective study with a 10-year follow-up period using the complete elderly cohort. During follow-up, the all-cause mortality rate was 89% and the cardiovascular mortality rate was 38%.

Methods

Subjects

The Leiden 85-plus Study is a population-based study in which all inhabitants of Leiden, The Netherlands, aged 85 years and over were invited to take part.[30] Of a total of 1258 eligible subjects, 221 died before enrolment, which lasted from December 1, 1986, to March 1, 1988. Of the 1037 remaining subjects, 977 (94%) participated and were medically interviewed at home. Diabetes was diagnosed on the basis of a medical interview, a glucose level over 11.0 mmol/l in a non-fasting blood sample and/or the use of medication against diabetes. After the exclusion of subjects with a non-Dutch (n=29) or unknown (n=69) place of birth, sufficient cell material was available from 666 (188 men/478 women) subjects for the present genetic study. DNA was extracted by protein precipitation using potassium acetate followed by chloroform extraction. *ACE* genotypes were determined as previously described.[32] Since the *ACE* I/D genotype is erroneously mistyped as *ACE* D/D in up to 5% of the cases, the presence of the *ACE* D/D genotype was confirmed with an insertion-specific PCR amplification.[33] *PAI1* 4G/5G genotypes were determined using an allele-specific PCR amplification described by Falk *et al.*[34] It was not possible to determine the *ACE* genotype in 26 and the *PAI1* genotype in 22 cases for technical reasons. The *ACE* and PAI genotypes were independently assessed by two observers. The study was approved by the Medical Ethics Committee of the Leiden University and informed consent was obtained from all participants.

Cross-sectional analysis

ACE and *PAI1* genotype distributions were compared among elderly subjects aged 85 years and over and young controls. To avoid false associations with the *ACE* and *PAI1* polymorphisms due to differences in geographical origin rather than age, only those subjects aged 85 years and over who were born in Leiden (n=356; 55%) were compared with a control population which consisted of 250 (139 men/111 women) blood donors aged 18-40 years of Dutch descent 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 place of birth of their grandparents was obtained from a written questionnaire.

genotype was estimated at 0.8 (95% CI, 0.5-1.2) as compared with the *ACE* I/I genotype.

Ten-year survival of the complete cohort of elderly subjects according to *ACE* genotype is shown in figure 1. During follow-up, the all-cause mortality rate was 89% and the cardiovascular mortality rate was 38%. The all-cause mortality risk of elderly *ACE* D/D carriers was 0.9 (95% CI, 0.7-1.1) compared with *ACE* I/I carriers (table 2). The *ACE* D/D genotype was not associated with the risk of death from cardiovascular disease (RR, 0.8; 95% CI, 0.6-1.2) (table 2). Similar estimates were obtained for men and women, except for the *ACE* I/D genotype. In men, the heterozygous genotype was associated with an increased mortality risk. This result, however, is not compatible with a recessive or (co-)dominant effect.

PAI1 4G/5G polymorphism

The *PAI1* genotype frequencies were 19.2% (5G/5G), 52.9% (4G/5G) and 27.9% (4G/4G) in the cohort of elderly subjects aged 85 years and over (n=646). The *PAI1* genotype distributions in the elderly subjects born in Leiden (n=354) and in young subjects whose families originated from the Leiden area (n=250) were in Hardy-Weinberg equilibrium (table 3). The genotype frequencies in young subjects were similar to those previously reported for white subjects.[23,25] No overall significant differences were observed in *PAI1* genotype distribution (P=0.37), nor for men and women separately (P=0.11 and P= 0.78, respectively). The mortality risk up to the age of 85 years associated with the *PAI1* 4G/4G genotype was estimated at 0.8 (95% CI, 0.5-1.2) compared with the *PAI1* 5G/5G genotype.

Figure 1 shows the 10-year survival of elderly subjects according to *PAI1* genotype. Elderly carriers of the *PAI1* 4G/4G genotype were not at an increased risk for death from any cause (RR, 0.9; 95% CI, 0.7-1.1) or from cardiovascular disease (RR, 0.9; 95% CI, 0.6-1.3) compared with carriers of the *PAI1* 5G/5G genotype (table 4). Similar estimates were obtained for men and women.

Among men, the *PAI1* 4G/4G genotype was associated with an increased risk of fatal ischaemic heart disease (table 4). Assuming a recessive effect (i.e. combining *PAI1*

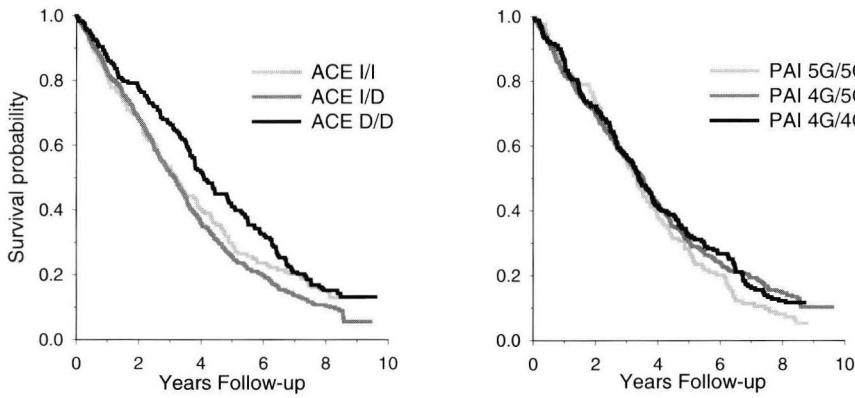

Figure 1. Kaplan-Meier estimate of 10-year cumulative survival according to *ACE* and *PAI1* genotype for subjects aged 85 years and over.

Table 3. *PAI1* genotype distributions in subjects aged 85 years and over and young subjects whose families originated from the same geographical region.

***PAI1* Genotype**	**Subjects**			
	Elderly[a] (%)		**Young**[b] (%)	
all subjects	354		250	
5G/5G	67	(18.9%)	59	(23.6%)
4G/5G	190	(53.7%)	125	(50.0%)
4G/4G	97	(27.4%)	66	(26.4%)
men only	111		139	
5G/5G	17	(15.3%)	36	(25.9%)
4G/5G	61	(55.0%)	70	(50.4%)
4G/4G	33	(29.7%)	33	(23.7%)
women only	243		111	
5G/5G	50	(20.6%)	23	(20.7%)
4G/5G	129	(53.1%)	55	(49.5%)
4G/4G	64	(26.3%)	33	(29.7%)

[a] Median age: 89 years (range 85-100).
[b] Median age: 31 years (range 18-40).

4G/5G and 5G/5G as a single reference group), the relative risk was estimated at 3.1 (95% CI, 1.2-7.6; P=0.015). None of the men carrying the *PAI1* 4G/4G genotype who died of ischaemic heart disease were diagnosed as having diabetes at baseline.

The analyses were repeated for elderly subjects with diabetes (n=69) because they can be expected to have elevated plasma triglycerides, insulin and glucose. In this subset, the all-cause mortality risks associated with the *PAI1* 4G/4G and 4G/5G genotypes were 0.4 (95% CI, 0.2-0.9) and 0.6 (95% CI, 0.3-1.2), respectively, as compared with the 5G/5G genotype.

Table 4. 10-Year all-cause and cardiovascular disease mortality risks according to *PAI1* genotype in subjects aged 85 years and over.

***PAI1* Genotype**		**all cause**		**cardiovascular disease**		**ischaemic heart disease**		**cerebrovascular disease**	
	N	RR	95% CI	RR	95% CI	RR	95% CI	RR	95% CI
all subjects	644		n=575		n=242		n=61		n=78
5G/5G	124	1		1		1		1	
4G/5G	340	0.8	(0.7-1.0)	0.9	(0.6-1.2)	0.9	(0.5-2.0)	0.9	(0.5-1.6)
4G/4G	180	0.9	(0.7-1.1)	0.9	(0.6-1.3)	1.6	(0.8-3.3)	0.7	(0.4-1.4)
men only	181		n=159		n=61		n=19		n=19
5G/5G	32	1		1		1		1	
4G/5G	95	1.9	(0.8-1.8)	1.3	(0.6-2.6)	3.3	(0.4-27)	2.5	(0.6-11)
4G/4G	54	1.2	(0.8-2.0)	1.6	(0.7-3.4)	8.2	(1.0-64)	1.9	(0.4-10)
women only	463		n=416		n=181		n=42		n=59
5G/5G	92	1		1		1		1	
4G/5G	245	0.7	(0.6-0.9)	0.8	(0.5-1.1)	0.7	(0.3-1.5)	0.7	(0.4-1.3)
4G/4G	126	0.8	(0.6-1.0)	0.7	(0.5-1.1)	0.9	(0.4-2.2)	0.6	(0.3-1.2)

RR indicates the mortality risk as estimated with a Cox proportional hazard model adjusted for age at baseline.

Combined effects of ACE I/D and PAI1 4G/5G polymorphisms

The prevalence of homozygosity for both the *ACE* and the *PAI1* risk allele was similar among elderly subjects and young subjects of Leiden origin (6.3% and 7.6%, respectively; data not shown). Separate analysis of men and women did not reveal significant differences.

The 10-year mortality risk of elderly subjects carrying both the *ACE* D/D and *PAI1* 4G/4G genotype was 0.8 (95% CI, 0.6-1.2) from any cause and 0.9 (95% CI, 0.5-1.5) from cardiovascular disease as compared with a reference group of double heterozygous subjects and subjects homozygous for the *ACE* I- and/or the *PAI1* 5G-allele. It was not possible to test for an association with death from ischaemic heart disease because the number of subjects was too small.

Discussion

ACE I/D polymorphism

No evidence was obtained for increased (cardiovascular) mortality among carriers of the *ACE* D/D genotype in the cross-sectional comparison of young and elderly subjects or in the 10-year follow-up study among elderly subjects. Other cross-sectional studies also showed similar *ACE* D/D frequencies in elderly subjects of about the same age as those in our study.[11,37] A decreased prevalence of the *ACE* D/D genotype in hypertensive subjects aged 60 years and over was reported suggesting that the *ACE* D/D genotype may confer an increased mortality risk in this high-risk subgroup.[38] Increased mortality in the population at large has, however, not been observed in our study.

Although the neutral *ACE* I/D polymorphism explains 14 to 25% of the variance in ACE level,[1,2] there is much debate about the significance of the relation between the *ACE* polymorphism and cardiovascular disease. The conflicting results of the studies performed thus far can be attributed to differences in clinical phenotype (such as myocardial infarction and coronary artery disease), differences in age of the subjects, representativeness of control subjects and differences in environmental and genetic background. Moreover, a meta-analysis of the association between the *ACE* I/D polymorphism and myocardial infarction suggested a publication bias towards positive results for the smaller studies.[36]

A number of relatively large studies reported an association of the *ACE* D/D genotype with a history of myocardial infarction.[4-7] In some studies the association was found to be stronger[4,39] or present only[9,15] in subgroups otherwise at a low risk using different criteria. Furthermore, the prevalence of *ACE* D/D genotype was increased among patients who had a fatal myocardial infarction.[40] However, most larger studies, eight in total including the only prospective study, found no evidence for an association with myocardial infarction[8-14] or ischaemic heart disease.[15] Also, in most studies no association was observed in low-risk subjects.[5,8,10,12,13] Our observation - that the *ACE* D/D genotype was not associated with mortality or fatal cardiovascular disease in old age - indicates that the overall effect of the *ACE* polymorphism on fatal disease is probably limited.

Schächter *et al.*[16] reported an increased prevalence of the *ACE* D/D genotype in French centenarians compared with subjects aged 20 to 70 years. They suggested that the *ACE* D/D genotype might be deleterious in middle age, but beneficial to survival in very old age. Recently, the same group developed a mathematical model predicting trajectories of genotype frequencies in ageing cohorts as a consequence of differential population mortality rates for the genotypes.[41] This model indicated that a genotype with an effect on survival before the age of 95 years exhibits an opposite effect after this age. The increased frequency of the *ACE* D/D genotype in French centenarians was thus explained by assuming an increased mortality of *ACE* D/D carriers before 95 years. Neither in our cross-sectional analysis nor in the prospective study among subjects aged 85 years and over could we find support for this assumption. Moreover, no increased *ACE* D/D frequency was found in 187 Danish centenarians.[42]

PAI1 4G/5G polymorphism

We found that men aged 85 years and over carrying the *PAI1* 4G/4G genotype were at a 3-fold increased risk of death from ischaemic heart disease during a 10-year follow-up period. This was not reflected in the risk of death from all cardiovascular causes combined or an increased mortality rate before the age of 85 years. The indication that the *PAI1* polymorphism may contribute to the development of ischaemic heart disease in old age significantly extends the previous findings that the *PAI1* 4G/4G genotype is a risk factor for premature myocardial infarction, which has a much lower incidence (patients younger than 45 years[18] and with a mean age of 58 years[24,26]). It should, however, be noted that, although our study is prospective and thus not prone to bias, our findings are based on the analysis of a small subset and therefore require confirmation in more extensive studies.

In contrast to our studies and those mentioned earlier, as well as a study showing a higher prevalence of the *PAI1* 4G/4G genotype among subjects with a family history of coronary heart disease,[25] the *PAI1* polymorphism was not associated with myocardial infarction in the ECTIM study (men aged 25 to 64 years),[19] and the Physicians' Health Study (men aged 40 to 84 years; mean follow-up 8 years).[23] The variable outcomes of these studies may have resulted from chance, but may also point to population differences with respect to the pathogenesis of myocardial infarction or the prevalence of environmental factors modifying the effect of the *PAI1* polymorphism.

The current study could not provide an explanation for the gender-specificity of the association with fatal ischaemic heart disease. One previous study also reported data on both sexes separately and these showed that the *PAI1* 4G/4G genotype was a risk factor for myocardial infarction in men only.[26] Hormonal differences with respect to oestrogen are not likely to play a role because the elderly women were well beyond their menopause. Smoking has been suggested to modify the effect of the polymorphism.[43] Different smoking habits between men and women might have explained our observation, but the numbers were too small to test for this possibility. Another explanation could have been a different prevalence of diabetes in men and women because the effect of the *PAI1* 4G/4G genotype is known to be more pronounced in individuals with features of insulin resistance (elevated plasma levels of triglycerides,

insulin and glucose).[20,21] However, none of the men who carried the apparently deleterious *PAI1* genotype and died of ischaemic heart disease had been diagnosed as having diabetes. Overall, the (cardiovascular) mortality was not increased in elderly subjects carrying the 4G/4G genotype with diabetes. In contrast, the mortality risk in these patients was even significantly reduced. We do not have an explanation for this finding.

Interaction

Recent studies showed that not only the *PAI1* 4G/4G but also the *ACE* D/D genotype is associated with increased PAI-1 levels.[28,29] Although the possible deleterious effect on mortality of a decreased fibrinolytic capacity might have been more readily observed in subjects carrying both putative risk genotypes, none of our analyses indicated an increased mortality in this subgroup.

Study limitations

Our study indicates that it is unlikely that the putative *ACE* and *PAI1* risk genotypes have a major influence on mortality in the population at large. On de basis of the 95% confidence intervals, the overall mortality risks can be expected to be less than 1.2-fold increased. The current study does not, however, have sufficient power to exclude associations between the risk genotypes and specific causes of death, owing to the relatively small number of subjects in these subsets. Furthermore, non-differential misclassification of causes of death could be expected to have occurred since they were not confirmed at necropsy, which leads to an underestimation of the risk estimates. We have previously reported associations with single specific causes of death, indicating that the registry data have significant power to discriminate between the various causes of death.[30,31,44] Furthermore, it has been shown in the United Kingdom that underreporting of cerebrovascular and cardiovascular mortality as an underlying cause of death occurs relatively infrequently.[45] In the case of more than 90% of the individuals who died within four weeks of hospital admission because of stroke or ischaemic heart disease, the disease was mentioned on the death certificate.

Conclusions

We conclude that the influence on disease risk of moderately increased ACE and PAI-1 activities that are associated with the *ACE* D/D and the *PAI1* 4G/4G genotype is not large enough to affect mortality in the general population. Our data suggest, however, that the PAI 4G/4G genotype may play a role in fatal ischaemic heart disease in elderly men. This observation requires confirmation in larger prospective studies.

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Chapter 8

A common variant of the methylenetetrahydrofolate reductase gene (*MTHFR*, 1p36) is associated with an increased risk of cancer

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Abstract

Folate metabolism is thought to play an important role in carcinogenesis through its involvement in both DNA methylation and nucleotide synthesis. A common Ala/Val variant in the methylenetetrahydrofolate (*MTHFR*) gene leads to a mildly disturbed folate metabolism. We previously reported that the *MTHFR* Val/Val genotype was associated with increased cancer mortality in men from a population-based cohort of subjects aged 85 years and over. In order to further explore the deleterious effects of the *MTHFR* genotype, we studied the association of the genotype with overall cancer risk and the risk of cancer of specific organs in 860 men aged 65-84 years who were followed over 10 years (Zutphen Elderly Study). In addition, we compared *MTHFR* genotype frequencies between a series of 211 lung cancer patients and healthy blood donors.

During follow-up, 150 new cases of cancer occurred among the 793 men without cancer at baseline. The risk of cancer was 1.81-fold (95% CI, 1.09-3.00) increased among men with the Val/Val genotype as compared to men with the Ala/Ala genotype. The higher incidence of cancer could be attributed to an increased risk of cancer of the prostate (RR, 3.53; 95% CI, 1.06-11.7), the colorectum (RR, 3.69; 95% CI, 1.08-12.6) and the kidney and bladder (RR, 5.51; 95% CI, 1.68-18.1). Neither in the cohort of elderly men nor in the series of lung cancer patients we did find any indication that the Val/Val genotype contributed to the risk of lung cancer. The risks of cancer appeared to be particularly increased among men with lower folate intake, higher alcohol consumption and of an older age. In conclusion, our current and previous studies in two independent populations indicate that a common Ala/Val variant in the *MTHFR* gene may have a deleterious effect on the risk of cancer in the general population.

Submitted for publication

Introduction

Epidemiological studies implicated low folate status in the development of cancer in several organs including the cervix, colorectum, lung, brain, pancreas and breast.[1] These observations may be explained by the crucial role of folate as the donor of one-carbon groups in both DNA methylation and nucleotide synthesis. In humans, folate deficiency induces decreased DNA methylation,[2] which is a nearly universal feature of early tumorigenesis.[1] Insufficient methylation of DNA may promote carcinogenesis by the derepression of proto-ocogenes[3,4] or by the induction of genomic instability.[5] Folate is further required for the conversion of the nucleotide dUMP to dTMP. An imbalanced nucleotide pool caused by folate deficiency is associated with an increased occurrence of chromosome breaks as a result of the simultaneous removal and repair of adjacent misincorporated uracil bases on opposing DNA strands[6,7] and may thereby contribute to cancer risk.[8]

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism and converts 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF) (figure 1). The latter form of folate is used for the remethylation of homocysteine to methionine. DNA methylation is dependent on the synthesis of methionine because its activated form, S-adenosyl-methionine, is the methyl donor in this reaction. If not reduced to 5-methylTHF, 5,10-methyleneTHF can transfer its methylene group to dUMP to synthesise dTMP, or may contribute to purine synthesis. A common alanine-to-valine (Ala/Val) variant of the *MTHFR* gene was found to decrease the activity of the enzyme by 70% in homozygotes for the Val-allele[9] (about 10% of the general population) and leads to a shift in the distribution of different forms of THF at the expense of 5-methylTHF.[10] As is consistent with a diminished availability of 5-methylTHF, the Val/Val genotype is associated with elevated plasma homocysteine levels[11] and decreased genomic DNA methylation.[12]

In a previous report we presented evidence that the Val/Val genotype was associated with a higher mortality rate in men but not women aged 85 years and over and that this observation could be attributed to an increased risk of cancer.[13] In contrast, the Val/Val genotype was observed to reduce the risk of colorectal cancer[14,15] and acute lymphocytic leukaemia[16] in other studies. It thus remains unclear which of the putative effects of the genotype – either the deleterious influence on methylation or the advantageous influence on nucleotide synthesis - prevails in determining cancer risk in the general population. Therefore, we examined the association of the *MTHFR* genotype with the overall risk of cancer and the risk of cancer of the lung, prostate, colorectum and kidney or bladder in a population-based prospective study among elderly men (the Zutphen Elderly Study[17]). In addition, *MTHFR* genotypes were determined in a series of patients with lung cancer.

Methods

Zutphen Elderly Study

The Zutphen Elderly Study is a population-based, longitudinal investigation of risk factors for chronic diseases in elderly men.[17] It is an extension of the Dutch contribution to the Seven Countries Study. In 1985 the 555 survivors of the original cohort of 878 and

a random sample of 711 men of the same age (65-84 years) also living in Zutphen were approached. Of those invited 74% (939/1266) entered the study: 62 had moved or could not be reached, 109 could not be examined because of serious illness and 156 refused. Complete information on genotype and standard risk factors was available for 860 men; for 804 men information on diet was also available.

Baseline medical and diet examinations were carried out between March and June, 1985.[17] Information on smoking status and the occurrence of cancer was obtained by a standard medical questionnaire. Usual food intake of the participants in the month before the interview was recorded by trained dietitians who used a cross-check dietary history method adapted to the Dutch setting.[18] This procedure included a one hour interview with the participant and the person who prepared the food and an assessment of the quantities of food bought per week. On the basis of these data the daily folate and alcohol intake were estimated. Folate intake was calculated as previously described.[19]

Non-fasting venous blood samples were taken and serum stored at –20°C. No cells were stored. Serum total homocysteine was measured as previously described.[20] Genomic DNA was extracted from 400 µl serum using the QIAamp DNA blood mini kit (Qiagen) and dissolved in 200 µl 10mM Tris/0.1 mM EDTA. The DNA yield from serum was too low to allow direct PCR amplification. Therefore, 10 µl of the DNA solution was used in a whole genome PCR amplification using a mixture of 15-base random

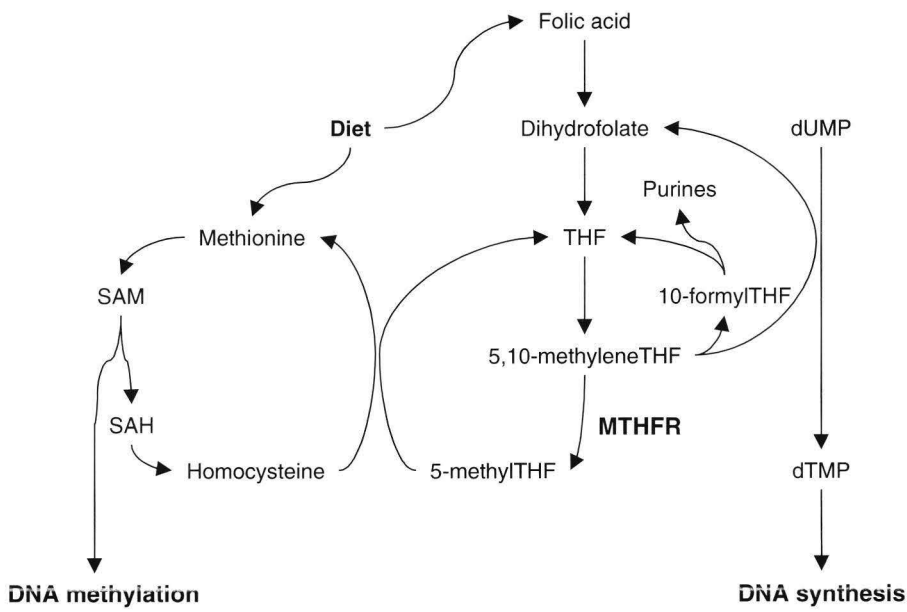

Figure 1. Competing pathways in folate metabolism.
THF Tetrahydrofolate
SAM S-adenosyl-methionine
SAH S-adenosyl-homocysteine

oligonucleotides.[21] Next, *MTHFR* genotypes were determined on 2 µl of the 50 µl whole genome amplification product by PCR-amplification of a 198 bp fragment containing the Ala/222Val polymorphism followed by digestion with *Hin*fI as previously described.[9] Two observers independently assessed all *MTHFR* genotypes and samples were reamplified if differences were observed. The reliability of the genotypes obtained from DNA extracted from serum was checked in two ways. First, twenty percent of the samples were also genotyped by performing a double PCR amplification using the standard genotyping protocol thus circumventing the whole genome amplification step. No differences were observed. Second, blood samples collected in 1990 were available for 14% of the population. DNA was extracted from lymphocytes and *MTHFR* genotypes were determined using the standard genotyping protocol. These genotypes were compared with the genotypes obtained from DNA extracted from serum that was subjected to a whole genome amplification step. Four inconsistencies were observed, which were removed from the data set. These differences did not follow a specific pattern.

Information on vital status of the participants until January 1995 was obtained from municipal population registries. One man was lost to follow-up in 1989 and three men were lost to follow-up in 1991 because they had moved abroad or moved to an unknown destination. These men were included in the analyses, but censored at 20th July 1989 or 31st December 1990. The prevalence of disease at baseline and clinical diagnoses of disease during follow-up were recorded at examinations in 1985, 1990 and 1995 using standardised questionnaires for responders and non-responders. Information on the primary causes of death was obtained from the Netherlands Central Bureau of Statistics for deaths that occurred between the baseline assessment and June 1990 and from the subjects' general practitioners for deaths that occurred thereafter. Data on baseline prevalence and incidence of all cancers were verified with hospital discharge data and written information from the general practitioner. The causes of death were coded according to the 9th revision of the International Classification of Diseases (ICD) by a single physician. Death from cancer was defined by ICD-9 codes 140-239, from lung cancer 162, from prostate cancer 185, from colorectal cancer 153-154 and from kidney/bladder cancer 188-189.

Lung cancer patients

Between 1984 and 1999, 232 squamous cell carcinoma patients were admitted to the Leiden University Medical Centre from whom paraffin embedded tissue samples were stored. Tissue samples were inspected by a pathologist and normal tissue samples were selected for DNA extraction. DNA was extracted from 5 to 6 5 µm thick sections by deparaffination using xylol followed by digestion of proteins with proteinase K and addition of chelex.[22] *MTHFR* genotypes were successfully determined in 211 patients. The *MTHFR* genotype distribution observed was compared to that in 250 blood donors aged 18-40 years who originated from the Leiden area, which was reported previously.[13]

Statistical analysis

For the cohort study, differences in baseline characteristics according to *MTHFR* genotype were evaluated by use of ANOVA for normally distributed variables, the Kruskal-Wallis test for variables with a skewed distribution and an overall χ^2 test for categorical variables. Odds ratios for cancer at baseline were estimated using logistic regression. In the follow-up study, associations between *MTHFR* genotypes and cancer risk were tested using Cox proportional hazards models. For evaluating the previously suggested interaction between the *MTHFR* genotype and the intake of folate and alcohol,[14,15] prevalent and incident cases of cancer were combined to increase the power of the analysis. Prevalent and incident cases had a similar folate (P=0.26) and alcohol (P=0.62) intake, which indicates that no major changes in diet occurred after the diagnosis of cancer. Possible changes in diet towards higher folate and lower alcohol intakes would lead to an underestimation of the interaction. Subjects were divided according to the tertile of folate and alcohol intake, and subjects in the two highest tertiles of folate intake and the two lowest tertiles of alcohol intake were grouped. To evaluate the influence of age, the same approach was used but now the subjects were divided according to the median age. *MTHFR* genotype frequencies in the lung cancer patients and controls were compared using a χ^2 test. All tests were two-sided and values of $P<0.05$ were considered statistically significant. The analyses were performed using SAS version 6.12.

Results

Table 1 shows the baseline characteristics of the studied cohort of men aged 65-84 years according to *MTHFR* genotype. The *MTHFR* genotype distribution was 49.4% (Ala/Ala), 42.1% (Ala/Val) and 8.5% (Val/Val) and was in Hardy-Weinberg equilibrium. The distributions of risk factors for cancer were similar for the *MTHFR* genotypes.

As expected, the Val/Val genotype was strongly associated with elevated total

Table 1. Baseline characteristics of participants of the Zutphen Elderly Study according to *MTHFR* genotype.

	***MTHFR* genotype**			
Characteristic	**Ala/Ala**	**Ala/Val**	**Val/Val**	**P-value**
Number	425	362	73	
Age, years[a]	71.5 (5.3)	71.4 (5.5)	71.2 (5.3)	0.90
Body mass index, kg/m^2	25.5 (3.2)	25.5 (3.2)	25.2 (2.9)	0.75
Smoking status[b]				0.36
Current smokers, %	29.5	30.9	32.9	
Former smokers, %	50.9	50.3	57.5	
Never smokers, %	19.6	18.8	9.6	
Alcohol intake, grams/day[c]	13.5 (17.4)	12.7 (16.4)	16.5 (18.4)	0.24
Folate intake, µg/day[c]	201 (61)	202 (63)	192 (55)	0.49

Values in parentheses for variables are SDs.
[a] range 65-84 years.
[b] Missing data on smoking for 1/860 subject.
[c] Missing data on folate and alcohol intake for 56/860 subjects.

Table 2. The relationship of *MTHFR* genotypes with total plasma homocysteine levels depending on folate intake in the Zutphen Elderly Study.

Folate intake	N	Homocysteine levels by *MTHFR* genotype			P-value
		Ala/Ala	Ala/Val	Val/Val	
all subjects	804	14.8 (0.4)	15.5 (0.4)	21.6 (0.9)	0.0001
folate tertiles					
<169.8 μg/day	268	15.6 (0.7)	17.2 (0.7)	25.1 (1.4)	0.0001
169.8-214.8 μg/day	167	15.0 (0.7)	14.6 (0.7)	24.3 (1.8)	0.0001
>214.8 μg/day	269	13.6 (0.7)	14.7 (0.7)	14.7 (1.6)	0.38
				Interaction	0.0006

Values in parentheses for variables are SEs.
Folate intake is estimated as described in methods.

plasma homocysteine levels (P=0.0001; table 2). For subjects homozygous for the Ala-allele, the mean level was 14.8 nmol/ml, whereas it was 21.6 nmol/ml among subjects homozygous for the Val-allele (Table 2). This association was significantly modulated by folate intake (P, interaction=0.0006; table 2).

The prevalence of cancer among subjects homozygous for the Val-allele (11.0%) tended to be higher than among those homozygous for the Ala-allele (6.1%), although this was not statistical significant (P=0.17; table 3). The estimated age-adjusted risk of cancer associated with the Val/Val genotype was 1.95 (95% CI, 0.84-4.54) as compared with the Ala/Ala genotype

During the 10-year follow-up period, 150 new cases of cancer occurred among the 793 men without cancer at baseline. The most common sites where cancer developed were the lung (29.3%), prostate (14.0%), colorectum (12.0%) and the kidney or bladder (10.7%). Compared with men homozygous for the common Ala-allele, the age adjusted risk of cancer was 1.81-fold (95% CI, 1.09-3.00) increased among men with the Val/Val genotype (table 4). Further adjustment for smoking status (RR, 1.77; 95% CI, 1.06-2.94) or smoking status, body mass index, alcohol and folate intake (RR, 1.63; 95% CI, 0.96-2.80; missing data for 54 subjects) did not appreciably alter this risk estimate. The analysis of the different forms of cancer revealed that the higher incidence of cancer was due to significantly increased risks of cancer of the prostate, colorectum and kidney or bladder, whereas the risk of lung cancer remained unaffected (table 3).

On the basis of previous published reports,[14,15] we next evaluated the influence of folate and alcohol intake on the association between the Val/Val genotype and cancer risk (table 5). To increase the power of the analysis we combined prevalent and incident

Table 3. The prevalence of cancer at baseline according to MTHFR genotype in the Zutphen Elderly Study.

Cancer	*MTHFR* genotype			P-value
	Ala/Ala	Ala/Val	Val/Val	
All cancer, % (n)	6.1 (26)	9.1 (33)	11.0 (8)	0.17
Lung cancer, % (n)	1.7 (7)	1.4 (5)	1.4 (1)	0.95
Prostate cancer, % (n)	0.9 (4)	1.4 (5)	4.1 (3)	0.10
Colorectal cancer, % (n)	0.2 (1)	1.7 (6)	1.4 (1)	0.11
Kidney and bladder cancer, % (n)	0.5 (2)	1.9 (7)	0.0 (0)	0.087

cases of cancer (see methods). Among men whose folate intake was ≤169.8 μg/day, the Val/Val genotype was associated with a 2.64-fold increased risk of cancer as compared with a 1.60-fold increased risk among men with a higher folate intake. Among men who consumed >14g alcohol per day, the Val/Val genotype was associated with a 2.27-fold increased risk of cancer as compared with a 1.63-fold increased risk among men with a lower alcohol consumption. Furthermore, the risk of cancer associated with the Val/Val genotype was 3.14-fold increased among men older than the median age of 71 years, whereas the risk was only 1.37-fold increased among younger men (table 5).

In addition to our investigations in the Zutphen Elderly Study, *MTHFR* genotypes were determined in a series of 211 lung cancer patients. The genotype distribution in this group was 53.1% (Ala/Ala), 35.5% (Ala/Val) and 11.4% (Val/Val) as compared to 40.0% (Ala/Ala), 47.6% (Ala/Val) and 12.4% (Val/Val) in a population of 250 healthy subjects from the same geographical region. The frequency of the Val/Val genotype was similar in both populations (P=0.74).

Discussion

We studied the influence of a common *MTHFR* Ala/Val polymorphism on the risk of cancer in a population-based cohort of men aged 65-84 years old and a series of patients with lung cancer. In the elderly men, the Val/Val genotype was associated with considerably elevated levels of plasma homocysteine as compared to the other genotypes, particularly among those with lower folate intakes. This is consistent with the hypothesis that the genotype contributes to a disturbed folate metabolism. Over a 10-year follow-up period, the Val/Val genotype was associated with an about 2-fold

Table 4. Relative risks of cancer according to *MTHFR* genotype among subjects without cancer at baseline in the Zutphen Elderly Study between 1985-1995.

End point	*MTHFR* genotype		
	Ala/Ala	Ala/Val	Val/Val
all cancers			
Cases/at risk	71/399	60/329	19/65
Relative risk	1 (reference)	1.01 (0.71-1.42)	1.81 (1.09-3.00)
lung cancer			
Cases/at risk	23/399	17/329	4/65
Relative risk	1 (reference)	0.88 (0.47-1.66)	1.16 (0.40-3.35)
prostate cancer			
Cases/at risk	8/399	9/329	4/65
Relative risk	1 (reference)	1.26 (0.49-3.27)	3.57 (1.07-11.9)
colorectal cancer			
Cases/at risk	7/399	7/329	4/65
Relative risk	1 (reference)	1.19 (0.42-3.38)	3.69 (1.08-12.6)
kidney and bladder cancer			
Cases/at risk	6/399	5/329	5/65
Relative risk	1 (reference)	1.01 (0.31-3.30)	5.51 (1.68-18.1)

Relative risks are adjusted for age.
Values in parentheses are 95% CIs.

increased risk of cancer. A similar 2-fold increase in risk was observed in the comparison of cancer at baseline although this was not statistically significant. These results could be attributed to significantly increased risks of cancer of the prostate, colorectum and kidney or bladder. Because of the small numbers, these observations require confirmation in larger studies. Neither in the cohort of elderly men nor in the series of lung cancer patients, did we find any indication that the Val/Val genotype contributed to the risk of lung cancer. The association of the Val/Val genotype and the overall risk of cancer appeared to be particularly increased among men of an older age, a lower folate intake or a higher alcohol consumption, which is in line with the hypothesis that the genotype influences cancer risk through its effect on folate metabolism. Our findings indicate that the *MTHFR* variant has a deleterious effect on cancer risk in the general population and confirm our earlier observation in a population-based study among subjects aged 85 years and over.[13]

Table 5. Odds ratios for cancer[a] depending on folate intake and alcohol consumption according to *MTHFR* genotype in the Zutphen Elderly Study.

Subgroup	*MTHFR* genotype		
	Ala/Ala	**Ala/Val**	**Val/Val**
folate intake[b,d]			
≤ 169.8 μg/day			
Cases/at risk	30/131	31/110	11/27
Relative risk	1 (reference)	1.37 (0.76-2.48)	2.64 (1.08-6.43)
> 169.8 μg/day			
Cases/at risk	63/261	59/236	13/39
Relative risk	1 (reference)	1.04 (0.69-1.57)	1.60 (0.77-3.30)
alcohol intake[c,d]			
≤ 14 g/day			
Cases/at risk	62/267	55/242	12/37
Relative risk	1 (reference)	0.99 (0.65-1.50)	1.63 (0.77-3.43)
> 14 g/day			
Cases/at risk	31/125	35/104	12/29
Relative risk	1 (reference)	1.41 (0.78-2.55)	2.27 (0.95-5.42)
age[e]			
≤ 71 years			
Cases/at risk	51/228	40/203	14/47
Relative risk	1 (reference)	0.87 (0.55-1.40)	1.37 (0.68-2.97)
> 71 years			
Cases/at risk	46/197	53/159	13/26
Relative risk	1 (reference)	1.62 (1.01-2.58)	3.14 (1.36-7.29)

Odds ratios are adjusted for age.
Values in parentheses are 95% CIs.
[a] In these analyses prevalent and incident cases of cancer are combined (see methods).
[b] Stratified according to lowest tertile of folate intake and the two highest tertiles.
[c] Stratified according to two lowest tertiles of alcohol intake and highest tertile.
[d] For 53 subjects data on alcohol and folate intake were missing.
[e] Subjects are stratified according to median age.

In contrast, several studies observed a protective effect of the Val/Val genotype on the risk of colorectal cancer[14,15] and acute lymphocytic but not myeloid leukaemia.[16] These outcomes were attributed to a beneficial effect of the genotype on the dUMP/dTMP balance owing to the accumulation of 5,10-methyleneTHF, which is postulated to be associated with the Val/Val genotype. The opposite effect of the Val/Val genotype in our studies may be explained by a deleterious influence of the genotype on DNA methylation owing to the depletion of 5-methylTHF.[10] The discrepancies between the results of the studies might be due to the influence of environmental factors. The reduced risk of colorectal cancer associated with the Val/Val genotype found in previous studies was abolished or even reversed among men with lower folate status or higher alcohol consumption.[14,15] Alcohol interferes with folate absorption and utilisation[23,24] and that alcohol is a methyl group antagonist.[25] Similarly, our analyses indicated that the overall cancer risk was particularly increased among men with a lower folate or higher alcohol consumption. The protective associations with colorectal cancer were observed in male American health professionals and physicians who are considered to be relatively health-conscious and well-nourished.[14,15] Indeed, the median folate intake of the health professionals was approximately 2-fold higher than that of the participants in our population-based study.[14] It may be hypothesised that such factors suppressed the deleterious effects of the Val/Val genotype. Additionally, age-differences might play a role. Our analyses suggested that the adverse effects of the Val/Val genotype may increase with age. The subjects in our current and previous[13] study were 65-84 and 85-100 years old, respectively, which is substantially higher than those in the studies on colorectal cancer (40-75[14] and 40-84[15] years) and leukaemia (16-70[16] years).

The *MTHFR* gene is located on chromosome 1p36.3. Several genome-wide searches provided evidence for a prostate cancer locus in this chromosomal region with peak LOD-scores at 1p36[26,27] and 1p35.1.[28] The patients in these studies had a positive family history of prostate cancer and the mean age of onset was 65 years (range 42-85[26,27] and 42-91[28] years). Linkage was especially high among subsets of families with a history of both prostate and brain cancer[26,27] or both prostate and breast cancer[28] and in those with an onset of prostate cancer before the age of 61 years.[27] The chromosomal region was also implicated in prostate cancer aggressiveness.[29] It would be interesting to test whether variation at the *MTHFR* gene had contributed to these outcomes. To our knowledge, no genome-wide searches have been published for cancer of the colorectum, bladder and kidney.

On the basis of the linkage data several candidate genes were suggested (TP73, FGR, TNFRSF1B, NBL1, ID3, CDC2L1).[27] Linkage disequilibrium between the *MTHFR* polymorphism and variation at these loci might explain our findings. This is, however, unlikely. Levels of linkage disequilibrium that are sufficiently high to be compatible with this explanation are thought to be limited to about 50 kb,[30-32] whereas the approximate physical distance between *MTHFR* and most candidate genes exceeds 10 Mb except for TNFRSF1B, which is located about 100 kb centromeric of *MTHFR* (http://www.ncbi.nlm.nih.gov/genome/guide/HsChr1.shtml). Since linkage disequilibrium is an improbable cause of the association, the *MTHFR* polymorphism is functional and there is a plausible mechanism underlying the association with cancer, it

is likely that the *MTHFR* polymorphism itself contributes to cancer risk.

In conclusion, our studies in two independent populations indicate that homozygosity for the *MTHFR* Ala/Val polymorphism increases the risk of cancer in elderly men from the general population. This finding might primarily have resulted from a deleterious effect on the development of prostate, colorectal, kidney and bladder cancer. The mechanism underlying this association might involve a decreased DNA methylation as a result of a disturbed folate metabolism.

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Chapter 9

Summary and general discussion

9.1 Summary and discussion of results

Biological pathways with a major role in human ageing have yet to be discovered. Genetic research may contribute to the identification of some of these pathways by revealing gene variants that affect disease risk and mortality. Over the last years, a large number of common gene variants have been suggested to contribute to cardiovascular disease risk, the major cause of death in Western societies. Very few studies, however, investigated whether such gene variants are also associated with fatal cardiovascular disease or mortality in the population at large. Moreover, in the majority of these studies, gene frequencies in centenarians were compared to those in younger groups. Centenarian studies may, however, not be suitable for testing the contribution of gene variants to mortality in the population at large because, firstly, extreme longevity may well be determined by other genetic and environmental factors than population mortality and, secondly, geographical and thereby genetic heterogeneity of groups of centenarians hampers the collection of appropriate control populations (see chapter 1.3.3).

We explored the contribution to mortality of common gene variants affecting different pathways involved in cardiovascular disease in two study designs based on the Leiden 85-plus Study.[1] Firstly, genotype distributions were compared between persons aged 85-years and over and subjects aged 18-40 years whose families originated from the same geographical region (cross-sectional analysis). Secondly, the population-based cohort of persons aged 85 years and over was followed for all-cause and cause-specific mortality over a 10-year period (prospective study). In addition, associations of gene variants with morbidity in old age were investigated if suggested by previous studies. Gene variants investigated in our study had previously been associated with altered gene function or expression, or level of the gene product in plasma indicating that the variants are either functional themselves or in linkage disequilibrium with the actual but yet unknown functional variant. Table 1 summarises the associations found for the gene variants tested in the Leiden 85-plus Study. The associations observed will be discussed in the next sections.

9.1.1 Methylenetetrahydrofolate reductase (MTHFR)

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the folate and the methionine/homocysteine metabolism (see chapter 8, figure 1)). In chapter 2 the association between a common Ala222/Val variant in the gene encoding MTHFR and mortality is described. The frequency of homozygosity for the variant, the Val/Val genotype, was significantly reduced among persons aged 85 years and over as compared with young persons whose families originated from the same geographical area, particularly among men. These findings were supported by the reduction of the Val/Val

genotype in French centenarians[2] and the gradual decline in prevalence of the genotype with age in Japanese subjects.[3] Other cross-sectional studies did, however, not observe an underrepresentation of the mutation in old age.[4-7]

The prospective study confirmed our cross-sectional data: during the 10-year follow-up period, homozygosity for the Val variant was associated with a significantly increased mortality risk in men aged 85 years but not in women. Our study could not provide an explanation for the gender dependence of the association. It might be that the deleterious influence of the *MTHFR* variant is aggravated by factors that have adverse effects on the folate and the methionine/homocyteine metabolism and that were more common among men than women. Such factors may be alcohol consumption, low folate intake and smoking.[8-15]

The analysis of specific causes of death in the prospective study indicated that the increased risk of mortality among old men with the Val/Val genotype was due to an increased risk of cancer mortality. However, the numbers in this analysis were small. To gain more insight in the validity of this outcome, we set out to replicate this finding

Table 1. Summary of results obtained in the Leiden 85-plus Study.

Gene and variant	Mortality <85 years cross-sectional	Mortality >85 years prospective	Cause of death >85 years prospective	Morbidity >85 years cross-sectional
MTHFR				
Ala222/Val	+ men	+ men	+ men/cancer	– dementia[a]
APOE				
ε2/ε3/ε4	+	–	–	+ dementia
-219G/T	–	–	–	+ dementia
-491A/T		–	–	
PON1				
Met55/Leu	–	–	–	
Gln192/Arg	–	–	–	
TNFA				
-308G/A	–	–	–	+ diabetes
2 STRs	–	–	–	– diabetes
F5				
Gln506/Arg	–	–	–	
ACE				
intron16(I/D)	–	–	–	
PAI1				
-675(4G/5G)	–	–	+ men/ischaemic heart disease	

+ indicates positive association, – absence of association, an empty cell indicates that no associations were investigated or the frequency of the variant was too low to allow for analysis (-491A/T); when applicable, the cause of death, the specific disease or the subset for which the association was found is indicated.

ACE = angiotensin I-converting enzyme *APOE* = apolipoprotein E *F5* = factor V *MTHFR* = 5,10-methylenetetrahydrofolate reductase *PAI1* = plasminogen activator inhibitor 1 *PON1* = paraoxonase *TNFA* = tumour necrosis factor α

[a] Gussekloo *et al.* J Neurol Neurosurg Psychiatry 1999; 67: 535-8.

(chapter 8). The *MTHFR* variant was measured in a population-based cohort of 860 men aged 65-84 years who were followed over a 10-year period (Zutphen Elderly Study[16]). Although the effect on all-cause mortality was found to be less pronounced in this population than the Leiden 85-plus Study, the Val/Val genotype was associated with a 2-fold increased risk of cancer. This finding could be attributed to an increased risk of cancer of the prostate, colorectum and kidney and bladder, whereas the risk of lung cancer remained unaffected. The replication of our initial findings in an independent population provides strong evidence for a role of the *MTHFR* variant in the development of cancer. Additionally, the link with prostate cancer but also the other cancers, which are more common among men than women, may - apart from lifestyle differences - partly explain why the deleteriousness of the genotype was restricted to men in the Leiden 85-plus Study.

Interestingly, genome-wide searches implicated the chromosomal region of *MTHFR*, 1p36.3, in prostate cancer.[17-20] In these studies, linkage was especially high among subsets of families, namely in those with a history of both prostate and brain cancer[17,18] or both prostate and breast cancer[19] and in those with an onset of prostate cancer before the age of 61 years.[18] It would be interesting to test whether variation at the *MTHFR* locus, either the Ala222/Val variant or mutations with a more severe effect, had contributed to these findings.

In contrast to our observations, several studies indicated a protective effect of the Val variant against the risk of colorectal cancer[21,22] and acute lymphocytic leukaemia.[23] These apparently paradoxical findings may be related to the fact that MTHFR acts as a switch between two competing folate-dependent pathways: the synthesis of the nucleotide dTMP from dUMP and the methylation of DNA and other molecules. MTHFR converts 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate. The Val variant causes a decreased MTHFR activity, which shifts the balance between these two forms of folate in favour of 5,10-methyltetrahydrofolate.[24] This may be beneficial to dTMP synthesis, which depends on this form of folate. A larger dTMP pool may reduce dUMP misincorporation in DNA and thereby decrease the risk of chromosome breaks, which can result from the simultaneous removal and repair of adjacent uracil bases on opposing DNA strands. This effect would be expected to reduce the risk of cancer.[25] At the same time, however, a diminished availability of 5-methyltetrahydrofolate may be detrimental because it could compromise the methylation of DNA for which it supplies the methyl-groups. Decreased DNA methylation may promote the development of cancer by inducing the expression of proto-oncogenes[26] and inducing an increased mutation rate.[27,28] Interestingly, decreased genomic DNA methylation was indeed observed in lymphocytes from subjects homozygous for the Val variant of the *MTHFR* gene.[29]

Possibly, environmental factors determine whether the beneficial or the detrimental influence of the *MTHFR* variant on cancer risk predominates. Collectively, previous[21,22] and our own studies suggested that lower folate status, higher alcohol intake and older age added to the adverse effects of the Val variant. The participants in our studies were older, had a lower folate intake and were likely to be less health-conscious than male United States health professionals[22] and physicians[21] among who the *MTHFR* variant appeared to protect against colorectal cancer. In addition, it may be speculated that the

use of aspirin by 30% of the health professionals[30] and by 50% of the physicians[31] influenced the effect of the *MTHFR* variant, since aspirin was shown to suppress the deleterious effects on colon cancer risk of high alcohol, low methionine and low folate intake, which are thought to disturb the folate and the methionine/homocysteine metabolisms.[30]

Apart from cancer, the *MTHFR* variant might also have contributed to cardiovascular mortality in the Leiden 85-plus Study. The Val/Val genotype leads to increased plasma homocysteine levels,[32] which are considered to be a risk factor for cardiovascular disease.[33] However, the Val/Val genotype did not predict cardiovascular mortality in old persons. This is in agreement with a large number of previous studies in which no evidence was found for an association between the *MTHFR* variant and the risk of myocardial infarction or coronary artery disease (see appendix). In the two initial positive studies,[34,35] moreover, the association was mainly due to a low frequency of the Val/Val genotype in the control groups and not to an increased frequency in the case groups. Since persons homozygous for the mutation are exposed to an, on average, 25% elevated homocysteine level[32] during their whole life, the absence of an association between the genotype and cardiovascular disease risk is important evidence against homocysteine being a causal risk factor for this disease. In this regard it was surprising that the *MTHFR* variant was associated with an increased risk of ischaemic heart disease in the Zutphen Elderly Study. Adjusting for homocysteine levels in plasma using a logistic regression model did, however, not influence the association. This indicates that the increased risk of ischaemic heart disease among men with the Val/Val genotype was due to other factors than an elevated level of plasma homocysteine. Such factors might include a disturbed methylation of DNA and proteins.[36,37]

Not only genetic data challenge whether homocysteine is causally involved in cardiovascular disease. Elevated levels of homocysteine in plasma are a poor predictor of future cardiovascular events in prospective studies, especially when the follow-up periods are longer.[38-42] Why cardiovascular patients are consistently found to have mildly elevated levels of plasma homocysteine,[33] remains to be determined but may be related to the observed increase of homocysteine levels in the period after a myocardial infarction[43] or stroke.[44] Taken together, these data indicate that elevated homocysteine levels may not be the cause but the consequence of cardiovascular disease.[38]

In conclusion, our studies in two independent populations indicate that homozygosity for the *MTHFR* Ala/Val variant increases the risk of cancer in elderly men from the general population.

9.1.2 Apolipoprotein E (APOE)

In chapter 3 the contribution of genetic variation at the *APOE* locus to mortality and dementia is investigated. The strong associations of the ε2 and ε4 alleles with a decreased and increased risk of dementia, respectively, in previous studies[45,46] were confirmed in the population-based cohort of subjects aged 85 years and over. To further characterise the role of *APOE* in dementia, two functional promoter variants, -291G/T and the -491A/T,[47] were studied. The promoter variants were found to be in linkage disequilibrium with the ε2/ε3/ε4 variation but not with each other. The -291T/T

genotype was identified as an additional risk factor for dementia independent of the ε2 and ε4 alleles thereby confirming the initial report in a series of hospital patients.[48] The -491A/T variant was too infrequent to allow for detailed analyses.

Despite the association with dementia in old age, the *APOE* variants did not confer increased all-cause or cardiovascular mortality after the age of 85 years during a 10-year follow-up period. In contrast, the results from the cross-sectional study indicated that the ε4/ε4 genotype was associated with an increased mortality risk prior to the age of 85 years. The data were also compatible with minor contributions to mortality of heterozygosity for the ε2 and ε4 alleles. These findings would be in line with previous prospective studies showing that the ε4 allele influenced mortality only before the age of ~75 years.[49-53]

It is currently not clear how to account for the age-dependent effects of *APOE* alleles. Associations of the *APOE* ε2/ε3/ε4 alleles variant with mortality are generally attributed to their contribution to the risk of cardiovascular diseases (see appendix) and dementia.[45] However, cardiovascular diseases are the leading cause of death both prior to and beyond the age of 75 years and the incidence of dementia starts to rise rapidly only after the age of 75 years. Hence, it might have been expected that *APOE* alleles also contributed to overall mortality at ages greater than 75 years. As yet ill-defined age-dependent differences in the pathogenesis of cardiovascular diseases and dementia might underlie these findings.

Our study of *APOE* variants emphasised the importance of controlling for geographical differences in genotype distribution. Both the old and the young population indicated that the frequencies of the ε2 and ε4 allele differed between persons with a Leiden and a non-Leiden background. This was not observed for eight other variants studied in this thesis nor for the two *APOE* promoter variants (the majority of rare promoter alleles occurred on a haplotype with the ε3 allele, the frequency of which was not dependent on geographical region). Not accounting for these differences would have overestimated the ε4-effect on mortality and would have indicated the ε2 allele as a risk factor for dementia. Previous cross-sectional studies that compared old and young populations did not extensively control for possible geographical differences in genotype distribution.[54-60] In addition, part of these studies selected relatively healthy old persons,[54-56] which is expected to cause an underrepresentation of the ε4 allele as a consequence of its association with cognitive impairment. Part of the previous cross-sectional studies may, therefore, have overestimated the impact of the ε4 allele on mortality.

9.1.3 Paraoxonase (PON1)

Both the paraoxonase Met55/Leu and the Gln192/Arg variants were associated with the ability of paraoxonase to protect LDL from oxidation *in vitro*.[61,62] Our study, however, suggested that the variants do not have a major effect on the risk of fatal cardiovascular disease (chapter 4). Our finding might be explained by a less important role of paraoxonase in protecting LDL from oxidation *in vivo* than studies *in vitro*[63-65] suggested. An alternative explanation is suggested by previous studies that indicated an association between the paraoxonase gene variants and the presence of coronary atherosclerosis, but

not myocardial infarction (see appendix). At the level of the general population, protection of LDL against oxidation might be particularly important in the early development of an atherosclerotic plaque whereas other factors influencing the stability and thrombogenicity of the plaque determine whether a plaque is prone to cause fatal complications.

Interestingly, all of the studies in diabetic patients performed thus far supported a role for paraoxonase gene variants in coronary artery disease.[66-69] In our study, the variants were associated with a non-significantly increased (cardiovascular) mortality among diabetic patients in the prospective study. Diabetes patients are exposed to higher levels of oxidative stress[63] and have an activated acute-phase response,[70,71] which is related to a decline in HDL-associated paraoxonase activity.[72] It may be hypothesised that these factors aggravate the effects of the paraoxonase gene variants.

9.1.4 Tumour necrosis factor α (TNFA)

The -308G/A *TNFA* promoter variant, which was indicated to increase transcriptional activity,[73-75] was strongly associated with the risk of diabetes (chapter 5). The investigation of two short tandem repeats indicated that the association with diabetes was independent of other genetic variation at the *TNF* locus. In addition, linkage disequilibrium with HLA-DR3 and DR4 could be excluded as an underlying cause of this finding. The association with diabetes is in line with extensive evidence that TNFα may contribute to insulin resistance by inhibiting the insulin induced tyrosine kinase activity of the insulin receptor.[76-78]

TNFα may be involved in the pathogenesis of cardiovascular diseases either through its contribution to the risk of diabetes or more directly, by contributing to foam cell formation, to T-lymphocyte activation and to the expression of matrix metalloproteinases.[79,80] The *TNFA* promoter variant was, however, not associated with (cardiovascular) mortality after the age of 85 years nor was a different genotype distribution observed in old and young persons (the latter analysis was not presented in chapter 5). These results support the previously reported absence of an association of the promoter polymorphism with myocardial infarction[81,82] and coronary artery disease.[83] The strong association with diabetes and not cardiovascular mortality may reflect that the aetiology of cardiovascular diseases is much more complex than that of type 2 diabetes.

9.1.5 Factor V (F5)

The Arg506/Gln mutation in the gene encoding coagulation factor V, referred to as factor V Leiden, causes a poor response to activated protein C,[84,85] the primary inhibitor of coagulation, and is consistently associated with an increased risk of venous thrombosis.[86,87] As is consistent with other studies,[88,89] the mutation did not confer an increased mortality risk (chapter 6). This finding substantiates the fact that the mutation does not contribute to the risk of cardiovascular disease in the population at large (see appendix) and that the mutation is of limited relevance to the risk of fatal complications of venous thrombosis, namely pulmonary embolism.[90-93]

Recent studies suggest that the mutation might play a role in the occurrence of a myocardial infarction in young women[94] and subjects without coronary stenosis as assessed by coronary angiography.[95] In these specific groups without substantial atherosclerosis, coagulation may be a more important determinant of complete occlusion of the coronary artery than in the population at large.

9.1.6 Angiotensin I-converting enzyme (ACE)

The *ACE* I/D variant is a marker for ACE activity in plasma[96,97] and tissue.[98] The consequences of the variant for cardiovascular disease risk are uncertain. Only 12 out of 37 investigations indicated a positive association with myocardial infarction as well as coronary heart disease (see appendix). The association of the variant with left ventricular mass and hypertrophy is also equivocal.[99-101] Furthermore, the observation that the *ACE* variant is especially associated with disease risk among persons otherwise at low risk[102-105] is refuted by the majority of studies.[83,106-109] Moreover, it is important to note that the positive results were obtained in the smaller studies thus far performed (appendix). This suggests that small studies with positive results are published more often than small studies with negative results (publication bias). A bias toward positive results for smaller studies has also been suggested by a previous meta-analysis.[110] The continuous accumulation of studies investigating a variety of cardiovascular end-points has thus failed to provide conclusive answers. Our study indicated that the *ACE* I/D variant does not contribute to mortality in the population at large (chapter 7). Taken together, it is likely that the *ACE* variant is of limited relevance to the overall risk of fatal disease.

9.1.7 Plasminogen activator inhibitor 1 (PAI1)

PAI-1 is the primary inhibitor of fibrinolysis. A 4G/5G variant in the promoter of the *PAI1* gene influences gene expression and PAI-1 levels in plasma.[111-114] Although the numbers were relatively small, we found that the variant predicted death from ischaemic heart disease in men aged 85 years and over (chapter 7). This association was not reflected in an increased overall mortality before or after the age of 85 years. The association with fatal cardiovascular disease has recently been confirmed in an autopsy study of men aged 33-69 years.[115] Our results suggest that the *PAI1* gene variant is not only a risk factor for premature ischaemic heart disease[111,116,117] but also for ischaemic heart disease at older ages when the incidence of is much greater.

9.2 Evolutionary theories of ageing

Our studies indicated the presence of common gene variants with late-acting deleterious effects, which is compatible with the predictions of evolutionary theories of ageing.[118,119] Whether these variants only have late deleterious effects (mutation accumulation theory, chapter 1.2) or additional favourable effects early in life (theory of antagonistic pleiotropy, chapter 1.2) remains unclear. Since even a small and hardly detectable fitness benefit early in life may outweigh a substantial deleterious effect later on, it is difficult, if not impossible to determine whether the high frequency of a variant is the result of genetic drift or natural selection.

However, for the two gene variants that were found to contribute to mortality, the presence of additional deleterious effects early in life have been suggested. The *MTHFR* variant was indicated as a risk factor for birth defects, specifically, neural tube defects.[120,121] The analysis of *APOE* haplotypes and comparisons to the chimpanzee *APOE* sequence indicated that the putatively deleterious *APOE* ε4 allele is the ancestral one.[122] The relatively low frequency of the ε4 allele (~15%) as compared to the ε3 allele is compatible with negative selection of the ε4 allele. The early deleterious effects that would be responsible for this selection have yet to be identified. Thus, the *MTHFR* and *APOE* gene variants that may raise mortality at older ages might do so at younger ages as well and thus decrease fitness. This would imply that the *MTHFR* and *APOE* gene variants are not examples of gene variants that combine a neutral or even beneficial fitness effect in young age with a deleterious effect late in life as are predicted to exist by evolutionary theories of ageing.

9.3 Study limitations

We identified several common gene variants as risk factors for late-onset disease and mortality. By studying a population-based cohort with a high response rate and carefully accounting for possible geographical differences in genotype distribution, we managed to minimise the sources of bias. However, the limitations of the study need to be discussed.

The analysis of mortality before the age of 85 years using a cross-sectional design entails that no data were available on specific causes of death. Especially for gene variants implicated in multiple diseases, this may limit the biological interpretation of the results. Furthermore, we may not have detected gene variants that were associated with a single cause of death that comprises only a smaller part of the total mortality. Still, associations with mortality in the population at large observed in our cross-sectional study warrant further in-depth studies of the gene variants in prospectively followed cohorts of individuals younger than 85 years. A practical problem of these studies is that younger cohorts with a relatively low mortality rate need to be substantially larger or need to be followed over a longer period than cohorts of elderly subjects to allow a reliable estimation of mortality risks.

Data on specific causes of death were analysed in the prospective study among subjects aged 85 years and over. The modest effects of a gene variant on cause-specific mortality may, however, not have been detected. The relatively small number of cases per cause of death led to a relatively low statistical power. In addition, the strength of associations may have been underestimated as the causes of death were not confirmed at necropsy, which is expected to lead to non-differential misclassification. The reported associations with single specific causes of death in this thesis and in previous studies[1,123] in the Leiden 85-plus Study indicate that the registry data have nevertheless sufficient power to discriminate between various causes of death. In addition, it has been shown that underreporting of cardiovascular diseases occurs relatively infrequently.[124] Although the analysis of specific causes of death may provide vital clues to the biological mechanism underlying an association, it should not obscure the fact that in the end it is

the overall mortality that counts. Particularly in old cohorts with a high prevalence of co-morbidity, a risk factor for a very specific pathology may cause an exchange in the ultimate cause of death without affecting lifespan.[125,126] This notion favours the investigation of gene variants affecting pathways that may affect a broad spectrum of pathologies.

An important limitation – as for any association study - is that false positive and false negative findings may occur. False positive findings may be excluded by performing replication studies. Replication was partly integrated into our study design by evaluating mortality both cross-sectionally and prospectively. The *MTHFR* variant was indicated to be associated with increased mortality in both parts of the study. The ultimate test for replication, however, is the investigation of an independent population. Therefore, the *MTHFR* variant was measured in an additional cohort as described in chapter 8 and section 9.1.1, which confirmed that the mutation was associated with an increased risk of cancer, although its influence on overall mortality seemed to be less pronounced in this population.

The significance of replication was illustrated by our studies on the effects of a variable number of tandem repeats (VNTR) polymorphism in the 5′ flanking region of the insulin gene (*INS*). The VNTR was shown to influence transcription of the *INS* gene[5,127-131] and the long class III alleles had been associated with an increased risk of insulin resistance[132] and type 2 diabetes.[133-135] The investigation of this gene variant in relation to mortality was made even more appealing by the evidence for a role of insulin and insulin signaling in ageing obtained from the genetic analysis of ageing in the nematode *Caenorhabditis elegans*,[136-140] from the study of the effects of caloric restriction[141-143] and from the analysis of age-related changes in the gene expression profile in mice.[144] In the Leiden 85-plus Study, homozygosity for class III alleles of the *INS* VNTR was associated with a 6-fold increased risk of cardiovascular mortality in smokers ($P<0.0001$) while the risk was 1 among non-smokers (table 2). Furthermore, the *INS* VNTR did not affect the mortality risk for non-cardiovascular causes, nor did it show an interaction with other classical cardiovascular risk factors measured in the study. Moreover, the association was supported by reduced frequencies of the risk genotype in old smokers as compared with young persons. Nevertheless, this association could not be replicated in the Zutphen Elderly Study; the risk of cardiovascular mortality was even somewhat decreased among class III homozygous subjects (table 2). These data suggest that, although the results from the Leiden 85-plus Study were compelling, they may have constituted a false positive finding.

In principle, performing additional studies would also resolve the occurrence of false negative findings. However, this is unlikely to occur since priority is given to the replication of positive findings and the absence of a specific research question hampers the design of replication studies. Therefore, false negative findings may be even more problematic than false positive findings are.

Table 2. Risk of cardiovascular mortality among *INS* VNTR class III homozygous persons stratified according to smoking status in the Leiden 85-plus Study and the Zutphen Elderly Study.

Cause of Death	All		Smokers		Non-smokers	
	RR	95% CI	RR	95% CI	RR	95% CI
Leiden 85-plus Study[a]		n=654		n=106		n=526
All cardiovascular diseases	1.0	(0.6-1.5)	6.1	(2.7-14.1)	0.6	(0.4-1.1)
Ischaemic heart disease	1.8	(0.9-3.6)	8.1	(2.0-33.0)	1.2	(0.5-2.9)
Zutphen Elderly Study[b]		n=876		n=263		n=613
All cardiovascular diseases	0.6	(0.3-1.0)	0.5	(0.2-1.2)	0.6	(0.3-1.3)
Ischaemic heart disease	0.3	(0.1-1.1)	0.2	(0.0-1.6)	0.4	(0.1-1.8)

[a] Risk associated with III/III genotype calculated with I/I and I/III as a reference group.
[b] Risk associated with III/III genotype calculated with I/I as a reference group.

9.4 Gene-gene and gene-environment interactions

It has been frequently stressed that interactions between genes and between genes and environment are pivotal to the occurrence of complex phenotypes as disease and mortality. In this thesis evidence is presented in support of this hypothesis. Persons homozygous for an Ala-to-Val variant of the *MTHFR* gene were suggested to be particularly at risk of developing cancer if their folate intake was relatively low or their alcohol consumption relatively high. If confirmed by more extensive studies, this interaction would imply that changes in diet or folate supplementation might suppress the deleterious effects of this mutation in persons homozygous for the mutation. However, lengthy and expensive intervention studies would be necessary to definitively prove or reject the existence of such benefits.

Detecting interactions is complicated, since it requires large sample sizes. In our study of 666 persons aged 85 years and over, for example, a reliable assessment of gene-gene interactions was not feasible. Even in the most favourable case, the possible interaction between the *PAI1* and *ACE* risk genotypes (both ~25%; chapter 7), the frequency of persons with both risk genotypes was only 6%, greatly reducing the power of the analyses. An additional complication is that not only large populations, but also many additional data need to be available to study gene-environment interactions.

The importance of interactions has major implications for replication studies. Different distributions of unknown interacting factors between genetic studies may cause conflicting outcomes. This can be illustrated by the association between the *MTHFR* variant and cancer risk: it may be possible that the deleterious effect of the mutation is no longer detectable in the United States because since a few years cereals are fortified with folic acid. Without taking such factors into account one might wrongfully refute a role for the gene variant and, thereby, folate metabolism in carcinogenesis. Hence, only if gene-gene and gene-environment interactions are characterised, will true replication be possible and can replication studies become more successful.

9.5 Conclusion

Our studies provided evidence that common variants in the genes encoding MTHFR and APOE contribute to mortality in the population at large. These data suggest that folate metabolism and APOE-dependent mechanisms involving lipid transport or other yet unidentified functions of APOE may represent critical pathways in human ageing.

The hunt for common gene variants contributing to disease and mortality has only just begun. Already new technologies are speeding up the detection of novel gene variants, particularly of single nucleotide polymorphisms (SNPs).[145-147] A consortium of academic laboratories and pharmaceutical companies is currently producing a SNP archive, which is expected to encompass 600,000-800,000 SNPs in April 2001 (http://snp.cshl.org). Together with the expected further acceleration in the development of high-throughput SNP scoring methods[117,148-152] this will allow genotypings to be performed for thousands of genetic markers in thousands of individuals. The application of these emerging technologies may spur on the identification of pathways high in the hierarchy of physiological processes that influence the onset of a broad spectrum of common age-related diseases and mortality rate in humans.

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Chapter 10

Nederlandse samenvatting

Inleiding

Een steeds groter deel van de bevolking krijgt een steeds groter deel van het leven te maken met de gevolgen van het ouder worden. Zo wordt er verwacht dat in 2050 26% van de Nederlandse bevolking ouder is dan 65 jaar en 11% ouder dan 80 tegenover ongeveer 14% en 3% nu. Dit betekent dat ook steeds meer mensen te maken krijgen met ziekten die optreden bij het ouder worden, zoals hart- en vaatziekten, diabetes, dementie en kanker. Dit geldt echter niet voor iedereen in dezelfde mate. Er zijn mensen die op negentigjarige leeftijd nog steeds gezond zijn en zelfstandig leven, terwijl er ook mensen zijn die al voor de zeventig jaar kampen met diverse ernstige kwalen. Wat de onderliggende oorzaken van zulke verschillen zijn, is grotendeels onbekend. Zelfs bekende risicofactoren voor ziekte op middelbare leeftijd, zoals roken en een verhoogde cholesterolspiegel in het bloed, lijken op hogere leeftijd van minder belang. Wel is bekend dat erfelijke aanleg in belangrijke mate bijdraagt aan het ontstaan van ziekten. Dit komt tot uiting in het feit dat erfelijke (genetische) verschillen ongeveer een derde tot de helft van de verschillen in levensduur tussen mensen verklaren. Dit biedt een belangrijk uitgangspunt: als deze genetische verschillen in kaart kunnen worden gebracht, dan komt er een schat aan informatie vrij over processen die een rol spelen bij de achteruitgang van lichamelijke functies en het ontstaan van ziekten bij het ouder worden. Het is pas sinds enkele jaren mogelijk om hier een begin mee te maken onder meer als gevolg van een stormachtige ontwikkeling in de technologie voor genetisch onderzoek. In het onderzoek dat beschreven staat in dit proefschrift hebben we gebruik gemaakt van deze ontwikkelingen en zijn we op zoek gegaan naar genetische verschillen die ten grondslag liggen aan de variatie in levensduur bij mensen.

Van alle naar schatting 50 tot 100 duizend menselijke genen bestaan verschillende varianten die naast elkaar in de bevolking voorkomen. Soms functioneert de ene variant van het gen beter dan de andere. Zo is er een gen dat codeert voor een eiwit waarmee de lever cholesterol uit het bloed kan halen, de zogenaamde LDL-receptor. Een relatief klein deel van de mensen (<0.5%) heeft echter een variant van dit gen waardoor de LDL-receptor niet goed functioneert. Hierdoor kan het cholesterol niet goed uit het bloed worden opgenomen. Als gevolg daarvan hebben mensen met deze variant een sterk verhoogde cholesterolspiegel in het bloed. Deze mensen hebben dan ook een veel groter risico om op jonge leeftijd hart- en vaatziekten te krijgen dan mensen met de goed functionerende versie van het gen. Dit is een voorbeeld van een ernstig gendefect dat al op jonge leeftijd tot ziekte kan leiden, maar tegelijkertijd slechts relevant is voor een klein deel van de bevolking. In de algemene bevolking zullen naar verwachting niet zozeer zeldzame, maar juist veelvoorkomende genvarianten in belangrijke mate bijdragen aan verschillen in levensduur (zie kader).

Het onderzoek dat beschreven staat in dit proefschrift, was gebaseerd op de hypothese dat er veel voorkomende genvarianten bestaan die bepalend zijn voor het

hart- en vaatziekten risicoprofiel en daarmee voor verschillen in levensduur. Uit onderzoek waarbij patiënten met hart- en vaatziekten werden vergeleken met gezonden mensen, zijn al ongeveer 50 genen naar voren gekomen die het hart- en vaatziekten risicoprofiel lijken te beïnvloeden. Vaak is onderzocht of een genvariant het risico verhoogd op een specifiek aspect van hart- en vaatziekten, zoals vernauwing van de kransslagaders. Er werd nog nauwelijks bestudeerd of zulke genvarianten ook bijdragen aan fataal verlopende hart- en vaatziekten en, meer algemeen, sterfte in de algemene bevolking. Deze vraag is in dit proefschrift onderzocht.

Studie-opzet

Het onderzoek is grotendeels verricht in de Leiden 85-plus Studie. Voor deze studie werden in 1986 alle inwoners van Leiden ouder dan 85 jaar gevraagd mee te doen. Hierdoor kwam het DNA van 666 ouderen beschikbaar. Met dit cohort ouderen werd de relatie tussen genvarianten en sterfte vanuit twee invalshoeken onderzocht. Eerst werd het voorkomen van genvarianten in deze groep oude mensen vergeleken met een groep van 250 mensen met een leeftijd tussen de 18 en 40 jaar. Als een genvariant vaker voorkomt bij de oude groep dan bij de jongere groep, dan wijst dit erop dat dragers van deze variant een grotere kans hebben om een hoge leeftijd te bereiken. Het kan dan bijvoorbeeld zo zijn dat de genvariant beschermt tegen hart- en vaatziekten. Voor een lagere frequentie van een genvariant in de oudere groep geldt dat dragers waarschijnlijk

Zeldzaam en ernstig of veel voorkomend en mild?

Wat zijn de kenmerken van genvarianten die bijdragen aan verschillen in levensduur tussen mensen? Het antwoord op deze vraag is van belang, omdat het gevolgen heeft voor de manier waarop zulke genvarianten kunnen worden gevonden. Het kunnen genvarianten zijn die het risico op ziekte in sterke mate verhogen, of juist genvarianten met een beperkt nadelig effect. Genvarianten met een ernstig effect, zoals de besproken varianten van het LDL-receptor gen, leiden weliswaar vaak tot ziekte bij mensen met deze variant, maar zijn zeldzaam in de algemene bevolking. Dit komt doordat dragers van deze variant, als gevolg van hun grote risico om op relatief jonge leeftijd ziek te worden, minder nakomelingen kregen dan mensen zonder deze variant. De genvariant werd daardoor zelden aan volgende generaties doorgegeven en heeft zich niet verspreiden kunnen verspreiden in de bevolking. Dit fenomeen wordt natuurlijke selectie genoemd. Nu voorspellen theorieën die het ontstaan van veroudering proberen te verklaren, dat er ook genvarianten bestaan die wél het risico op ziekte verhogen, maar niet of nauwelijks worden beïnvloed door natuurlijke selectie. Dit komt doordat tijdens de evolutie van de mens, de levensverwachting werd bepaald door bedreigingen uit de omgeving zoals honger, infectieziekten en ongelukken. Zolang het nadelige effect van genvarianten maar niet voor deze levensverwachting tot uiting kwam, ontsnapten ze aan de werking van natuurlijke selectie en konden ze in de loop van de generaties een hoge frequentie bereiken in de algemene bevolking. Vaak zullen dit genvarianten zijn met een relatief mild nadelig effect, bijvoorbeeld doordat alleen langdurige blootstelling aan dit effect het ontstaan van ziekten bevordert, of doordat een dergelijk effect pas tot uiting komt onder condities die laat in het leven optreden. Hoewel zulke genvarianten op zichzelf het risico op ziekte niet sterk verhogen, kunnen ze een als groep een substantieel deel van de genetische variatie in levensduur bepalen omdat een groot deel van de bevolking drager is van deze varianten.

een verhoogd risico hadden om voor de 85 jaar te overlijden. Omdat het zou kunnen dat een genvariant niet in elke geografische regio even vaak voorkomt, werd voor deze vergelijking alleen gebruik gemaakt van de ouderen die geboren waren in Leiden (55% van de 666), en jongeren van wie de familie eveneens afkomstig was uit de regio Leiden. De tweede invalshoek van waaruit genvarianten werden onderzocht bestond uit het testen of de genvariant sterfte voorspelde binnen het oude cohort. Dit was mogelijk omdat het Leidse 85-plus cohort 10 jaar lang gevolgd is. Zo kon worden vastgesteld of dragers van een variant eerder kwamen te overlijden dan niet-dragers. Bovendien kon nu worden nagegaan of een genvariant het risico op sterfte aan een bepaalde doodsoorzaak beïnvloedde. Omdat deze manier van onderzoek niet wordt verstoord door regionale verschillen in de frequentie van een genvariant, konden hiervoor de gegevens van alle 666 ouderen worden gebruikt.

In deze studie-opzet werd van twaalf varianten van zeven genen bestudeerd wat de invloed is op mortaliteit. De onderzochte varianten werden gekozen op basis van een relatief hoge frequentie in de algemene bevolking (5-50%) en op basis van voorgaande wetenschappelijke studies waarin was aangetoond dat de varianten de functie van het gen beïnvloedden. Bovendien werden genen gekozen op basis van hun rol in verschillende biologische mechanismen die betrokken zijn bij het ontstaan van hart- en vaatziekten, zoals het cholesterolmetabolisme, de bloedstolling, het samentrekken van de vaten, de ontsteking en het foliumzuurmetabolisme.

Cholesterolmetabolisme

Cholesterol speelt een centrale rol in het ontstaan van atherosclerose ('aderverkalking'). Cholesterol wordt in het bloed met name getransporteerd in LDL-deeltjes. Onder bepaalde omstandigheden kunnen deze deeltjes zich ophopen in de wand van een slagader waardoor er een zogenaamde atherosclerotische plaque ontstaat.

Vijftien procent van de bevolking heeft een variant van het apolipoproteïne E gen (APOE) waardoor één aminozuur anders is in het APOE eiwit. Het gevolg is dat deze personen een gemiddeld hogere cholesterolspiegel hebben dan mensen zonder deze variant. Eerder onderzoek heeft uitgewezen dat zij ook een verhoogd risico hebben om hart- en vaatziekten te ontwikkelen. Interessant is dat deze variant tegelijkertijd het risico op dementie verhoogt. Welk mechanisme hieraan ten grondslag ligt, is vooralsnog onbekend. In ons onderzoek vonden wij dat ouderen die drager waren van deze variant, inderdaad vaker leden aan dementie. Daarnaast was de frequentie van deze variant verlaagd in de groep oude mensen in vergelijking met de groep jonge mensen. Deze resultaten wijzen erop dat dragers van de APOE variant een kleinere kans hebben in goede gezondheid een hoge leeftijd te bereiken. Naast deze variant die leidt tot een aminozuurverandering, werden twee varianten bestudeerd die de activiteit (expressie) van het APOE gen beïnvloeden. Ook één van deze varianten bleek geassocieerd met het risico op dementie, maar niet met sterfte. Onze bevindingen tonen aan dat niet alleen de goede werking van het APOE eiwit, maar ook de hoeveelheid van het eiwit een rol speelt bij het ontstaan van dementie (hoofdstuk 3).

Er werden ook varianten onderzocht van het gen dat codeert voor het enzym paraoxonase. Paraoxonase beschermt LDL-deeltjes tegen oxidatie ('roesten'). Dit wordt

verondersteld een belangrijke rol te spelen bij het optreden van atherosclerose, omdat alleen geoxideerde LDL-deeltjes zich kunnen ophopen in de vaatwand. Door genetische varianten te bestuderen die de paraoxonase-activiteit beïnvloeden, werd indirect het belang van LDL-oxidatie in het optreden van fatale hart- en vaatziekten onderzocht. Dit belang bleek gering te zijn aangezien deze genetische varianten niet geassocieerd waren met een verhoogd sterfterisico of het fataal verlopen van hart- en vaatziekten (hoofdstuk 4).

Bloedstolling

Atherosclerose hoeft op zichzelf geen ernstige aandoening te zijn. Veelal treden er pas klinische problemen op als een atherosclerotische plaque scheurt. Het scheuren van een plaque leidt, zoals bij iedere wond, tot het vormen van een bloedstolsel, maar nu in een slagader. Een dergelijk bloedstolsel kan het gehele vat afsluiten en de zuurstoftoevoer naar de achterliggende organen blokkeren met als gevolg bijvoorbeeld een hartinfarct of beroerte. Zowel de snelheid en duur van het bloedstollingsproces als de efficiëntie waarmee het gevormde stolsel vervolgens wordt opgelost (fibrinolyse) kunnen bepalend zijn voor het al dan niet fataal verlopen van hart- en vaatziekten. Een variant van het factor V gen, genaamd de factor V Leiden mutatie, veroorzaakt een grotere stollingsneiging en is de meest frequente genetische risicofactor voor veneuze trombose. Plasminogen activator inhibitor (PAI-1) is de belangrijkste remmer van de fibrinolyse. De spiegel van PAI-1 in het bloed wordt beïnvloed door een variant in de promoter van het gen (het deel waarmee de activiteit van een gen wordt gereguleerd). Deze variant komt voor met een frequentie van maar liefst 50% in de algemene bevolking. De PAI-1 genvariant was geassocieerd met verhoogde sterfte aan een hartinfarct en vergelijkbare doodsoorzaken bij mannen ouder dan 85 jaar (hoofdstuk 7), maar een associatie van de factor V Leiden mutatie met vroegtijdige sterfte was niet meetbaar (hoofdstuk 6). Mogelijk speelt in de algemene bevolking een effectieve fibrinolyse een grotere rol bij het fataal verlopen van hart- en vaatziekten dan een beperkte verstoring van de bloedstolling.

Er is veelvuldig gespeculeerd over de mogelijkheid om dragers van de factor V Leiden mutatie preventief met anti-stollingsmedicatie te behandelen. Dit lijkt niet verstandig aangezien deze personen geen lagere levensverwachting hebben, terwijl anti-stollings therapie gepaard gaat met een aanzienlijk risico op complicaties.

Het samentrekken van vaten

Niet alleen de vorming van een stolsel kan een slagader afsluiten, maar ook het plotseling samentrekken van het vat zelf. Dit kan met name een probleem zijn, omdat juist het ontstaan van een wond, zoals het scheuren van een atherosclerotische plaque, het samentrekken stimuleert en daarmee de afsluiting van het vat door een stolsel kan verergeren. Het enzym angiotensin I-converting enzyme (ACE) bevordert de samentrekking van vaten en een veel voorkomende variant van het ACE gen gaat samen met een verhoogd niveau van het enzym in het bloed. Er bestaat grote belangstelling voor deze variant, omdat medicijnen die de activiteit van ACE remmen zeer effectief zijn

in de behandeling van hoge bloeddruk en hypertrofie van de hartspier. De vele wetenschappelijke studies die tot nog toe zijn verricht en waarin een veelheid aan cardiovasculaire eindpunten is onderzocht, hebben echter geen uitsluitsel gegeven over het belang van de variant voor het ontstaan van hart- en vaatziekten. Ons onderzoek in de Leiden 85-plus Studie toonde aan dat de variant geen of minimale invloed heeft op het sterfterisico en dus waarschijnlijk een gering effect heeft op het fataal verlopen van hart- en vaatziekten (hoofdstuk 7). Hoewel dit niet uitsluit dat deze ACE variant een beperkte rol speelt in sommige aspecten van hart- en vaatziekten, lijkt het raadzaam om in gedetailleerder vervolgonderzoek prioriteit te geven aan genvarianten die wel in belangrijke mate bijdragen aan het fatale verloop van hart- en vaatziekten.

Ontsteking

Ontsteking speelt een centrale rol in het ontstaan van atherosclerose. De opeenhoping van cholesterol in de vaatwand wordt geïnitieerd door een ontstekingsreactie van de bekleding van de vaatwand (het endotheel). Bovendien bepaalt de ontsteking in een later stadium de stabiliteit van de atherosclerotische plaque. Een atherosclerotische plaque wordt omgeven door een laag van gladde spiercellen en bindweefsel. Ontsteking van een atherosclerotische plaque en daarmee samenhangende processen leiden er toe dat deze laag wordt afgebroken en de plaque eerder geneigd is te scheuren.

Tumour necrosis factor α (TNFα) stimuleert de ontsteking. Een promotervariant van het gen dat codeert voor TNFα, is geassocieerd met een verhoogde expressie van het gen. Deze genvariant bleek echter niet bij te dragen aan het risico op fatale hart- en vaatziekten of sterfte (hoofdstuk 5).

TNFα is niet alleen betrokken bij de ontstekingreactie, maar kan ook de respons van cellen op insuline verstoren. Hierdoor zijn cellen niet meer in staat om glucose adequaat uit het bloed op te nemen. In overeenstemming met dit effect van TNFα vonden wij dat een variant van het gen, die leidt tot een hogere TNFα expressie, geassocieerd was met een verhoogd risico op diabetes op hoge leeftijd (hoofdstuk 5).

Foliumzuurmetabolisme

Een variant van het methyleentetrahydrofolaatreductase gen (MTHFR) leidt tot een alanine-naar-valine aminozuursubstitutie, die een verstoring van het foliumzuur metabolisme veroorzaakt. Daarmee is deze variant mogelijk een risicofactor voor zowel hart- en vaatziekten als kanker. Personen die dubbel drager zijn van de variant (homozygoot zijn) hebben gemiddeld een 25% verhoogd niveau van homocysteïne in hun bloed, doordat in hun lichaam minder foliumzuur beschikbaar is om deze stof af te breken. Omdat patiënten met hart- en vaatziekten een hogere homocysteïnespiegel in hun bloed hebben dan gezonde mensen, wordt wel gedacht dat homocysteïne één van de oorzaken is van deze ziekten. Het onderliggende mechanisme is echter onduidelijk. Naast de relatie met hart- en vaatziekten, is het een verstoring van het foliumzuurmetabolisme mogelijk betrokken bij het ontstaan van kanker. Het foliumzuurmetabolisme is onder andere noodzakelijk voor de methylering van DNA. Een verminderde methylering van DNA zou het ontstaan van kanker kunnen

bevorderen, omdat methylering werkt als een rem op de expressie van genen die normale cellen kunnen veranderen in kankercellen. Niet-gemethyleerd DNA is bovendien gevoelig voor het optreden van mutaties. Het onderzoek in dit proefschrift wees uit dat op jonge leeftijd 12% van de mannen homozygoot was voor de variant, terwijl dit percentage was gedaald tot 4% boven de 85 jaar. Dit resultaat geeft aan dat homozygote mannen een substantieel grotere kans hadden om voor hun 85ste jaar te overlijden dan mannen die niet homozygoot waren voor de variant. Ook na de 85 jaar bleken de mannen die homozygoot waren voor de variant, eerder te overlijden dan andere mannen. Deze sterfte op hoge leeftijd leek vooral een gevolg te zijn van een hoger risico op kanker en niet op hart- en vaatziekten. Opvallend was dat de variant bij vrouwen niet gecorreleerd was met vroegtijdige sterfte (hoofdstuk 2).

Dit resultaat gaf aanleiding tot drie vragen. Is het effect op kankerrisico eveneens aanwezig in een onafhankelijke populatie, zodat uitgesloten kan worden dat het een toevalsbevinding was? Waarom was het risico op overlijden alleen onder mannen verhoogd? En: aan welke ziekten draagt de MTHFR variant bij voor het 85ste levensjaar? Daartoe hebben we het effect van de variant bestudeerd in de Zutphen Ouderen Studie. Dit is een studie onder 860 mannen woonachtig in Zutphen met een leeftijd variërend van 65 tot 85 jaar, die gedurende 10 jaar gevolgd zijn (hoofdstuk 8). Ook in deze studie was de MTHFR variant geassocieerd met een verhoogd risico op kanker. Dit bleek vooral het risico op prostaat-, dikke darm- en nier- en blaaskanker te betreffen, maar niet het risico op longkanker. Dus het antwoord op de eerste vraag luidt: de MTHFR variant lijkt inderdaad het risico van kanker te verhogen. Een van de redenen waarom de nadelige effecten van de variant vooral bij mannen tot uiting kwamen, kan zijn de variant het risico verhoogd om prostaatkanker te ontwikkelen. Daarbij komen ook dikke darm-, blaas- en nierkanker vaker voor bij mannen dan bij vrouwen. In deze leeftijdsgroep van 65 tot 85 jaar hadden mannen die homozygoot waren voor de variant, echter niet alleen een hoger risico om kanker te ontwikkelen, maar ook om hart- en vaatziekten te krijgen.

Onze bevindingen hebben mogelijk implicaties voor de preventie van kanker en hart- en vaatziekten. Eerdere studies gaven aan dat de inname van extra foliumzuur een deel van de nadelige effecten van de MTHFR variant kan opheffen. Misschien kan het extra ziekterisico van personen die homozygoot zijn voor de MTHFR variant, worden genormaliseerd met deze relatief eenvoudige en goedkope maatregel.

Conclusie

In dit proefschrift werd onderzocht of veel voorkomende varianten van acht genen de mortaliteit beïnvloeden in de algemene bevolking. Voor varianten van drie van deze genen bleek dit inderdaad het geval: APOE, dat betrokken is bij het cholesterolmetabolisme; PAI-1, de belangrijkste remmer van de fibrinolyse; en MTHFR, dat een sleutelrol speelt in het foliumzuurmetabolisme. Het nadelige effect van de MTHFR variant op ziekterisico kon worden bevestigd in een onafhankelijke studie, wat de waarde van de bevindingen onderstreept. Opvallend is dat sommige genvarianten het risico op meer dan één ziekte beïnvloeden. Een variant van het APOE gen verhoogt het risico op hart- en vaatziekten en dementie en een variant van het MTHFR gen

verhoogt het risico op hart- en vaatziekten en kanker. Nieuwe methoden van preventie die gebaseerd zijn op de uitkomsten van dit of soortgelijk onderzoek, zouden daarom een gunstig effect kunnen hebben op het risico op meer dan één ziekte die veel voorkomen op hogere leeftijd.

Appendix

Overview of studies investigating the association between common gene variants and various cardiovascular endpoints

This overview is based on Medline searches and is updated until September 2000. Its goal is to be as extensive as possible. Despite this, some studies may unintentionally not have been included.

Legend

Endpoints

MI = myocardial infarction
CAD = coronary artery disease
CHD = coronary heart disease
CVD = cerebrovascular disease
CS = carotid stenosis
Othr = other, includes PAD, peripheral arterial disease, and CAS, coronary artery spasm

Indications for study design, study size and outcome

+ indicates presence of association between gene variant and end point; – indicates absence of association; ± indicates trend.
1 and 2 indicate study design, 1 = prospective or population-based; 2 = hospital-based or cases and controls from different populations.
a, b and c indicate number of cases and controls, a: n≥300, b: 200≤n<300, c: 100≤n<200. Example: ab = more than 300 cases and between 200-300 controls.

Abbreviations genes

ACE = angiotensin I-converting enzyme *AGT* = angiotensinogen *AGRT1B* = angiotensin II type 1 receptor *ADRB3* = β_3 adrenergic receptor *APOA1* = apolipoprotein A1 *APOB* = apolipoprotein B *APOE* =apolipoprotein E *CD14* = monocyte differentiation antigen CD14 *CETP* = cholesteryl ester transfer protein *CX73* = connexin 37 *CYBA* = cytochrome β α subunit = $p22^{phox}$ *CYP11B2* = aldosterone synthase *ENDRA* = endothelin receptor A *F2* = prothrombin *F5* = factor V *F7* = factor VII *F13* = factor XIII *FGA* = fibrinogen α *FGB* = fibrinogen β *GP1BB* = platelet glycoprotein Ib *GSTM* = glutathione S-transferase μ *GSTT* = glutathione S-transferase τ *HFE* = haemochromatosis gene *HSPG2* = heparan sulfate proteoglycan *IL1A* = interleukin-1α *IL1B* = interleukin-1β *IL1RN* = interleukin-1 receptor antagonist *ITGA2* = platelet glycoprotein Iα *ITGB3* = platelet glycoprotein IIIα *LACI* = tissue factor pathway inhibitor *LIPC* = hepatic lipase *LPA* = apolipoprotein (a) *LPL* = lipoprotein lipase *MMP3* = matrix metalloproteinase 3 = stromelysin 1 *MMP9* = matrix metalloproteinase 9 = gelatinase B *MMP12* = matrix metalloproteinase 12 *MTHFR* = methylenetetrahydrofolate reductase *MTR* = methionine synthase *NOS3* = endothelial nitric oxide synthase *PAFAH* = platelet-activating factor acetylhydrolase *PAI1* = plasminogen activator inhibitor 1 *PON1* = paraoxonase *SELE* = E-selectin *SELP* = P-selectin *SERT* = serotonin transporter *TFA* = tissue factor *TGFB1* = transforming growth factor β *TNFA* = tumour necrosis factor α *TPA* = tissue plasminogen activator *WRN* = Werner helicase

Gene and variant	Endpoint					
	MI	CAD	CHD	CVD	CS	Othr
Lipid metabolism						
APOE						
ε2/ε3/ε4	$+^{1}_{1ba}$ $\pm^{2}_{2aa}$ $-^{3}_{2aa}$ $-^{4}_{2aa}$ $+^{5}_{2ba}$ $+^{6}_{2ca}$	$+^{7}_{1ac}$ $-^{8}_{2aa}$ $+^{9}_{2ba}$ $-^{10}_{2ca}$ $-^{11}_{2cc}$ $+^{12}_{2cc}$ $+^{13}_{2c}$	$+^{14}_{1aa}$ $+^{15}_{1ca}$ $+^{16}_{1ca}$ $-^{17}_{1ca}$ $-^{18}_{2cb}$	$-^{19}_{1ca}$		
APOB						
I/D	$-^{20}_{2aa}$ $\pm^{21}_{2aa}$ $-^{22}_{2cc}$	$-^{8}_{2aa}$ $-^{21}_{2aa}$ $+^{24}_{2bb}$ $+^{10}_{2ca}$ $-^{25}_{2cc}$ $-^{22}_{2cc}$				
XbaI	$\pm^{23}_{2ba}$ $+^{22}_{2cc}$	$-^{8}_{2aa}$ $-^{24}_{2bb}$ $-^{25}_{2cc}$ $-^{26}_{2cc}$ $+^{22}_{2cc}$	$-^{27}_{2cc}$ $-^{28}_{2cc}$			
EcoRI (Glu/Lys)		$-^{8}_{2aa}$ $-^{25}_{2cc}$ $+^{26}_{2cc}$	$+^{27}_{2cc}$ $+^{28}_{2cc}$			
APOA1						
-75G/A		$+^{30}_{2a}$	$+^{32}_{2cc}$			
PstI	$-^{29}_{2aa}$	$-^{8}_{2aa}$ $-^{31}_{2bc}$	$-^{33}_{2cc}$			
LPA						
isoforms	$-^{34}_{1ba}$ $-^{35}_{2aa}$		$\pm^{36}_{1cc}$ $+^{37}_{2aa}$ $+^{38}_{2cc}$		$-^{39}_{1cb}$	
CETP						
TaqI	$\pm^{40}_{2aa}$	$+^{41}_{1a}$			$\pm^{42}_{1a}$	
LPL						
HindIII	$+^{43}_{2aa}$	$+^{44}_{2ca}$ $-^{45}_{2a}$ $+^{46}_{2cc}$				
PvuII	$-^{43}_{2aa}$	$\pm^{44}_{2ca}$ $+^{45}_{2a}$ $-^{46}_{2cc}$	$+^{49}_{2cc}$			
Ser447Ter	$-^{43}_{2aa}$	$+^{47}_{1a}$ $-^{48}_{2a}$ $-^{46}_{2cc}$				
LIPC						
-480C/T		$\pm^{50}_{2aa}$ $-^{51}_{2bb}$				
HSPG2						
BamHI		$-^{52}_{2ab}$				
LDL oxidation						
PON1						
Met55/Leu		$-^{57}_{2ba}$ $+^{58}_{2cb}$	$-^{64}_{2cc}$	$-^{57}_{2ba}$	$+^{66}_{1a}$	
Gln192/Arg	$-^{53}_{2aa}$ $-^{54}_{2ca}$ $-^{55}_{2ca}$ $-^{56}_{2cc}$	$-^{54}_{2aa}$ $+^{57}_{2ba}$ $+^{59}_{2bb}$ $-^{60}_{2ac}$ $-^{61}_{2ac}$ $-^{62}_{2bb}$ $+^{63}_{2cb}$ $+^{56}_{2cc}$	$+^{65}_{2cc}$	$+^{57}_{2ba}$	$-^{66}_{1a}$	
PAFAH						
Val279/Phe			$+^{67}_{2aa}$			
Inflammation						
SELE						
Ser128/Arg		$+^{68}_{2cc}$				
SELP						
Thr715/Pro	$+^{69}_{2aa}$					
TNFA						
-308G/A	$-^{70}_{2aa}$	$-^{71}_{2ac}$ $-^{72}_{2a}$				
IL1B						
-511C/T		$-^{71}_{2ac}$				
IL1A						
-889C/T		$-^{71}_{2ac}$				

Gene and variant	Endpoint					
	MI	CAD	CHD	CVD	CS	Othr
Inflammation						
IL1RN						
VNTR		±[71] 2ac				
TGFB1						
-509C/T	−[73] 2aa	−[75] 2ab				
Leu10/Pro	−[73] 2aa +[74] 2bb	−[75] 2ab				
Arg25/Pro	+[73] 2aa					
CD14						
-260C/T	±[76] 2aa +[77] 2cc	−[78] 2aa				
Matrix degradation						
MMP3						
5A/6A	+[79] 2aa	+[80] 1a +[81] 1c				
MMP9						
-1562C/T	−[82] 2aa	±[82] 2cc				
MMP12						
-82A/G		−[83] 2a				
Homocysteine metabolism						
MTHFR						PAD
Ala222/Val	−[84] 1bb −[85] 2ab −[86] 2ba −[87] 2bb −[88] 2cc	−[89] 2aa −[90] 2aa −[91] 2aa −[76] 2ab −[92] 2bb −[86] 2ac	−[93] 1aa −[94] 1ba −[95] 1ca +[9] 2ba −[96] 2bb −[97] 2ca −[98] 2cc	+[99] 2ba −[100] 2ac	−[101] 1aa	−[102] 2ba
MTR						
Asp919/Gly		−[103] 2aa −[101] 2bb		−[101] 2bb		
Thrombus formation/lysis						
PAI1						
-675(4G/5G)	−[104] 1aa −[105] 2aa −[106] 2aa −[107] 2aa −[87] 2bb +[108] 2ca +[109] 2cc +[110] 2cc −[111] 2cc	+[105] 2aa −[112] 2ca −[110] 2a −[113] 2bc	+[114] 2cc			
TPA						
I/D (~-7351C/T)	−[115] 1aa +[116] 1cb −[117] 2aa					
F2						
20210G/A	−[118] 1aa −[119] 2aa −[120] 2aa −[121] 2aa ±[122] 2aa	−[119] 2aa ±[121] 2aa		−[118] 1ba		
F5						
Gln506/Arg	−[123] 1aa −[124] 2aa −[121] 2aa ±[122] 2aa −[125] 2ba +[126] 2ca	−[121] 2aa	−[127] 1aa		−[128] 1a	
F7						
Arg353/Gln	−[129] 2aa −[130] 2aa −[87] 2bb +[131] 2cb	−[132] 2a −[112] 2ca				
F13						
Val34/Leu	+[133] 2ca +[108] 2ca −[109] 2cc			+[134] 2aa		
TFA						
promoter haplotype	−[135] 2aa					

Gene and variant	Endpoint					
	MI	CAD	CHD	CVD	CS	Othr
Thrombus formation/lysis						
LACI						
Val264/Met	$-_{2aa}^{136}$					
FGA						
Thr312/Ala				$-_{2aa}^{137}$		
FBG						
-455G/A	$-_{2aa}^{138}$ $+_{2ca}^{139}$	$-_{1a}^{141}$ $-_{2a}^{132}$ $+_{2a}^{138}$ $+_{2ca}^{139}$ $-_{2ca}^{112}$	$-_{1aa}^{142}$			
BclI	$+_{2cc}^{140}$					
148C/T					$+_{1a}^{143}$	
ITGA2						
807C/T	$-_{2aa}^{144}$ $+_{2aa}^{145}$ $\pm_{2ba}^{146}$	$-_{2aa}^{145}$ $-_{2ab}^{146}$ $-_{2a}^{147}$				
GP1BB						
Thr145/Met	$-_{2bb}^{87}$	$-_{2cc}^{148}$	$+_{2cc}^{149}$	$+_{2cc}^{149}$		
ITGB3						
Leu33/Pro	$-_{1aa}^{150}$ $-_{2aa}^{151}$ $-_{2ba}^{152}$ $+_{2bb}^{87}$ $-_{2bb}^{153}$ $\pm_{2cb}^{154}$ $+_{2cc}^{109}$ $-_{2cc}^{155}$	$-_{2aa}^{152}$ $-_{2a}^{154}$ $-_{2ca}^{156}$ $-_{2cc}^{155}$		$-_{1aa}^{150}$ $-_{2aa}^{157}$ $-_{2ba}^{158}$ $-_{2ba}^{156}$	$-_{1a}^{159}$	
Vasoconstriction						
ACE						
intron16(I/D)	$-_{1aa}^{160}$ $-_{1aa}^{161}$ $-_{2aa}^{162}$ $-_{2aa}^{163}$ $-_{2aa}^{164}$ $+_{2aa}^{165}$ $-_{2aa}^{166}$ $-_{2ab}^{167}$ $+_{2ba}^{5}$ $+_{2bb}^{168}$ $-_{2ca}^{169}$ $+_{2ca}^{170}$ $+_{2ca}^{171}$ $-_{2bc}^{172}$ $+_{2cb}^{173}$ $+_{2bc}^{174}$ $-_{2cc}^{22}$ $+_{2cc}^{175}$	$-_{1aa}^{161}$ $-_{2aa}^{175}$ $-_{2aa}^{176}$ $-_{2aa}^{164}$ $\pm_{2aa}^{166}$ $-_{2a}^{169}$ $-_{2a}^{174}$ $-_{2bb}^{170}$ $+_{2bb}^{177}$ $-_{2bc}^{178}$ $+_{2bc}^{173}$ $-_{2bc}^{179}$ $+_{2ca}^{180}$ $+_{2cc}^{181}$ $-_{2cc}^{22}$	$-_{1aa}^{182}$ $\pm_{1aa}^{183}$ $-_{2aa}^{184}$ $-_{2cb}^{185}$	$-_{1aa}^{186}$	$-_{1a}^{187}$	
AGRT1B						
+1166A/C	$-_{2aa}^{188}$ $-_{2aa}^{189}$ $-_{2ab}^{167}$ $-_{2ba}^{190}$ $-_{2ca}^{169}$ $-_{2cc}^{109}$	$-_{2aa}^{188}$ $-_{2a}^{169}$ $-_{2bb}^{177}$	$-_{2cb}^{185}$			
-810T/A	$+_{2aa}^{191}$					
ATG						
Met235Thr	$-_{2aa}^{192}$ $-_{2aa}^{193}$ $-_{2ba}^{194}$ $-_{2ca}^{169}$ $-_{2cc}^{109}$	$-_{2aa}^{193}$ $-_{2a}^{169}$ $-_{2bc}^{178}$ $+_{2bb}^{177}$	$+_{1aa}^{182}$ $+_{2aa}^{184}$		$-_{1a}^{187}$	
(~-20A/C + ~-6G/A)						
CYP11B2						
-344T/C	$-_{2aa}^{195}$					
NOS3						CAS
Glu298/Asp	$+_{2ba}^{196}$ $+_{2ba}^{197}$ $+_{2bc}^{198}$	$+_{2bc}^{198}$		$-_{2bb}^{201}$ $-_{2ab}^{202}$		$+_{2cc}^{203}$
4a/b	$+_{2aa}^{199}$ $-_{2ba}^{197}$	$-_{1ca}^{95}$ $\pm_{2ac}^{200}$				
-786T/C						$+_{2cc}^{204}$
EDNRA						
-231A/G	$-_{2aa}^{205}$					

Gene and variant	Endpoint					
	MI	CAD	CHD	CVD	CS	Othr
Other						
ADRB3						
Trp64/Arg		$-_{2cb}^{206}$ $-_{2cc}^{207}$ $-_{2cc}^{208}$	$-_{1ba}^{209}$			
CYBA						
His72/Tyr		$+_{1c}^{210}$				
SERT						
I/D		$+_{2cb}^{212}$				
102T/C	$+_{2bb}^{211}$					
HFE						
Cys282/Tyr	$+_{1a}^{213}$ $+_{1a}^{214}$					
GSTM1						
null allele	$+_{2cc}^{215}$		$\pm_{1aa}^{216}$			
GSTT1						
null allele	$-_{2cc}^{215}$		$\pm_{1aa}^{216}$			
CX37						
Pro319/Ser		$+_{2bc}^{217}$				
WRN						
Cys1367Arg	$+_{2cc}^{218}$					

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Curriculum Vitae

Bas Heijmans werd geboren op 12 juni 1972 te Heemskerk. In 1990 behaalde hij het gymnasium β diploma aan het Augustinus College in Beverwijk. In hetzelfde jaar begon hij met de studie biologie aan de Universiteit Leiden. Tijdens zijn studie werd hij door dr. F.N.J. Droog geïntroduceerd in de moleculaire biologie (Instituut voor Moleculaire Plantkunde, Leiden) en door dr. P.E. Slagboom in onderzoek naar de genetica van complexe ziekten (TNO Preventie en Gezondheid, Leiden). In 1995 studeerde hij *cum laude* af met als specialisatie moleculaire biologie. Als assistent-in-opleiding verrichtte hij vanaf oktober 1995 onderzoek bij de afdeling Vaat- en Bindweefselonderzoek van TNO Preventie en Gezondheid (dr. P.E. Slagboom en prof. dr. C. Kluft) en sectie Gerontologie en Geriatrie van het Leids Universitair Medisch Centrum (prof. dr. R.G.J. Westendorp en prof. dr. D.L. Knook) in Leiden. De resultaten van dit promotieonderzoek staan beschreven in dit proefschrift. Sinds december 1999 is hij als postdoc werkzaam op een project dat is gericht op het vinden van genen die het hart- en vaatziekten risicoprofiel beïnvloeden. Het onderzoek betreft een samenwerking tussen de vakgroep Psychonomie van de Vrije Universiteit Amsterdam (prof. dr. D.I. Boomsma) en de afdelingen Anthropogenetica van de Universiteit Leiden (prof. dr. R.R. Frants en prof. dr. G.J.B. van Ommen) en Vaat- en Bindweefselonderzoek van TNO Preventie en Gezondheid (dr. P.E. Slagboom) en wordt gefinancierd door de Nederlandse Hartstichting en de *National Institutes of Health* uit de Verenigde Staten.

Nawoord

Het aantal mensen dat heeft bijgedragen aan de totstandkoming van dit proefschrift is groot. Het feit dat alleen mijn naam op de voorkant staat is dan ook een eer, maar doet afbreuk aan de dagelijkse werkelijkheid van wetenschappelijk onderzoek.

Het onderzoek dat beschreven staat in dit proefschrift, is voortgekomen uit de samenwerking tussen de afdeling Vaat- en Bindweefselonderzoek van TNO Preventie en Gezondheid (dr. P. Eline Slagboom en prof. dr. Kees Kluft) en de sectie Gerontologie en Geriatrie van het Leids Universitair Medisch Centrum (prof. dr. Rudi G.J. Westendorp en prof. dr. Dick L. Knook). Het onderzoek kon worden uitgevoerd dankzij een subsidie van de Nederlandse Hartstichting (project 94.047). Financiële ondersteuning van Zorg Onderzoek Nederland maakte het mogelijk een replicatiestudie te verrichten in de Zutphen Ouderen Studie (project 2100.0023).

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Bij de sectie Gerontologie en Geriatrie werken en werkten de mensen die de Leiden 85-plus Studie hebben opgezet en uitgevoerd. Met name de bijdrage van Jacobijn, die altijd geduld leek te hebben met mijn ongeduld, was van direct belang voor de totstandkoming van dit proefschrift. Dankzij de stimulerende en daarbij succesvolle samenwerking met Edith en Jolanda, die verbonden zijn aan het Centrum voor Chronische Ziekten Epidemiologie van het Rijksinstituut voor Volksgezondheid en Milieuhygiëne, was het mogelijk replicatiestudies uit te voeren. De donorassistenten van de Bloedbank Leidsenhage en vooral Marjo Dirven hebben het verzamelen van de controlegroep op de rails gezet en gehouden.

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