Genes, inflammation, and age-related diseases

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Chapter 1

General introduction

A dramatic increase in mean and maximal life span, coupled with a significant reduction in early mortality has lead to a large increase in the number of elderly people in modern societies. Progression of age is associated with a reduction of the response to environmental stimuli and, in general, is associated with an increased predisposition to illness and death. The high incidence of death due to infections, cardiovascular disease, and cancer underlies a common feature in these pathologies that is represented by dysregulation of both systemic and innate immunity (1). Ageing is accompanied by a chronic low-grade inflammation state clearly showed by 2-4-fold increase in serum levels of pro-inflammatory mediators (2). This pro-inflammatory state, interacting with the genetic background, potentially triggers the onset of age-related inflammatory diseases like atherosclerosis, dementia, and cancer (1). Genetic epidemiology is an important tool to investigate the association between innate immunity and age-related diseases.

Inflammation and cardiovascular disease

Atherosclerosis, a progressive disease characterized by the accumulation of lipid and fibrous elements in the large arteries, is the most important contributor of cardiovascular disease (3). Advances in medical science have established a fundamental role for inflammation in mediating all stages of atherosclerosis from initiation through progression (4-6). For example, macrophages and blood leucocytes, mediators of host defenses and inflammation, are found in the earliest lesions of atherosclerosis. Macrophage-derived foam cells and leucocytes drive lesion progression by secreting inflammatory stimuli (7).

Inflammatory stimuli, like the pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor-alpha (TNFα), are implicated in the development of atherosclerosis (6;8). IL-6 stimulates endothelial activation, vascular smooth muscle cell proliferation and leucocyte recruitment, all of which lead to plaque growth or instability. Major effects of TNFα on the cardiovascular system include increased expression of adhesion molecules, release of endothelial cytokines and nitric oxide, and enhanced vascular permeability. Another pro-inflammatory cytokine is lymphotoxin-alpha (LTA), also known as tumor necrosis factor beta (TNFβ), which activates a cytokine cascade

by inducing IL-1 (9;10). LTA is expressed in atherosclerotic lesions and induces the expression of a number of molecules involved in atherogenesis (11;12). Moreover, atherosclerotic lesions in LTA knock-out mice are significantly smaller compared to LTA wild-type mice (12).

Besides these pro-inflammatory cytokines, anti-inflammatory cytokines like IL-10 may also play a role in the development of atherosclerosis (13). In IL-10 knockout mice, the absence of IL-10 leads to a marked increase in susceptibility of atherosclerosis (14). Furthermore, after occlusion of the middle cerebral artery, brain infarcts in IL-10 knockout mice are 30% larger compared to wild-type mice (15). However, the contribution of IL-10 to the modulation of the atherosclerotic process in humans remains largely to be elucidated.

Inflammation and cognitive function

Inflammation plays also an important role in the development of cognitive decline and dementia in old age (16). There is abundant evidence that inflammatory mechanisms contribute to cognitive impairment via cytokine-mediated interactions (16). Animal models expressing high levels of pro-inflammatory cytokines in the brain suffer from neurodegeneration (17). Furthermore, up-regulation of pro-inflammatory cytokines in tissue cultures leads to microglial activation and neuronal damage (18) and moreover, several markers of inflammation have been found in and around senile plaques in the brain (19).

The interleukin-1 signaling pathway is likely to have a prominent role in the development of cognitive decline and dementia (20-23). For example, in rodents peripheral administration of interleukin-1beta (IL-1β) induces various cognitive-behavioral effects (20). Furthermore, expression of IL-1β is increased in patients with Alzheimer's disease (22). One of the possible mechanisms by which IL-1β acts on cognitive function is by binding to IL-1 type-1 receptors which are abundantly expressed in the hippocampus (21), the area of the brain that has a critical role in memory and learning.

It has been shown that patients with sporadic Alzheimer's disease have lower IL-10 serum levels compared to healthy controls (24;25). Various studies have also investigated the association between genetic variation in the IL-10 gene and Alzheimer's disease (24;26-30). The majority of the studies found that the prevalences of variant alleles of IL-10 promoter polymorphisms, determinants of low IL-10 production capacity, were increased in patients with Alzheimer's disease compared to healthy controls (24;26;27;30). This indicates that besides pro-inflammatory processes also anti-inflammatory processes play a role in cognitive function and dementia in old age.

Inflammation and cancer

Cancer is now generally accepted as an age-related disease. In fact, incidence and mortality rates of most cancers increase consistently with age up to 90 years. Inflammatory responses are also thought to be critical in many aspects of promoting the growth and spread of cancers (31). Various studies support the hypothesis that inflammatory stimuli, like the pro-inflammatory cytokines IL-6, IL-1β, and TNFα, are involved in cancer pathogenesis (32-35). Moreover, elevated levels of various cytokines, like IL-1, IL-6, TNFα, fibroblast growth factor (FGF), and transforming growth factor (TGF) have been found in blood, urine, and ascites of cancer patients, suggesting that these cytokines are involved in incidence and growth and spread of cancer (36).

A recent study showed that cell lines of Lewis lung carcinoma had an increased production of the pro-inflammatory cytokines IL-6 and TNFα through activation of the Toll-like receptor (TLR) family members TLR2 and TLR6 (37). Moreover, pro-inflammatory cytokines are also involved in promoting tumor cell adhesion in metastatic sites which then activate local normal cells to produce tumor growth factors (36). Distant-site metastases are the leading cause of cancer-associated mortality. Furthermore, animal studies have suggested a role for pro-inflammatory cytokines in the generation of cancer-associated cachexia, which is the most important cause of morbidity among cancer patients (33;38-40).

Genetic risk factors

Various studies have reported only moderate associations between inflammatory markers and cognitive decline (20;23;41). Therefore, systemic markers are unlikely to be useful as risk predictors for cognitive decline (42). On the contrary, genetic variation in inflammatory genes is more likely to be a good marker for risk prediction, especially since associations between genetic variation and cognitive function are assumed to be unconfounded (43). Moreover, uncertainty exists whether levels of cytokines are risk factors for cognitive decline or whether they are a consequence of cognitive decline. Functional polymorphisms determine the level of cytokine plasma levels, therefore genetic variation can be used as useful marker to overcome this problem of reverse causality.

The innate production capacity of inflammatory markers has been shown to be under tight genetic control. An extended twin study found that over 50% of the variance in production capacity of cytokines is explained by genetic factors (44). IL-1 receptor antagonist (IL-1Ra) and TNFα had the lowest heritability (53%), IL-6 and IL-10 had a heritability of 57 and 62% respectively, whereas IL-1β had the highest heritability (86%). The gene encoding for interleukin-1β-converting enzyme (ICE) is likely to be one of the main genes influencing IL-1β. ICE mediates the cleavage of the inactive precursor of IL-1β into the biologically active form (45). Inhibition of ICE decreases the age-related increase in IL-1β levels (45). Genetic variation in the ICE gene is likely to be functional since patients with the 5352AA genotype in the ICE gene have an increased risk of developing restenosis after percutaneous coronary intervention, a process where inflammation plays a major role (46). Genotypic variation in the IL-10 gene is associated with significantly lower IL-10 responsiveness upon stimulation with bacterial lipopolysaccharide (LPS) (47). Since genetic variation in the promoter region of the IL-10 gene influences the production levels of IL-10, this variation can also be related to cardiovascular disease and/or cognitive function.

Other studies have also shown that genetic variation in genes involved in inflammatory processes are associated with vascular disease, like myocardial infarction (MI) and stroke (11;48-52). For

example, reports have appeared on the association between the LTA C804A polymorphism and the severity of atherosclerosis in patients with coronary artery disease (11). This indicates that genetic variation in genes involved in inflammation can lead to an increased risk for cardiovascular disease.

Epigenetics and ageing

While the relationship between inflammation and ageing has been widely investigated, other possible mechanisms influencing the progress of ageing and age-related diseases are not well understood. One of these mechanisms is epigenetics, the study of heritable changes in gene expression that occurs without a change in the sequence of DNA (53-56). The best known epigenetic modifications are DNA methylation and post-transcriptional histone modifications, including methylation, acetylation, ubiquitylation and phosphorylation. An increased DNA methylation will lead to less gene expression, whereas increased histone acetylation will lead to more gene expression. Recently it has been shown that aged organisms have modified epigenetic features. The best understood epigenetic modification in relation to age-related diseases is that of DNA methylation in relation with cancer (53). In cancer cells it is well established that DNA methylation patterns are abnormal. More specifically, a global state of hypomethylation is observed, along with a hypermethylation state in many gene promoters. In various malignancies an increased promoter methylation of the adhesion molecule E-cadherin, an important tumor suppressor, is observed. As a result, the hypermethylation state leads to the loss of expression of E-cadherin resulting in a predisposition to develop cancer.

Moreover, epigenetic processes modulate gene expression patterns and have profound effects on the cellular repertoire of expressed genes. Therefore, epigenetic regulators involved in histone acetylating and deacetylating activities can play an important role in extracellular matrix formation, inflammation, and proliferation, processes involved in cardiovascular pathologies such as atherosclerosis and restenosis (57;58). Another argument that epigenetics could play a role in the pathology of cardiovascular disease comes from the Dutch Hunger Winter Study. Subjects who were prenatally exposed to a period of famine during World War II had 5.2% less DNA methylation

of the IGF2 gene compared to their unexposed sibling (59). Furthermore, subjects exposed to famine during gestation had a higher cumulative incidence of coronary artery disease and an earlier onset of coronary heart disease compared to unexposed subjects (60). These two findings together indicate that changes in epigenetic information can play a role in the pathology of complex diseases like cardiovascular disease. Hence, epigenetics can help to explain the relationship between an individual's genetic background, the environment, ageing, and disease. Therefore, active investigations continue into potential functional relationships between epigenetic changes and disease pathology of age-related diseases.

Mendelian Randomization

One of the aims of observational research is to identify causes of disease. However, observational research has had several high-profile failures when risk factors of disease that were identified in observational studies were later shown to be non-causal in randomised controlled trials. Reasons for such failures include confounding or disturbance by reverse causality. Genetic epidemiology can contribute to establishing the causal nature of environmentally modifiable risk factors, through the application of Mendelian randomization approaches (43;61;62). Or in other words, Mendelian randomization provides an alternative way of dealing with the problem of causal inference that is inherent to observational studies (63).

This new genetic epidemiological tool is based on Mendel's law that inheritance of one trait is independent of inheritance of other traits. This means that the association between a genetically determined phenotypic trait and a disease is not susceptible to reverse causality or confounding, provided that the presence of the genotype that causes the trait does not influence the subject's lifestyle or environment. This condition will usually be fulfilled as long as subjects are unaware of their genotype.

There are three key assumptions for a genetic variant to be met before they can be used in Mendelian randomization (63). First, the genetic variant is associated with the exposure of interest. Second, the genetic variant is unrelated to all confounding factors and third, there is no effect of the

genetic variant on the outcome or any other mediated effect other than through the exposure of interest. These assumptions are graphically illustrated in figure 1.

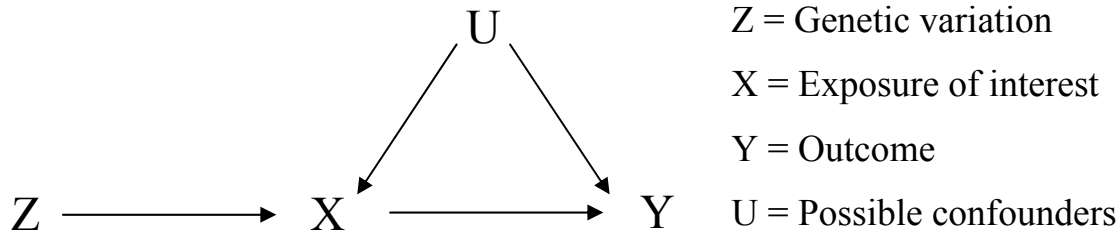

Figure 1: Graphical representation of the Mendelian randomization concept.

In 1986 Katan was the first to propose to investigate the causality of cholesterol in the relation with cancer by making use of genetic variation in the Apolipoprotein E (ApoE) gene (64). Observational studies had shown a relationship between low plasma cholesterol levels and an increased risk for cancer. However, randomized clinical trials with cholesterol lowering medications have not shown an increased risk for cancer (65). The results of the observational studies could be the result of confounding or reverse causality since cholesterol levels could also be lowered by the presence of latent tumours. The ApoE gene is known to affect plasma cholesterol levels, with rising cholesterol levels from isoform E2 to E3 to E4. Katan's idea was that individuals carrying the ApoE2 will naturally have lower plasma cholesterol levels from birth and that if naturally low cholesterol favours tumour growth, carriers of the ApoE2+ genotype should have an increased risk of cancer (64). This proposal constituted a prime example of what would later be named Mendelian randomization (66;67).

General objective

The general objective of this thesis was to investigate associations between variants in genes involved in inflammation and epigenetics and age-related diseased at old age to get more insights in the patho-physiological mechanisms involved in age-related diseases like cardiovascular disease,

dementia, and cancer. For all analyses we used data of the participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER).

The aim of PROSPER was to determine whether therapy with pravastatin, a cholesterol-lowering medication, would have a beneficial effect on cardiovascular disease risk in an elderly population. The PROSPER study was a multicenter, double-blind, placebo-controlled trial of pravastatin against placebo in 70-82 year old men and women with either pre-existing vascular disease or at elevated risk of such disease due to smoking, hypertension or diabetes. Between December 1997 and May 1999, subjects were screened and enrolled in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). A total number of 5804 subjects were randomly assigned to pravastatin or placebo. The trial concluded that pravastatin given for three years reduces the risk of coronary heart disease in the elderly. However, no beneficial effect of pravastatin was found on occurrence of stroke and cognitive performance. Therefore, cholesterol-lowering treatment with pravastatin is also recommended in the elderly for preventing coronary heart disease.

Aim of this thesis

First we present five studies investigating the association between genetic variation in inflammation related genes and age-related diseases like cardiovascular disease, cognitive decline, and cancer. In chapter 2 and 3 we assessed the relation between genetic variation in pro- and anti-inflammatory genes and cardiovascular disease. We first assessed the association between LTA and the risk of clinical stroke (chapter 2) and then the association between four promoter polymorphisms in the anti-inflammatory IL-10 gene and cardiovascular disease (chapter 3). In chapter 4 and 5 the results of the association between genetic variation in inflammatory genes and cognitive function are shown. We investigated the association between genetic variation in the ICE gene and cognitive function in chapter 4 and the relation between genetic variation in the anti-inflammatory IL-10 gene and cognitive function in chapter 5. In chapter 6 we describe the relation between pro-inflammatory cytokines, measured by systemic levels and innate production capacity, and cancer incidence and cancer death.

Second, we describe two studies investigating the relation between genetic variation in genes involved in epigenetics and age-related diseases. In chapter 7, we investigated the relation of the CREB gene, a histone acetyltransferase (HAT) involved in epigenetic control, with cognitive function in old age. Chapter 8 describes a study investigating the relation between the PCAF gene, a transcriptional co-activator with intrinsic HAT-activity, and cardiovascular outcomes in three different study populations, the PROSPER study, the WOSCOPS study and the GENDER study.

Third, we introduce an innovative approach to investigate the relation between a phenotype and outcome independent of confounding and reverse causality by using genetic variation, which is called Mendelian randomization. In chapter 9 we investigated whether low cholesterol is a risk factor for cancer by using the ApoE genotype, a typical example of a Mendelian randomization study. Finally, the main conclusions are summarized and discussed in chapter 10.

Reference List

(1) Licastro F, Candore G, Lio D, Porcellini E, Colonna-Romano G, Franceschi C, et al. Innate immunity and inflammation in ageing: a key for understanding age-related diseases. Immun Ageing 2005 May 18;2:8.

(2) Vasto S, Candore G, Balistreri CR, Caruso M, Colonna-Romano G, Grimaldi MP, et al. Inflammatory networks in ageing, age-related diseases and longevity. Mech Ageing Dev 2007 Jan;128(1):83-91.

(3) Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005 Apr 21;352(16):1685-95.

(4) Kaperonis EA, Liapis CD, Kakisis JD, Dimitroulis D, Papavassiliou VG. Inflammation and atherosclerosis. Eur J Vasc Endovasc Surg 2006 Apr;31(4):386-93.

(5) Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002 Mar 5;105(9):1135-43.

(6) Libby P. Inflammation in atherosclerosis. Nature 2002 Dec 19;420(6917):868-74.

(7) Packard RR, Libby P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. Clin Chem 2008 Jan;54(1):24-38.

(8) Ohsuzu F. The roles of cytokines, inflammation and immunity in vascular diseases. J Atheroscler Thromb 2004;11(6):313-21.

(9) Gray PW, Aggarwal BB, Benton CV, Bringman TS, Henzel WJ, Jarrett JA, et al. Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. Nature 1984 Dec 20;312(5996):721-4.

(10) McDevitt H, Munson S, Ettinger R, Wu A. Multiple roles for tumor necrosis factor-alpha and lymphotoxin alpha/beta in immunity and autoimmunity. Arthritis Res 2002;4 Suppl 3:S141-S152.

(11) Laxton R, Pearce E, Kyriakou T, Ye S. Association of the lymphotoxin-alpha gene Thr26Asn polymorphism with severity of coronary atherosclerosis. Genes Immun 2005 Sep;6(6):539-41.

(12) Schreyer SA, Vick CM, LeBoeuf RC. Loss of lymphotoxin-alpha but not tumor necrosis factor-alpha reduces atherosclerosis in mice. J Biol Chem 2002 Apr 5;277(14):12364-8.

(13) Mallat Z, Heymes C, Ohan J, Faggin E, Leseche G, Tedgui A. Expression of interleukin-10 in advanced human atherosclerotic plaques: relation to inducible nitric oxide synthase expression and cell death. Arterioscler Thromb Vasc Biol 1999 Mar;19(3):611-6.

(14) Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F, Bureau MF, et al. Protective role of interleukin-10 in atherosclerosis. Circ Res 1999 Oct 15;85(8):e17-e24.

(15) Grilli M, Barbieri I, Basudev H, Brusa R, Casati C, Lozza G, et al. Interleukin-10 modulates neuronal threshold of vulnerability to ischaemic damage. Eur J Neurosci 2000 Jul;12(7):2265-72.

(16) Wilson CJ, Finch CE, Cohen HJ. Cytokines and cognition--the case for a head-to-toe inflammatory paradigm. J Am Geriatr Soc 2002 Dec;50(12):2041-56.

(17) Campbell IL, Stalder AK, Chiang CS, Bellinger R, Heyser CJ, Steffensen S, et al. Transgenic models to assess the pathogenic actions of cytokines in the central nervous system. Mol Psychiatry 1997 Mar;2(2):125-9.

(18) Ho GJ, Drego R, Hakimian E, Masliah E. Mechanisms of cell signaling and inflammation in Alzheimer's disease. Curr Drug Targets Inflamm Allergy 2005 Apr;4(2):247-56.

(19) Eikelenboom P, Veerhuis R. The importance of inflammatory mechanisms for the development of Alzheimer's disease. Exp Gerontol 1999 Jun;34(3):453-61.

(20) Gibertini M, Newton C, Friedman H, Klein TW. Spatial learning impairment in mice infected with Legionella pneumophila or administered exogenous interleukin-1-beta. Brain Behav Immun 1995 Jun;9(2):113-28.

(21) Schneider H, Pitossi F, Balschun D, Wagner A, del Rey A, Besedovsky HO. A neuromodulatory role of interleukin-1beta in the hippocampus. Proc Natl Acad Sci U S A 1998 Jun 23;95(13):7778-83.

(22) Solfrizzi V, D'Introno A, Colacicco AM, Capurso C, Todarello O, Pellicani V, et al. Circulating biomarkers of cognitive decline and dementia. Clin Chim Acta 2006 Feb;364(1-2):91-112.

(23) Thomson LM, Sutherland RJ. Systemic administration of lipopolysaccharide and interleukin-1beta have different effects on memory consolidation. Brain Res Bull 2005 Sep 30;67(1-2):24-9.

(24) Arosio B, Trabattoni D, Galimberti L, Bucciarelli P, Fasano F, Calabresi C, et al. Interleukin-10 and interleukin-6 gene polymorphisms as risk factors for Alzheimer's disease. Neurobiol Aging 2004 Sep;25(8):1009-15.

(25) Remarque EJ, Bollen EL, Weverling-Rijnsburger AW, Laterveer JC, Blauw GJ, Westendorp RG. Patients with Alzheimer's disease display a pro-inflammatory phenotype. Exp Gerontol 2001 Jan;36(1):171-6.

(26) Lio D, Licastro F, Scola L, Chiappelli M, Grimaldi LM, Crivello A, et al. Interleukin-10 promoter polymorphism in sporadic Alzheimer's disease. Genes Immun 2003 Apr;4(3):234-8.

(27) Ma SL, Tang NL, Lam LC, Chiu HF. The association between promoter polymorphism of the interleukin-10 gene and Alzheimer's disease. Neurobiol Aging 2005 Jul;26(7):1005-10.

(28) Peila R, Launer LJ. Inflammation and dementia: epidemiologic evidence. Acta Neurol Scand Suppl 2006;185:102-6.

(29) Ramos EM, Lin MT, Larson EB, Maezawa I, Tseng LH, Edwards KL, et al. Tumor necrosis factor alpha and interleukin 10 promoter region polymorphisms and risk of late-onset Alzheimer disease. Arch Neurol 2006 Aug;63(8):1165-9.

(30) Scassellati C, Zanardini R, Squitti R, Bocchio-Chiavetto L, Bonvicini C, Binetti G, et al. Promoter haplotypes of interleukin-10 gene and sporadic Alzheimer's disease. Neurosci Lett 2004 Feb 12;356(2):119-22.

(31) Vasto S, Carruba G, Lio D, Colonna-Romano G, Di BD, Candore G, et al. Inflammation, ageing and cancer. Mech Ageing Dev 2009 Jan;130(1-2):40-5.

(32) Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G. Inflammation and cancer: how hot is the link? Biochem Pharmacol 2006 Nov 30;72(11):1605-21.

(33) de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer 2006 Jan;6(1):24-37.

(34) Dinarello CA. The paradox of pro-inflammatory cytokines in cancer. Cancer Metastasis Rev 2006 Sep;25(3):307-13.

(35) Tan TT, Coussens LM. Humoral immunity, inflammation and cancer. Curr Opin Immunol 2007 Apr;19(2):209-16.

(36) Dunlop RJ, Campbell CW. Cytokines and advanced cancer. J Pain Symptom Manage 2000 Sep;20(3):214-32.

(37) Kim S, Takahashi H, Lin WW, Descargues P, Grivennikov S, Kim Y, et al. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. Nature 2009 Jan 1;457(7225):102-6.

(38) Deans C, Wigmore SJ. Systemic inflammation, cachexia and prognosis in patients with cancer. Curr Opin Clin Nutr Metab Care 2005 May;8(3):265-9.

(39) Fearon KC, Barber MD, Falconer JS, McMillan DC, Ross JA, Preston T. Pancreatic cancer as a model: inflammatory mediators, acute-phase response, and cancer cachexia. World J Surg 1999 Jun;23(6):584-8.

(40) McNamara MJ, Alexander HR, Norton JA. Cytokines and their role in the pathophysiology of cancer cachexia. JPEN J Parenter Enteral Nutr 1992 Nov;16(6 Suppl):50S-5S.

(41) van Exel E, de Craen AJ, Remarque EJ, Gussekloo J, Houx P, Bootsma-van der Wiel A, et al. Interaction of atherosclerosis and inflammation in elderly subjects with poor cognitive function. Neurology 2003 Dec 23;61(12):1695-701.

(42) Schram MT, Euser SM, de Craen AJ, Witteman JC, Frolich M, Hofman A, et al. Systemic markers of inflammation and cognitive decline in old age. J Am Geriatr Soc 2007 May;55(5):708-16.

(43) Davey SG, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol 2003 Feb;32(1):1-22.

(44) de Craen AJ, Posthuma D, Remarque EJ, van den Biggelaar AH, Westendorp RG, Boomsma DI. Heritability estimates of innate immunity: an extended twin study. Genes Immun 2005 Mar;6(2):167-70.

(45) Gemma C, Fister M, Hudson C, Bickford PC. Improvement of memory for context by inhibition of caspase-1 in aged rats. Eur J Neurosci 2005 Oct;22(7):1751-6.

(46) Monraats PS, de Vries F, de Jong LW, Pons D, Sewgobind VD, Zwinderman AH, et al. Inflammation and apoptosis genes and the risk of restenosis after percutaneous coronary intervention. Pharmacogenet Genomics 2006 Oct;16(10):747-54.

(47) Westendorp RG, van Dunne FM, Kirkwood TB, Helmerhorst FM, Huizinga TW. Optimizing human fertility and survival. Nat Med 2001 Aug;7(8):873.

(48) Hagiwara N, Kitazono T, Kamouchi M, Kuroda J, Ago T, Hata J, et al. Polymorphisms in the Lymphotoxin Alpha Gene and the Risk of Ischemic Stroke in the Japanese Population. The Fukuoka Stroke Registry and the Hisayama Study. Cerebrovasc Dis 2008 Mar 17;25(5):417-22.

(49) Ozaki K, Tanaka T. Genome-wide association study to identify SNPs conferring risk of myocardial infarction and their functional analyses. Cell Mol Life Sci 2005 Aug;62(16):1804-13.

(50) Porto I, Leone AM, Crea F, Andreotti F. Inflammation, genetics, and ischemic heart disease: focus on the major histocompatibility complex (MHC) genes. Cytokine 2005 Mar 7;29(5):187-96.

(51) Szolnoki Z, Havasi V, Talian G, Bene J, Komlosi K, Somogyvari F, et al. Lymphotoxin-alpha gene 252G allelic variant is a risk factor for large-vessel-associated ischemic stroke. J Mol Neurosci 2005;27(2):205-11.

(52) Um JY, An NH, Kim HM. TNF-alpha and TNF-beta gene polymorphisms in cerebral infarction. J Mol Neurosci 2003;21(2):167-71.

(53) Fraga MF, Esteller M. Epigenetics and aging: the targets and the marks. Trends Genet 2007 Aug;23(8):413-8.

(54) Bjornsson HT, Fallin MD, Feinberg AP. An integrated epigenetic and genetic approach to common human disease. Trends Genet 2004 Aug;20(8):350-8.

(55) Feinberg AP. Epigenetics at the epicenter of modern medicine. JAMA 2008 Mar 19;299(11):1345-50.

(56) Kahn A, Fraga MF. Epigenetics and aging: status, challenges, and needs for the future. J Gerontol A Biol Sci Med Sci 2009 Feb;64(2):195-8.

(57) Pons D, de Vries FR, van den Elsen PJ, Heijmans BT, Quax PH, Jukema JW. Epigenetic histone acetylation modifiers in vascular remodelling: new targets for therapy in cardiovascular disease. Eur Heart J 2009 Feb;30(3):266-77.

(58) Turunen MP, Aavik E, Yla-Herttuala S. Epigenetics and atherosclerosis. Biochim Biophys Acta 2009 Sep;1790(9):886-91.

(59) Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A 2008 Nov 4;105(44):17046-9.

(60) Painter RC, de R, Sr., Bossuyt PM, Simmers TA, Osmond C, Barker DJ, et al. Early onset of coronary artery disease after prenatal exposure to the Dutch famine. Am J Clin Nutr 2006 Aug;84(2):322-7.

(61) Davey SG, Ebrahim S, Lewis S, Hansell AL, Palmer LJ, Burton PR. Genetic epidemiology and public health: hope, hype, and future prospects. Lancet 2005 Oct 22;366(9495):1484-98.

(62) Ebrahim S, Davey SG. Mendelian randomization: can genetic epidemiology help redress the failures of observational epidemiology? Hum Genet 2008 Feb;123(1):15-33.

(63) Sheehan NA, Didelez V, Burton PR, Tobin MD. Mendelian randomization and causal inference in observational epidemiology. PLoS Med 2008 Aug 26;5(8):e177.

(64) Katan MB. Apolipoprotein E isoforms, serum cholesterol, and cancer. Lancet 1986 Mar 1;1(8479):507-8.

(65) Kuoppala J, Lamminpaa A, Pukkala E. Statins and cancer: A systematic review and meta-analysis. Eur J Cancer 2008 Aug 14.

(66) Katan MB. Commentary: Mendelian Randomization, 18 years on. Int J Epidemiol 2004 Feb;33(1):10-1.

(67) Keavney B. Commentary: Katan's remarkable foresight: genes and causality 18 years on. Int J Epidemiol 2004 Feb;33(1):11-4.

Chapter 2

Lymphotoxin-alpha C804A polymorphism is a risk factor for stroke. The PROSPER study

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Abstract

Inflammation plays a prominent role in the development of atherosclerosis, which is the most important risk factor for vascular events. Lymphotoxin alpha (LTA) is a pro-inflammatory cytokine and is found to be expressed in atherosclerotic lesions. We investigated the association between the C804A polymorphism within the LTA gene and coronary and cerebrovascular events in 5804 participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER). The primary endpoint was the combined endpoint of death from coronary heart disease, non-fatal myocardial infarction, and clinical stroke. Secondary endpoints were the coronary and cerebrovascular components separately. All associations were assessed with a Cox-proportional hazards model adjusted for sex, age, pravastatin use, and country. Our overall analysis showed a significant association between the C804A polymorphism and the primary endpoint (p=0.03). After stratification for gender, this association was found only in males. Furthermore, we found that the association between the C804A polymorphism and the primary endpoint was mainly attributable to clinical strokes (p=0.02). The C804A polymorphism in the LTA gene associates with clinical stroke, especially in men. But further research is warranted to confirm our results.

Introduction

Inflammation plays a prominent role in the development of atherosclerosis, which is the most important risk factor for vascular events (1-3). Lymphotoxin-alpha (LTA), also known as tumor necrosis factor beta (TNFβ), is a pro-inflammatory cytokine which activates a cytokine cascade by inducing interleukin-1 (4;5). LTA is expressed in atherosclerotic lesions and induces the expression of a number of molecules involved in atherogenesis (6;7). Moreover, atherosclerotic lesions in LTA knock-out mice are significantly smaller compared to LTA wild-type mice (7).

Genetic variation in the LTA gene has been associated with vascular disease, like myocardial infarction (MI) and stroke (6;8-12). For example, Laxton *et al* have reported an association between the LTA C804A polymorphism and the severity of atherosclerosis in patients with coronary artery

disease (6). They found that carriers of the 804A variant had a higher risk for severe atherosclerosis. Furthermore, they found that only the male carriers had this higher risk.

Based on this evidence we hypothesized that genetic variation in the LTA gene is associated with vascular disease, especially in men. We assessed the association between the LTA C804A polymorphism and coronary and cerebrovascular events in participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER).

Methods

A detailed description of the protocol of the PROSPER study has been published elsewhere (13;14). Here a short outline is provided.

Participants

The PROSPER study was a prospective multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in elderly. Between December 1997 and May 1999, we screened and enrolled subjects in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. A total number of 5804 subjects were randomly assigned to pravastatin or placebo for an average 3.5-year intervention period. The primary endpoint in the study was the combined endpoint of death from coronary heart disease (CHD), non-fatal myocardial infarct (MI), and occurrence of clinical stroke, either fatal or non-fatal. When death occurred following a non-fatal stroke within a period of 28 days, it was regarded as a fatal stroke. Secondary endpoints were the separate coronary and cerebrovascular components of the primary endpoint. All endpoints were adjudicated by the study endpoint committee. More details about the diagnosis of the cerebrovascular and coronary events within the PROSPER study has been published elsewhere (13).

Genotyping

The single nucleotide polymorphism (SNP) C804A (rs1041981) in the LTA gene was selected based on its allele frequency and available literature. A genome wide scan showed two SNPs within the LTA gene that were associated with vascular disease (15). An additional study showed that the LTA C804A polymorphism is indeed functional and results in an amino-acid change T26N (8). The SNP was genotyped by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS), using the Sequenom MassARRAYtm methodology (Sequenom Inc, San Diego, CA, USA). Amplification reactions and parameters were based on the manufacturer's instructions. Genotyping of the LTA C804A polymorphism was successful in 5389 participants. The results of the remaining patients are missing due to lack of DNA or inconclusive genotyping.

Statistical analysis

The program Haploview (16) was used to estimate the allele frequency and to test the consistency of the genotype frequency at the SNP locus with Hardy-Weinberg equilibrium. Hazard ratios (HR) with 95% confidence intervals (CI) were calculated with Cox-proportional hazards model. Subjects who withdrew consent or deceased during the study were censored on the date they left the study. All analyses were adjusted for sex, age, pravastatin use, and country. All analyses were additionally sex-stratified performed. To assess whether the PROSPER study is large enough to gain statistical power in a sex-stratified analysis, we performed power calculations (Quanto software, http://hydra.usc.edu/gxe). Based on a total number of 124 cases with a fatal or non-fatal stroke in males (n=2617), we calculated that with a minor allele frequency (MAF) of 20% in a log-additive model, a baseline risk of fatal or non-fatal stroke of 4%, and a gene effect of 1.5, the statistical power to detect the association between the polymorphism and fatal or non-fatal stroke is 98% for a p-value of 5×10^{-2}.

The SPSS software (version 12.0.1, SPSS Inc, Chicago, Ill) was used for all statistical analyses. P-values lower than 0.05 were considered statistically significant.

Results

Genotyping of the LTA C804A polymorphism was successful for 5389 subjects, the results of the remaining subjects were missing because of insufficient DNA or incomplete genotyping (success rate 93.2%). Table 1 represents the baseline characteristics of all 5389 participants divided over categories of the C804A polymorphism. About 50% of the participants were male (N=2617) and the mean age of all subjects at study entry was 75.3 years. The mean follow-up time was 3.2 years (range 2.8-4.0) for participants who did not die or withdrew consent. There were no differences in baseline characteristics between genotype groups.

Table 1: Baseline characteristics of the participants of the Prosper study (N=5389).

	Lymphotoxin-alpha C804A		
	Wt/Wt (N=2102)	Wt/Var (N=2547)	Var/Var (N=740)
Continous variates (mean, SD)			
Age, (years)	75.4 (3.3)	75.3 (3.4)	75.2 (3.3)
Body Mass Index, (kg/m2)	26.8 (4.2)	26.9 (4.2)	26.7 (4.3)
Total cholesterol , (mmol/L)	5.7 (0.9)	5.7 (0.9)	5.6 (0.9)
LDL cholesterol, (mmol/L)	3.8 (0.8)	3.8 (0.8)	3.8 (0.8)
HDL cholesterol, (mmol/L)	1.3 (0.4)	1.3 (0.4)	1.3 (0.4)
Categorical variates (N, %)			
Female	1067 (51)	1317 (52)	388 (52)
Current smoker	547 (26)	716 (28)	193 (26)
History of diabetes	219 (10)	270 (11)	93 (13)
History of hypertension	1327 (63)	1550 (61)	440 (60)
History of angina	580 (28)	666 (26)	194 (26)
History of claudication	129 (6)	187 (7)	47 (6)
History of myocardial infarction	265 (13)	366 (14)	88 (12)
History of vascular disease	938 (45)	1127 (44)	313 (42)
History of stroke or TIA	253 (12)	279 (11)	75 (10)

The major allele frequency of the C804A polymorphism was 63% in all participants. The C804A polymorphism showed no significant deviation from Hardy-Weinberg equilibrium ($p=0.77$). The genotype frequencies between the three countries differed significantly ($p<0.01$, data not shown), for Scotland the major allele frequency was 62%, for Ireland 61% and for the Netherlands 66%. Therefore all analyses were adjusted for country to control for population stratification.

Figure 1 shows the association between the C804A polymorphism and the primary endpoint. In the overall analysis a significant relation with the primary endpoint was found (p=0.03). The significant association of the overall analysis was mainly due to homozygous carriers of the variant (HR 1.27, 95%CI 1.03-1.55). Furthermore, after stratification for gender, the relation with the primary endpoint was especially present in males (HR 1.36, 95%CI 1.04-1.77) and not in females (HR 1.15, 95%CI 0.84-1.58), although the interaction term for gender with genotype was not significant (p=0.60).

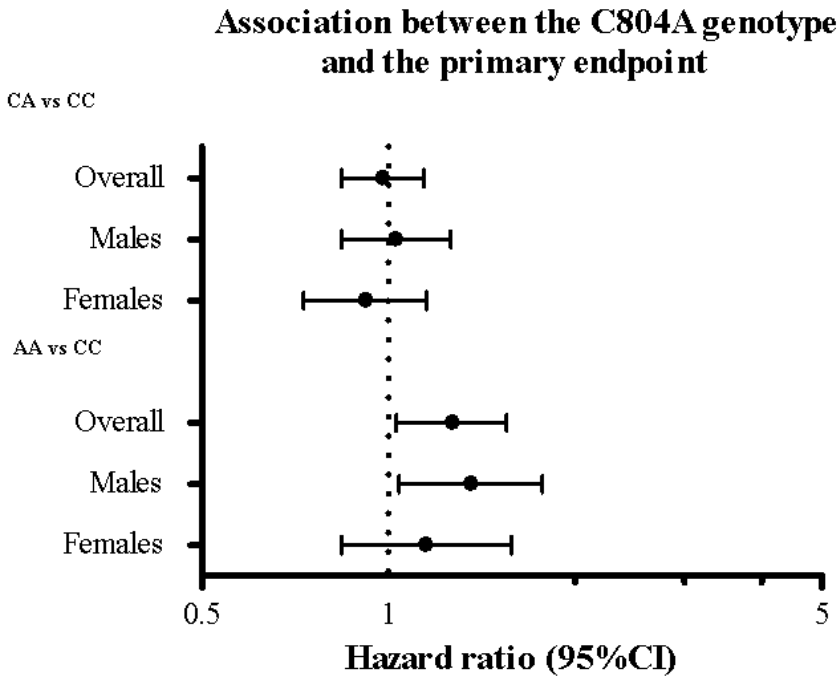

Figure 1: Association between the lymphotoxin-alpha C804A genotype and the primary endpoint in the participants of the Prosper study (n=5389).
The primary endpoint included coronary heart disease death, non-fatal myocardial infarct, and fatal or non-fatal stroke. In the overall group a significant association between the C804A genotype and primary endpoint was found (p=0.03), namely because an increased risk for the primary endpoint in males (HR 1.36, 95%CI 1.04-1.77).

We assessed the association of the C804A variant with the coronary and cerebrovascular endpoints separately. The association with the primary endpoint in men was mainly attributable to occurrence of clinical strokes and not to coronary events (figure 2). The increased risk for clinical stroke for the heterozygous carriers was 1.43 (95%CI 0.95-2.15) and for the homozygous male carries 2.07

(95%CI 1.24-3.44) (p-trend=0.02). In women, there was no significant association for clinical stroke for both the heterozygous carriers (HR 0.94, 95%CI 0.63-1.41) and the homozygous carries (HR 1.47, 95%CI 0.89-2.44).

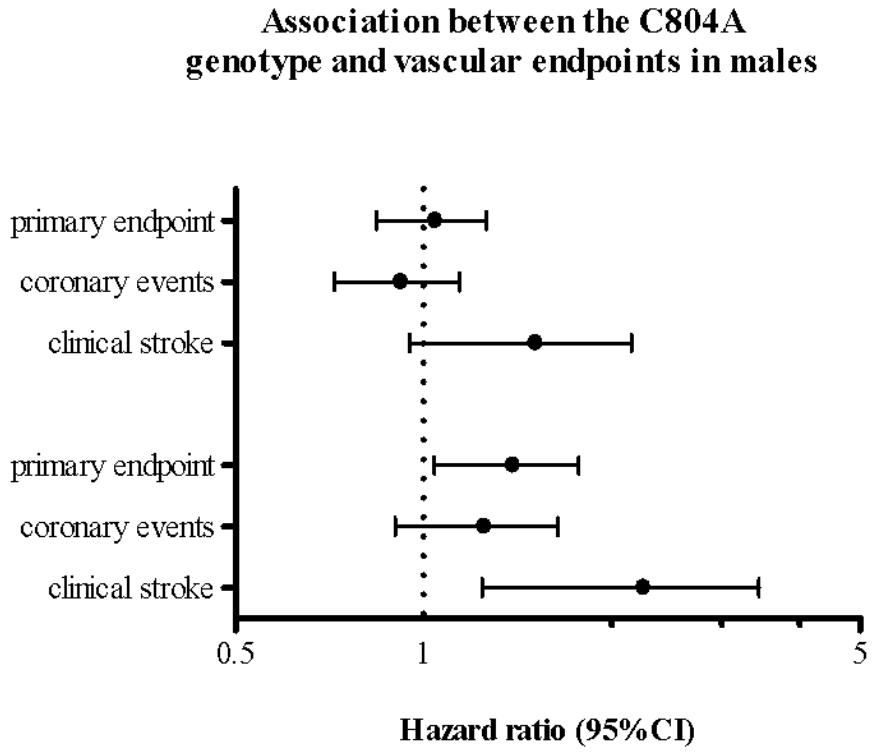

Figure 2: Association between the lymphotoxin-alpha C804A genotype and vascular endpoints in male participants (N=2617).
The primary endpoint included coronary heart disease death, non-fatal myocardial infarct, and fatal or non-fatal stroke. Coronary events are coronary heart disease death and non-fatal myocardial infarct. Clinical stroke consists of fatal or non-fatal stroke. The association in males with the primary endpoint is namely due to an increased risk of clinical stroke (p=0.02). The increased risk for clinical stroke for the heterozygous carriers was 1.43 (95%CI 0.95-2.15) and for the homozygous male carries 2.07 (95%CI 1.24-3.44).

Discussion

We assessed the association between the C804A polymorphism in the LTA gene and vascular events in an elderly population at risk for vascular disease. Our results indicate that carriers of the 804A allele have an increased risk for the primary study endpoint consisting of coronary events and clinical strokes. After stratification for gender, this association was only significant in men.

Furthermore, we found that the association between the C804A polymorphism and the primary endpoint in males was mainly attributable to incident strokes.

Although we found no statistically significant interaction with gender, the association between the C804A polymorphism and clinical strokes was only significant in men. Such a sex-specific effect has been reported previously (6). Men who were homozygous for the 804A allele were more likely to develop atherosclerosis than homozygous females. This finding is in line with our results and fits well within the widely recognized difference in susceptibility and severity of atherosclerosis between men and women. Men have a higher predisposition to atherosclerosis compared to females (17). Likewise, several other genes, like apolipoprotein E, have been shown to have gender specific effects on cardiovascular outcomes (18;19). However, further research is necessary to confirm our results.

Three studies have previously investigated the association between the LTA gene and the susceptibility for stroke (9;10;12). The study of Hagiwara *et al* found no higher frequency of the LTA C804A polymorphism in stroke patients (12). Um *et al* found an increase of the homozygous 252G allele in subjects with cerebral infarction compared to controls (9). Szolnoki *et al* also found that the homozygous LTA allele with the 252G and 804A SNPs is more frequent in stroke patients than in controls (10). These studies combined with our findings, indicate that carriers of the variant allele are indeed at a higher risk for the development of clinical strokes.

We do not have information about the separate ischemic and hemorrhagic strokes. In our study both types of strokes were combined into one clinical endpoint. Because we know from previous studies in elderly populations that approximately 80 percent of all strokes is attributable to ischemic events (20;21), the association between the C804A polymorphism and clinical stroke is probably driven by an association between the polymorphism and ischemic stroke. If there is no association with the polymorphism and hemorrhagic stroke, then the association we found is an underestimation of the true relative risk for ischemic stroke.

The whole LTA gene is in strong linkage disequilibrium, therefore the 252G allele naturally coexists with the 804A allele (22). Ozaki *et al* investigated the functionality of the A252G and C804A SNPs in the LTA gene (15).The C804A polymorphism causes an amino-acid change from threonine (T) to asparagine (N) at codon 26. They found that the variant protein 26N is associated with a two-fold increase in the induction of cell-adhesion molecules in vascular smooth muscle cells (15). Adhesion molecules are implicated in cardiovascular disease because elevated levels have been observed in atherosclerotic lesions (23;24). This might explain the association of the polymorphisms in the LTA gene and the increased risk for incident stroke.

In our study we found no association between the LTA polymorphism and myocardial infarction (MI). A genome-wide association study identified two functional polymorphisms in the LTA gene associated with MI (A252G and C804A) (8). A case-control association study by Ozaki *et al* found that subjects homozygous for the mutant allele (804AA) had an almost two-fold higher risk for MI (15). However, three observational studies did not find any association between the LTA polymorphisms and myocardial infarction (22;25;25;26). Moreover, a meta-analysis of six studies investigating this association found no significant result (22). The association between the LTA gene and incident stroke has not been replicated recently. Further research into this association is warranted before we can draw definite conclusions from our results.

That we found an association between the LTA C804A genotype and incident stroke and not with coronary events is understandable based on available literature (27). Recently, Vanderlaan *et al* suggested that the variation of lesion development at different vascular beds is sensitive to various parameters (28). For example, hypertension is one of the main risk factors for atherosclerosis in the carotid arteries and for incident stroke whereas smoking is a stronger risk factor for coronary atherosclerosis (27). This indicates that cerebrovascular disease has other risk factors than coronary disease, which also suggests a different genetic background. LTA 804AA carriers could therefore have an increased risk for incident stroke and not for coronary events.

A possible weakness of our study is that we have measured only one SNP in the LTA gene. But because the SNPs of the LTA gene are in strong linkage disequilibrium, information of one SNP is sufficient for analyses. Moreover, we have an enrichment of the variant allele in our study population compared to European populations reported in the NCBI database (www.ncbi.nlm.nih.gov). However, this does not affect the internal validity.

The strength of our study is that it is a prospective study which is not affected by population stratification (29). Because the genotype frequencies differed in the three countries we performed a stratified analysis for each country. This analysis showed consistent but not significant results, because of lack of statistical power. Another strength is our population size. We had sufficient cases of incident stroke to reach a high power for statistical analyses. Furthermore, all participants were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes, which makes this study population suitable for investigating coronary and cerebrovascular diseases.

In conclusion, we found an association of the C804A polymorphism in the LTA gene with the primary endpoint, which seems primarily due to an association in men. After separating the coronary and cerebrovascular events, we found that the association with the primary endpoint and the C804A variant was mainly attributable to clinical stroke. This study is a further argument that the LTA gene is associated with cerebrovascular disease, especially in males, but further research is warranted.

Acknowledgements

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Reference List

(1) Kaperonis EA, Liapis CD, Kakisis JD, Dimitroulis D, Papavassiliou VG. Inflammation and atherosclerosis. Eur J Vasc Endovasc Surg 2006 Apr;31(4):386-93.

(2) Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002 Mar 5;105(9):1135-43.

(3) Libby P. Inflammation in atherosclerosis. Nature 2002 Dec 19;420(6917):868-74.

(4) Gray PW, Aggarwal BB, Benton CV, Bringman TS, Henzel WJ, Jarrett JA, et al. Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. Nature 1984 Dec 20;312(5996):721-4.

(5) McDevitt H, Munson S, Ettinger R, Wu A. Multiple roles for tumor necrosis factor-alpha and lymphotoxin alpha/beta in immunity and autoimmunity. Arthritis Res 2002;4 Suppl 3:S141-S152.

(6) Laxton R, Pearce E, Kyriakou T, Ye S. Association of the lymphotoxin-alpha gene Thr26Asn polymorphism with severity of coronary atherosclerosis. Genes Immun 2005 Sep;6(6):539-41.

(7) Schreyer SA, Vick CM, LeBoeuf RC. Loss of lymphotoxin-alpha but not tumor necrosis factor-alpha reduces atherosclerosis in mice. J Biol Chem 2002 Apr 5;277(14):12364-8.

(8) Ozaki K, Tanaka T. Genome-wide association study to identify SNPs conferring risk of myocardial infarction and their functional analyses. Cell Mol Life Sci 2005 Aug;62(16):1804-13.

(9) Um JY, An NH, Kim HM. TNF-alpha and TNF-beta gene polymorphisms in cerebral infarction. J Mol Neurosci 2003;21(2):167-71.

(10) Szolnoki Z, Havasi V, Talian G, Bene J, Komlosi K, Somogyvari F, et al. Lymphotoxin-alpha gene 252G allelic variant is a risk factor for large-vessel-associated ischemic stroke. J Mol Neurosci 2005;27(2):205-11.

(11) Porto I, Leone AM, Crea F, Andreotti F. Inflammation, genetics, and ischemic heart disease: focus on the major histocompatibility complex (MHC) genes. Cytokine 2005 Mar 7;29(5):187-96.

(12) Hagiwara N, Kitazono T, Kamouchi M, Kuroda J, Ago T, Hata J, et al. Polymorphisms in the Lymphotoxin Alpha Gene and the Risk of Ischemic Stroke in the Japanese Population. The Fukuoka Stroke Registry and the Hisayama Study. Cerebrovasc Dis 2008 Mar 17;25(5):417-22.

(13) Shepherd J, Blauw GJ, Murphy MB, Cobbe SM, Bollen EL, Buckley BM, et al. The design of a prospective study of Pravastatin in the Elderly at Risk (PROSPER). PROSPER Study Group. PROspective Study of Pravastatin in the Elderly at Risk. Am J Cardiol 1999 Nov 15;84(10):1192-7.

(14) Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Cobbe SM, et al. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet 2002 Nov 23;360(9346):1623-30.

(15) Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T, et al. Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. Nat Genet 2002 Dec;32(4):650-4.

(16) Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005 Jan 15;21(2):263-5.

(17) Joakimsen O, Bonaa KH, Stensland-Bugge E, Jacobsen BK. Age and sex differences in the distribution and ultrasound morphology of carotid atherosclerosis: the Tromso Study. Arterioscler Thromb Vasc Biol 1999 Dec;19(12):3007-13.

(18) Desvarieux M, Schwahn C, Volzke H, Demmer RT, Ludemann J, Kessler C, et al. Gender differences in the relationship between periodontal disease, tooth loss, and atherosclerosis. Stroke 2004 Sep;35(9):2029-35.

(19) Reilly SL, Ferrell RE, Sing CF. The gender-specific apolipoprotein E genotype influence on the distribution of plasma lipids and apolipoproteins in the population of Rochester, MN. III. Correlations and covariances. Am J Hum Genet 1994 Nov;55(5):1001-18.

(20) Melcon CM, Melcon MO. Prevalence of stroke in an Argentine community. Neuroepidemiology 2006;27(2):81-8.

(21) Sagui E, M'Baye PS, Dubecq C, Ba FK, Niang A, Gning S, et al. Ischemic and hemorrhagic strokes in Dakar, Senegal: a hospital-based study. Stroke 2005 Sep;36(9):1844-7.

(22) Clarke R, Xu P, Bennett D, Lewington S, Zondervan K, Parish S, et al. Lymphotoxin-alpha gene and risk of myocardial infarction in 6,928 cases and 2,712 controls in the ISIS case-control study. PLoS Genet 2006 Jul;2(7):e107.

(23) Belch JJ, Shaw JW, Kirk G, McLaren M, Robb R, Maple C, et al. The white blood cell adhesion molecule E-selectin predicts restenosis in patients with intermittent claudication undergoing percutaneous transluminal angioplasty. Circulation 1997 Apr 15;95(8):2027-31.

(24) Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM, Jr., et al. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. Circulation 1997 Dec 16;96(12):4219-25.

(25) Kimura A, Takahashi M, Choi BY, Bae SW, Hohta S, Sasaoka T, et al. Lack of association between LTA and LGALS2 polymorphisms and myocardial infarction in Japanese and Korean populations. Tissue Antigens 2007 Mar;69(3):265-9.

(26) Sedlacek K, Neureuther K, Mueller JC, Stark K, Fischer M, Baessler A, et al. Lymphotoxin-alpha and galectin-2 SNPs are not associated with myocardial infarction in two different German populations. J Mol Med 2007 Sep;85(9):997-1004.

(27) Caprie Study group. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. Lancet 1996 Nov 16;348(9038):1329-39.

(28) VanderLaan PA, Reardon CA, Getz GS. Site specificity of atherosclerosis: site-selective responses to atherosclerotic modulators. Arterioscler Thromb Vasc Biol 2004 Jan;24(1):12-22.

(29) Beaty TH, Fallin MD, Hetmanski JB, McIntosh I, Chong SS, Ingersoll R, et al. Haplotype diversity in 11 candidate genes across four populations. Genetics 2005 Sep;171(1):259-67.

Chapter 3

Genetic variation in the interleukin-10 gene promoter and risk of coronary and cerebrovascular events: the PROSPER study.

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Abstract

Pro-inflammatory cytokines, like interleukin (IL)-6 and tumor necrosis factor-alpha, are implicated in the development of atherosclerosis. The role of anti-inflammatory cytokines, like IL-10, is largely unknown. We investigated the association of four single nucleotide polymorphisms (SNPs) in the promoter region of the IL-10 gene, (4259AG, -1082GA, -592CA and -2849GA), with coronary and cerebrovascular disease in participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) trial. All associations were assessed with Cox-proportional hazards models adjusted for sex, age, pravastatin use, and country. Haplotype analysis of the four SNPs showed a significant association between haplotype 4 (containing the -592A variant allele) and risk of coronary events (p=0.019). Moreover, analysis of separate SNPs found a significant association between -2849AA carriers with incident stroke (HR (95%CI): 1.50 (1.04-2.17), p-value = 0.02). Our study suggests that not only pro-inflammatory processes contribute to atherosclerosis, but that also anti-inflammatory cytokines may play an important role.

Introduction

Inflammatory stimuli, like the pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor-alpha (TNFα), are implicated in the development of atherosclerosis (1;2). Besides these pro-inflammatory cytokines, anti-inflammatory cytokines like IL-10 may also play a role in the development of atherosclerosis. In IL-10 knockout mice, the absence of IL-10 leads to a marked increase in susceptibility of atherosclerosis (3). Furthermore, after occlusion of the middle cerebral artery, brain infarcts in IL-10 knockout mice are 30% larger as in wild-type mice (4). These preclinical results indicate a possible role for IL-10 in the atherosclerotic process. However, the contribution of IL-10 to the modulation of the atherosclerotic process in humans remains largely to be elucidated.

IL-10 production levels are under tight genetic control. An extended twin study found that approximately two-thirds of the variance in production level of IL-10 is genetically determined (5).

Moreover, we have previously reported that genotypic variation in the IL-10 gene is associated with significantly lower IL-10 responsiveness upon stimulation with bacterial lipopolysaccharide (LPS)(6).

We performed a genetic association study of four IL-10 promoter SNPs (4259AG, -1082GA, -592CA, and -2849GA) with coronary and cerebrovascular events in participants at risk for vascular disease.

Methods

Study participants come from the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) trial. A detailed description of the protocol and results of the study has been published elsewhere (7). Here a short outline is provided.

Participants

PROSPER was a prospective multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in elderly individuals. Between December 1997 and May 1999, we screened and enrolled subjects in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. A total number of 5804 subjects were randomly assigned to pravastatin or placebo. The primary endpoint was the combined endpoint of fatal coronary heart disease (CHD), non-fatal myocardial infarct (MI), and occurrence of clinical stroke, either fatal or non-fatal. Secondary endpoints were the separate coronary and cerebrovascular components of the primary endpoint. All endpoints were adjudicated by a study endpoint committee.

Genotyping

We selected four SNPs in the promoter region of the IL-10 gene, 4259AG (rs3024498), -1082GA (rs1800896), -592CA (rs1800872), and -2849GA (rs6703630) based on the frequency of the minor

allele and possible functionality. All polymorphisms were genotyped by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS), using the Sequenom MassARRAYtm methodology (Sequenom Inc, San Diego, CA, USA). Amplification reactions and parameters were based on the manufacturer's instructions.

Statistical analysis

The program Haploview (8) was used to estimate allele frequencies, test the consistency of genotype frequencies at each SNP locus with Hardy-Weinberg equilibrium, and estimate and plot pairwise linkage disequilibrium (LD) between the SNPs examined. Haplotypes and haplotype frequencies were calculated using SNPHAP software (http://www-gene.cimr.cam.ac.uk/clayton/software). Haplotypes with a frequency of less than 5 % were combined and included in all analyses, without reporting the results. The posterior probabilities of pairs of haplotypes per subject as estimated by the SNPHAP were used as weights in all analyses. The haplotype analysis approach used in this study assumes an additive effect of the haplotypes, and details of this approach have been described elsewhere (9). Hazard ratios with 95% confidence intervals (CI) were calculated using a Cox-proportional hazards model. All analyses were adjusted for sex, age, pravastatin use, and country. The analyses were performed with STATA statistical software, version 9.0 (StataCorp LP, Texas, USA).

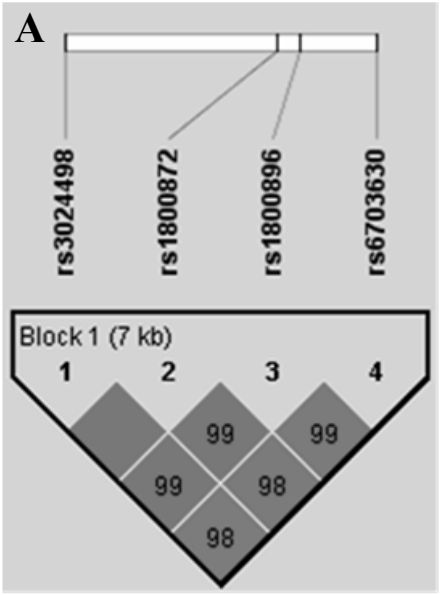
Results

The mean age of the participants was 75.3 years and approximately 50% were female (table 1). There were significant differences in minor allele frequencies between the countries (p-value Chi-square < 0.01, data not shown). The variants 4259G, -1082A and -2849A were more common in the Irish subjects compared with the subjects from Scotland and the Netherlands. Therefore, all analyses were adjusted for country. Mean follow-up of study subjects was 3.2 years (range 2.8-4.0).

Table 1: Baseline characteristics of the participants of PROSPER per country.

	Scotland (N=2520)	Ireland (N=2184)	The Netherlands (N=1100)
Continous variates (mean, SD)			
Age (years)	75.3 (3.4)	75.5 (3.3)	75.1 (3.3)
Body Mass index, (kg/m2)	26.7 (4.2)	27.0 (4.4)	26.7 (3.8)
Total cholesterol, (mmol/L)	5.7 (1.0)	5.6 (0.9)	5.8 (0.9)
LDL cholesterol, (mmol/L)	3.8 (0.8)	3.7 (0.8)	3.9 (0.8)
HDL cholesterol, (mmol/L)	1.3 (0.4)	1.3 (0.4)	1.3 (0.3)
Categorical variates (n, %)			
Female	1283 (51)	1197 (55)	520 (47)
Current smoker	708 (28)	583 (27)	267 (24)
History of diabetes	213 (9)	225 (10)	185 (17)
History of hypertension	1446 (57)	1441 (66)	705 (64)
History of angina	811 (32)	523 (24)	225 (21)
History of claudication	229 (9)	114 (5)	47 (4)
History of myocardial infarction	379 (15)	258 (12)	139 (13)
History of vascular disease	1239 (49)	849 (39)	477 (43)
History of stroke or TIA	265 (11)	222 (10)	162 (15)
Genotype, minor allele frequency (%)			
IL-10 4259AG	30	33	28
IL-10 -1082GA	51	55	50
IL-10 -592CA	21	20	21
IL-10 -2849GA	30	33	29

Genotyping of the four IL-10 polymorphisms was complete for 5786 subjects. All four SNPs were in Hardy-Weinberg equilibrium (all $p>0.05$). The four SNPs were in strong linkage disequilibrium (LD) and occurred together in one haploblock (figure 1). Six haplotypes were found in our study population (figure 1B). The four haplotypes with a frequency above 5% were included in analyses. We used haplotype2, with no variants present, as reference haplotype. Haplotype1, the most frequent, had three variant alleles, 4259G, -1082A and -2849A. Haplotype3 carried the -1082A variant and haplotype4 the-592A variant.



B

	4259AG	-592CA	-1082GA	-2849GA	Haplotype frequency
H1	2	1	2	2	0.304
H2	1	1	1	1	0.269
H3	1	1	2	1	0.209
H4	1	2	1	1	0.207
H5	1	1	2	2	0.009
H6	2	1	2	1	0.003

Figure 1: Haplotype information.

Figure A shows the linkage disequilibrium (LD) between the single nucleotide polymorphisms (SNPs) examined. All SNPs are in LD and occur together in one haploblock. Figure B shows the haplotype frequencies. Only the first four haplotypes (frequency> 5%) were included in the analyses.

Haplotype1 was associated with an increased risk for the primary endpoint compared to haplotype2 (HR (95%CI) 1.14 (1.01-1.29), p= 0.035) (table 2). Carriers of haplotype4, with the -592A variant allele, also had a significantly increased risk for the primary endpoint (HR (95%CI) 1.19 (1.04-1.36), p=0.012). To determine whether the significant haplotype associations with the primary endpoint were attributable to coronary events, strokes, or both, we subdivided the primary endpoint into coronary events and strokes. The significant association with haplotype4 was due to a significant relation with coronary events (HR (95%CI) 1.21 (1.03-1.41), p=0.019). The significant association with haplotype1 was not clearly due to coronary events or strokes (HR (95%CI) 1.13 (0.98-1.30), p=0.082 and HR (95%CI) 1.22 (0.96-1.54), p=0.097 respectively).

Therefore, we performed a single SNP analysis with the three SNPs present in haplotype1, 4259AG, -1082GA and -2849GA, to assess the association with coronary events or strokes. No consistent associations were found between the IL-10 4259AG and the -1082GA and any of the endpoints. The

IL-10 -2849AA genotype showed an increased risk of strokes (HR (95%CI): 1.50 (1.04-2.17), p-value = 0.02) (table 3). In each country a comparable trend was observed.

Table 2: Haplotype analysis with various endpoints in the overall group (n=5786)

	Haplotype 2 (1.1.1.1)	Haplotype 1 (2.1.2.2)		Haplotype 3 (1.1.2.1)		Haplotype 4 (1.2.1.1)	
	HR (95%CI)	HR (95%CI)	p-value	HR (95%CI)	p-value	HR (95%CI)	p-value
Primary endpoint	1.0 (ref)	**1.14 (1.01-1.29)**	**0.035**	1.14 (0.99-1.30)	0.065	**1.19 (1.04-1.36)**	**0.012**
Coronary events	1.0 (ref)	1.13 (0.98-1.30)	0.082	1.10 (0.94-1.29)	0.236	**1.21 (1.03-1.41)**	**0.019**
Clinical stroke	1.0 (ref)	1.22 (0.96-1.54)	0.097	1.23 (0.95-1.59)	0.114	1.14 (0.89-1.47)	0.291

Hazard ratios are assessed with Cox-proportional hazards model adjusted for sex, age, treatment, and country. The primary endpoint included coronary heart disease death, non-fatal myocardial infarct, and fatal/non-fatal stroke

Table 3: Association between IL-10 -2849GA genotype and stroke in separate countries (n=5786)

Clinical stroke	Genotypes			HR 2 vs 1	HR 3 vs 1	p-value
	Wt/Wt (1) n/N (%)	Wt/Var (2) n/N (%)	Var/Var (3) n/N (%)	(95% CI)	(95% CI)	
Scotland	51/1165 (4)	37/997 (4)	15/217 (7)	0.83 (0.55-1.27)	1.57 (0.88-2.80)	0.12
Ireland	41/926 (4)	38/887 (4)	15/234 (6)	0.96 (0.62-1.49)	1.45 (0.80-2.63)	0.37
The Netherlands	27/531 (5)	19/415 (5)	7/92 (8)	0.89 (0.49-1.60)	1.53 (0.66-3.51)	0.47
Overall	119/2622 (5)	94/2299 (4)	37/543 (7)	0.88 (0.67-1.16)	1.50 (1.04-2.17)	**0.02**

Hazard ratios (HR) are assessed with the Cox-proportional hazards model adjusted for sex, age, treatment, and country

Discussion

This study investigates the association between functional polymorphisms in the promoter region of the IL-10 gene with coronary and cerebrovascular events. The haplotype analysis showed an association of two haplotypes with the primary endpoint. When we looked at this association in more detail haplotype 4, with the -592A variant, was associated primarily with coronary events. The -2849AA in haplotype1 was found to be associated with an increased risk of clinical strokes.

Production of IL-10 is under tight genetic control, with heritability estimates between 50-70% (10). Part of this genetic variation comes from polymorphisms in its own promoter sequence (11). We have previously reported that carriers of the IL-10 -2849AA genotype have significantly lower IL-10 responsiveness upon stimulation with bacterial lipopolysaccharide (LPS)(6). Although it is unknown whether the -592GA SNP is functional by itself or that it is in linkage disequilibrium with another variant, the -592A variant has been associated with low IL-10 production rates (12).

We found that the -592GA polymorphism in the promoter region of the IL-10 gene was associated with coronary events. In IL-10 knock-out mice the absence of IL-10 leads to an increased susceptibility of atherosclerosis (3). Furthermore, a study with an overexpressing transgenic mice model and IL-10 null mice showed a marked difference in lesion size between the groups (13). IL-10 transgenic mice displayed significantly less atherosclerotic lesion formation compared to wild-type whereas IL-10 null mice had increased lesion formation (13). Moreover, in patients with acute coronary syndromes it was demonstrated that elevated IL-10 serum levels are associated with a significantly improved outcome (14).

We also showed that the -2849AA genotype was associated with an increased risk of incident stroke. The role of IL-10 in ischaemic brain damage has been evaluated before. Brain infarcts produced by occlusion of the middle cerebral artery were 30% larger in IL-10 knockout mice as compared with wild-type mice (4). Furthermore, exogenous administration of IL-10 induces neuroprotection in rat models of cerebral focal ischaemia (15). Moreover, we have earlier demonstrated that elderly subjects with low IL-10 production capacity have an increased risk of incident stroke (16).

The reported associations are relatively small. However, due to our large study population they are significant and consistent. The strength of our study is that we could replicate the increased risk for strokes for -2849AA carriers in three separate study populations. Because we randomized participants from three countries, each study group could be used separately. Although the separate

associations were not significant due to small numbers, similar trends were observed. Another strength of our study is that all subjects were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes, therefore genetic markers of coronary and cerebrovascular events may be identified easily.

In conclusion, genetic variation in the promoter region of the IL-10 gene is associated with vascular events. This provides evidence that not only pro-inflammatory processes contribute to atherosclerosis but that also anti-inflammatory cytokines are implicated. These findings support the hypothesis that genetic programming of the inflammatory response may be relevant to the pathogenesis of atherosclerosis. If these findings are confirmed and adequately explained on the basis of independent studies, screening patients for the IL-10 promoter polymorphisms may contribute to a better risk stratification of patients at increased risk for atherosclerosis and may improve individual treatment.

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Reference List

(1) Ohsuzu F. The roles of cytokines, inflammation and immunity in vascular diseases. J Atheroscler Thromb 2004;11(6):313-21.

(2) Libby P. Inflammation in atherosclerosis. Nature 2002 Dec 19;420(6917):868-74.

(3) Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F, Bureau MF, et al. Protective role of interleukin-10 in atherosclerosis. Circ Res 1999 Oct 15;85(8):e17-e24.

(4) Grilli M, Barbieri I, Basudev H, Brusa R, Casati C, Lozza G, et al. Interleukin-10 modulates neuronal threshold of vulnerability to ischaemic damage. Eur J Neurosci 2000 Jul;12(7):2265-72.

(5) de Craen AJ, Posthuma D, Remarque EJ, van den Biggelaar AH, Westendorp RG, Boomsma DI. Heritability estimates of innate immunity: an extended twin study. Genes Immun 2005 Mar;6(2):167-70.

(6) Westendorp RG, van Dunne FM, Kirkwood TB, Helmerhorst FM, Huizinga TW. Optimizing human fertility and survival. Nat Med 2001 Aug;7(8):873.

(7) Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Cobbe SM, et al. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet 2002 Nov 23;360(9346):1623-30.

(8) Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005 Jan 15;21(2):263-5.

(9) Wallenstein S, Hodge SE, Weston A. Logistic regression model for analyzing extended haplotype data. Genet Epidemiol 1998;15(2):173-81.

(10) Reuss E, Fimmers R, Kruger A, Becker C, Rittner C, Hohler T. Differential regulation of interleukin-10 production by genetic and environmental factors--a twin study. Genes Immun 2002 Nov;3(7):407-13.

(11) Kurreeman FA, Schonkeren JJ, Heijmans BT, Toes RE, Huizinga TW. Transcription of the IL10 gene reveals allele-specific regulation at the mRNA level. Hum Mol Genet 2004 Aug 15;13(16):1755-62.

(12) Crilly A, Hamilton J, Clark CJ, Jardine A, Madhok R. Analysis of the 5' flanking region of the interleukin 10 gene in patients with systemic sclerosis. Rheumatology (Oxford) 2003 Nov;42(11):1295-8.

(13) Pinderski Oslund LJ, Hedrick CC, Olvera T, Hagenbaugh A, Territo M, Berliner JA, et al. Interleukin-10 blocks atherosclerotic events in vitro and in vivo. Arterioscler Thromb Vasc Biol 1999 Dec;19(12):2847-53.

(14) Heeschen C, Dimmeler S, Hamm CW, Fichtlscherer S, Boersma E, Simoons ML, et al. Serum level of the antiinflammatory cytokine interleukin-10 is an important prognostic determinant in patients with acute coronary syndromes. Circulation 2003 Apr 29;107(16):2109-14.

(15) Spera PA, Ellison JA, Feuerstein GZ, Barone FC. IL-10 reduces rat brain injury following focal stroke. Neurosci Lett 1998 Jul 31;251(3):189-92.

(16) van Exel E, Gussekloo J, de Craen AJ, Bootsma-van der Wiel A, Frolich M, Westendorp RG. Inflammation and stroke: the Leiden 85-Plus Study. Stroke 2002 Apr;33(4):1135-8.

Chapter 4

Genetic variation in the interleukin-1 beta-converting enzyme associates with cognitive function. The PROSPER study

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Abstract

Inflammation is thought to play an important role in the development of cognitive decline and dementia in old age. The interleukin-1 signaling pathway may play a prominent role in this process. The gene encoding for interleukin-1β-converting enzyme (ICE) is likely to influence IL-1β levels. Inhibition of ICE decreases the age-related increase in IL-1β levels and may therefore improve memory function. We assessed whether genetic variation in the ICE gene associates with cognitive function in an elderly population. All 5804 participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) were genotyped for the 10643GC, 9323GA, 8996AG, and 5352GA polymorphisms in the ICE gene. Cross-sectional associations between the polymorphisms and cognitive function were assessed with linear regression. Longitudinal associations between polymorphisms, haplotypes and cognitive function were assessed with linear mixed models. All associations were adjusted for sex, age, education, country, treatment with pravastatin, and version of test where appropriate. Subjects carrying the variants 10643C and 5352A allele had significantly lower IL-1β production levels ($p<0.01$). Furthermore, we demonstrated that homozygous carriers of the 10643C and the 5352A allele performed better on all executive function tests at baseline and during follow-up compared to homozygous carriers of the wildtype allele (all $p<0.02$). The haplotype with two variants present (10643C and 5352A) was associated with better executive function (all $p<0.02$) compared to the reference haplotype without variants. For memory function the same trend was observed, although not significant. Genetic variation in the ICE gene is associated with better performance on cognitive function and lower IL-1β production levels. This suggests that low levels of IL-1β are protective for memory and learning deficits. Inhibition of ICE may therefore be an important therapeutic target for maintaining cognitive function in old age.

Introduction

Inflammation plays an important role in the development of cognitive decline and dementia in old age (1). The interleukin-1 signaling pathway is likely to have a prominent role in this process (2-5).

For example, in rodents peripheral administration of interleukin-1beta (IL-1β) induces various cognitive-behavioral effects (2). Furthermore, expression of IL-1β is increased in patients with Alzheimer's disease (5). One of the possible mechanisms by which IL-1β acts on cognitive function is by binding to IL-1 type-1 receptors which are abundantly expressed in the hippocampus (3), the area of the brain that has a critical role in memory and learning.

IL-1β production capacity is under tight genetic control. An extended twin study found that over 80% of the variance in production capacity of IL-1β is explained by genetic factors (6). The gene encoding for interleukin-1β-converting enzyme (ICE) is likely to be one of the main genes influencing IL-1β. ICE mediates the cleavage of the inactive precursor of IL-1β into the biologically active form (7). Inhibition of ICE decreases the age-related increase in IL-1β levels (7). Genetic variation in the ICE gene is likely to be functional since patients with the 5352AA genotype in the ICE gene have an increased risk of developing restenosis after percutaneous coronary intervention, a process where inflammation also plays a key role (8).

Since genetic variation in the gene coding for ICE influences expression and function of IL-1β, we assessed the association between four polymorphisms within the ICE gene and cognitive function in an elderly population.

Methods

All data come from the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER). A detailed description of the study has been published elsewhere (9;10). A short summary is provided here.

Participants

PROSPER was a prospective multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in elderly. Between December 1997 and May 1999, we screened and enrolled subjects in Scotland (Glasgow), Ireland

(Cork), and the Netherlands (Leiden). Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. A total number of 5804 subjects were randomly assigned to pravastatin or placebo. A large number of prospective tests were performed including Biobank tests and cognitive function measurements.

Cognitive function

The Mini-Mental State Examination (MMSE) was used to measure global cognitive function. The MMSE scores range from zero points (very severe cognitive impairment) to 30 points (optimal cognitive function). Participants with poor cognitive function (MMSE< 24) were not eligible for inclusion in the PROSPER study. Four neuropsychological performance tests were used to measure various cognitive domains. The Stroop-Colour-Word-test for attention and the Letter-Digit Coding Test (LDT) for processing speed were used to measure executive functioning. The outcome parameter for the Stroop test was the total number of seconds to complete the third Stroop card containing 40 items. The outcome variable for the LDT was the total number of correct entries in 60 seconds. Memory was assessed with the 15-Picture Learning test (PLT) testing immediate and delayed recall. The main outcome parameters were the accumulated number of recalled pictures over the three learning trials and the number of pictures recalled after 20 minutes. The six correlation coefficients between the four neuropsychological performance tests varied between 0.29 ($p<0.001$) for the Stroop test and the Picture Learning test delayed and 0.77 ($p<0.001$) for the Picture Learning test immediate and delayed. Reliability and sensitivity of these tests in an elderly population have been published elsewhere (11).

Cognitive function was tested at six different time points during the study, before randomization, at baseline, after 9, 18, and 30 months, and at the end of the study. The time point of this last measurement was different for the participants (at 36-48 months) therefore we performed the analyses with their individually varying time-point but report the results for the mean of these time points (at 42 months). The pre-randomized measurement was discarded in the analysis to preclude

possible learning effects. Since the MMSE is not suitable for longitudinal research because of learning and ceiling effects, MMSE scores are not reported here.

Compound cognitive test scores were constructed by transforming individual test scores into standardized *Z*-scores (*Z*-score= (individual score–mean population score)/standard deviation of the population score) for global cognitive function, executive function and memory function. Global cognitive function was calculated by averaging the *Z*-scores of the Stroop-Colour-Word-test, the Letter-Digit Coding test, and the 15-Picture Learning Test immediate and delayed recall. Executive function included the *Z*-scores of the Stroop-Colour-Word-test and the Letter-Digit Coding test. Memory function included the *Z*-scores of the 15-Picture Learning Test immediate and delayed recall.

Genotyping

We selected four single nucleotide polymorphisms in the ICE gene, 10643GC (rs554344), 9323GA (rs488992), 8996AG (rs1977989), and 5352GA (rs580253) based on its minor allele frequency (>5%) and to cover the genomic region of the ICE gene for haplotype analyses. Using the HapMap database (http://www.hapmap.org) we identified these SNPs as tagSNPs for the existing haplotypes within the gene. All SNPs were genotyped by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS), using the Sequenom MassARRAYtm methodology (Sequenom Inc, San Diego, CA, USA). Amplification reactions and parameters were based on the manufacturer's instructions.

IL-1β production levels

Cytokine production levels were measured in a randomly chosen subgroup of 411 participants at baseline. Whole blood samples were stimulated with 10 ng/ml of lipopolysaccharide (LPS) to assess the innate IL-1β production capacity. Unstimulated baseline samples were obtained to serve as a control for contamination.

Statistical analysis

The program Haploview (12) was used to estimate the allele frequencies, test the consistency of the genotype frequencies at each SNP locus with Hardy-Weinberg equilibrium, and estimate and plot pairwise linkage disequilibrium (LD) between the SNPs examined. Haplotypes and haplotype frequencies were calculated using SNPHAP software (http://www-gene.cimr.cam.ac.uk/clayton/software). We used multiple imputation analysis to deal with incomplete data and to account for many haplotype probabilities per subject. This method has been described elsewhere in more detail (13). Haplotypes with a frequency of less than 5 % were combined and included in all analyses, without reporting the results. The haplotype analysis approach used in this study assumes an additive effect of the haplotypes, and details of this approach have been described elsewhere (14). Cross-sectional associations between the four ICE polymorphisms and cognitive function were assessed with linear regression, adjusted for sex, age, education, country, and version of test where appropriate. The associations between the four genotypes, the ICE haplotypes and cognitive function during follow-up were assessed with a linear mixed model for repeated measurements without an interaction term for time and genotype. The estimate for time represents the cognitive decline per year. The results for the genotypes represent the mean difference over time between the genotypes. All longitudinal analyses were adjusted for sex, age, education, country, use of pravastatin, and version of test where appropriate. The SPSS software (version 12.0.1, SPSS Inc, Chicago, Ill) was used for all statistical analyses. P-values lower than 0.05 were considered statistically significant.

Results

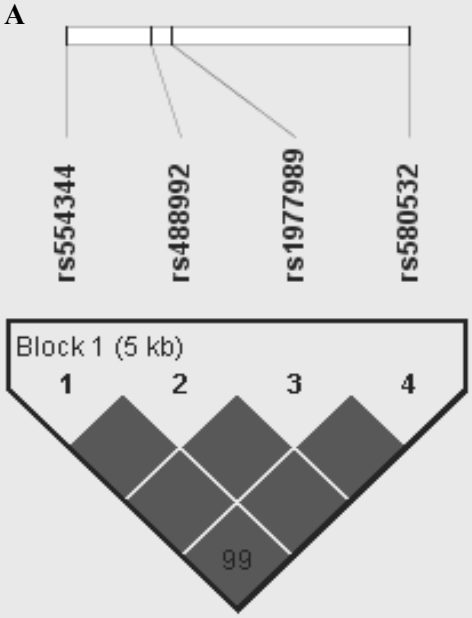
Table 1 shows the baseline characteristics of the 5804 participants divided over the three countries. The mean age of all subjects at study entry was 75.3 years and about 50% of the participants were female. There were significant differences in minor allele frequencies between the countries (p-value Chi-square < 0.01, data not shown). The variants 10643C and 5352A were more common in the Dutch subjects compared with the subjects from Scotland and Ireland. Therefore, all analyses were adjusted for country. Mean follow-up of study subjects was 42 months (range 36-48 months).

Table 1: Baseline characteristics of the participants of the PROSPER study per country.

	Scotland (N=2520)	Ireland (N=2184)	The Netherlands (N=1100)
Continous variates (mean, SD)			
Age (years)	75.3 (3.4)	75.5 (3.3)	75.1 (3.3)
Body Mass index, (kg/m2)	26.7 (4.2)	27.0 (4.4)	26.7 (3.8)
Total cholesterol, (mmol/L)	5.7 (1.0)	5.6 (0.9)	5.8 (0.9)
LDL cholesterol, (mmol/L)	3.8 (0.8)	3.7 (0.8)	3.9 (0.8)
HDL cholesterol, (mmol/L)	1.3 (0.4)	1.3 (0.4)	1.3 (0.3)
Categorical variates (n, %)			
Female	1283 (51)	1197 (55)	520 (47)
Current smoker	708 (28)	583 (27)	267 (24)
History of diabetes	213 (9)	225 (10)	185 (17)
History of hypertension	1446 (57)	1441 (66)	705 (64)
History of angina	811 (32)	523 (24)	225 (21)
History of claudication	229 (9)	114 (5)	47 (4)
History of myocardial infarction	379 (15)	258 (12)	139 (13)
History of vascular disease	1239 (49)	849 (39)	477 (43)
History of stroke or TIA	265 (11)	222 (10)	162 (15)
Genotype, minor allele frequency (%)			
ICE 10643GC	17	15	20
ICE 9323GA	10	9	11
ICE 8996AG	28	28	25
ICE 5352GA	17	15	20

Genotyping of the four ICE polymorphisms was complete for at least 5403 subjects. The results of the remaining subjects were missing because of insufficient DNA or incomplete genotyping. All four SNPs were in Hardy-Weinberg equilibrium (all $p>0.3$). The four SNPs were in strong linkage disequilibrium (LD) and occurred together in one haploblock (figure 1A). Four haplotypes were found in our study population (figure 1B). All haplotypes with a frequency above 5% were included in analyses. We used H1111, with no variants present, as reference haplotype. H1121 carried the 9323G variant, H2212 carried three variant alleles, 10643C, 8996A, and 5352A, and H2112 carried two variants, 10643C and 5352A.

A



B

	10643GC	9323GA	8996AG	5323GA	Haplotype Frequency
H1111	1	1	1	1	0.555
H1121	1	1	2	1	0.274
H2212	2	2	1	2	0.102
H2112	2	1	1	2	0.069

Figure 1: Haplotype information.

Figure 1A shows the linkage disequilibrium (LD) between the single nucleotide polymorphisms (SNPs) examined. All SNPs are in LD and occur together in one haploblock. Figure 1B shows the haplotype frequencies. All four haplotypes (frequency> 5%) were included in the analyses.

Table 2: Association between four ICE polymorphisms and IL-1β production levels (n=398).

	Wt/Wt	Wt/Var	Var/Var	p-value trend
ICE 10643GC				
N	254	112	16	
Log IL-1 β	3.94 (0.30)	3.86 (0.31)	3.81 (0.31)	**0.009**
ICE 9323GA				
N	310	78	3	
Log IL-1 β	3.91 (0.31)	3.88 (0.30)	3.96 (0.21)	0.468
ICE 8996AG				
N	217	135	36	
Log IL-1 β	3.89 (0.31)	3.92 (0.30)	3.96 (0.26)	0.128
ICE 5352GA				
N	258	115	17	
Log IL-1 β	3.94 (0.31)	3.86 (0.31)	3.84 (0.32)	**0.008**

P-value for trend was assessed with linear regression. Data is presented as mean (SD)

To determine the functionality of the four polymorphisms in our study sample, we assessed the difference in IL-1β production capacity over the three genotypes. Subjects carrying the variant 10643C and 5352A allele had significantly lower IL-1β production levels compared to carriers of the wild-type allele ($p<0.01$) (table 2). We found no significant result for the 8996AG

polymorphism, although a trend was seen that the variant allele had a higher IL-1β production capacity compared to the wildtype allele.

Table 3: Cross-sectional association between four ICE polymorphisms and cognition on baseline

	Wt/Wt	Wt/Var	Var/Var	p-value trend
ICE 10643GC (N)	3885	1522	173	
Attention	67.57 (0.46)	65.01 (0.71)	61.31 (1.71)	**<0.001**
Processing speed	22.77 (0.13)	23.56 (0.21)	23.97 (0.60)	**0.001**
Immediate memory	9.30 (0.03)	9.33 (0.05)	9.66 (0.15)	0.096
Delayed memory	10.09 (0.04)	10.15 (0.07)	10.67 (0.21)	**0.041**
Global cognition	-0.01 (0.05)	0.29 (0.08)	0.81 (0.24)	**<0.001**
Executive function	-0.03 (0.03)	0.01 (0.05)	0.39 (0.15)	**<0.001**
Memory function	-0.04 (0.03)	0.18 (0.05)	0.34 (0.13)	**0.045**
ICE 9323GA (N)	4555	1031	58	
Attention	67.10 (0.42)	64.84 (0.86)	56.77 (2.34)	**0.005**
Processing speed	22.92 (0.12)	23.55 (0.25)	24.51 (0.98)	**0.032**
Immediate memory	9.30 (0.03)	9.39 (0.06)	9.57 (0.28)	0.185
Delayed memory	10.10 (0.04)	10.25 (0.08)	10.79 (0.38)	**0.041**
Global cognition	0.04 (0.05)	0.35 (0.10)	1.10 (0.39)	**0.002**
Executive function	-0.03 (0.03)	0.08 (0.06)	0.38 (0.29)	**0.001**
Memory function	-0.01 (0.03)	0.18 (0.06)	0.61 (0.18)	0.069
ICE 8996AG (N)	2935	2210	428	
Attention	65.68 (0.48)	67.11 (0.62)	69.73 (1.64)	**0.004**
Processing speed	23.14 (0.15)	22.97 (0.17)	22.76 (0.42)	0.429
Immediate memory	9.33 (0.04)	9.30 (0.04)	9.31 (0.09)	0.710
Delayed memory	10.16 (0.05)	10.10 (0.06)	10.12 (0.13)	0.560
Global cognition	0.17 (0.06)	0.0.7 (0.07)	-0.11 (0.17)	0.102
Executive function	0.08 (0.03)	0.01 (0.04)	-0.11 (0.10)	0.052
Memory function	0.01 (0.04)	-0.03 (0.04)	-0.02 (0.09)	0.597
ICE 5352GA (N)	3739	1499	165	
Attention	67.46 (0.47)	64.44 (0.69)	60.18 (1.73)	**<0.001**
Processing speed	22.85 (0.13)	23.65 (0.21)	24.22 (0.59)	**<0.001**
Immediate memory	9.29 (0.03)	9.32 (0.05)	9.70 (0.15)	0.061
Delayed memory	10.10 (0.04)	10.14 (0.07)	10.71 (0.22)	**0.043**
Global cognition	-0.01 (0.05)	0.31 (0.08)	0.95 (0.23)	**<0.001**
Executive function	-0.03 (0.03)	0.21 (0.05)	0.41 (0.12)	**<0.001**
Memory function	-0.03 (0.03)	0.00 (0.05)	0.43 (0.15)	**0.038**

All p-values for trend were assessed with linear regression adjusted for sex, education, age, country, and where appropriate test of version use

The results of the cross-sectional association between the ICE polymorphisms and cognitive function at baseline are presented in table 3. Significant associations were found between the 10643C variant and all cognitive function tests (all $p<0.05$), the same results were found for the association between the 5352A variant and cognitive function (all $p<0.05$). For the 9323A allele comparable results were found, although the effects with memory function were not so strong. Subjects with these variant alleles had a better cognitive performance at baseline compared to the wild-type allele. Carriers of the 8996G variant performed worse on the Stroop-colour-word test for attention ($p=0.004$). Excluding subjects with a history of stroke did not materially change our results.

Table 4 shows the results of the longitudinal association between the ICE polymorphisms and cognitive function. Follow-up was complete for 4283 subjects, loss to follow-up was for most of the subjects due to mortality. The term for time was significant for all domains of cognitive function, indicating that all domains declined over time. The estimates represent the mean difference over time between the genotypes. Carriers of the 10643C and 5352A alleles significantly performed better on the global and executive function tests (all $p< 0.01$). For memory function the same trend was seen but not statistically significant. The 9323GA was positively associated with attention and executive function ($p=0.03$) but with the other cognitive performance test no association was found. Carriers of the 8996G variant performed worse over time on the test for attention ($p=0.023$).
Results remained similar when the analyses were done without adjusting for education (data not shown).

To assess whether the association between genotypes and cognition was dependent on development of stroke, we investigated the occurrence of clinical strokes during follow-up. There was an equal division of the occurrence of clinical stroke between the genotypes (all $p>0.5$). The results of the longitudinal association between ICE polymorphisms and cognitive function did not materially change after excluding all subjects with stroke or TIA in their history or during follow-up (data not shown).

The results of the longitudinal analysis between the ICE polymorphisms and executive function are graphically displayed in figure 2. A dose-dependent association was present for the 10643GC, 9323GA, and the 5352GA polymorphisms with executive function (all p<0.03). With the 8996AG polymorphism no dose-dependent association was found (p=0.194).

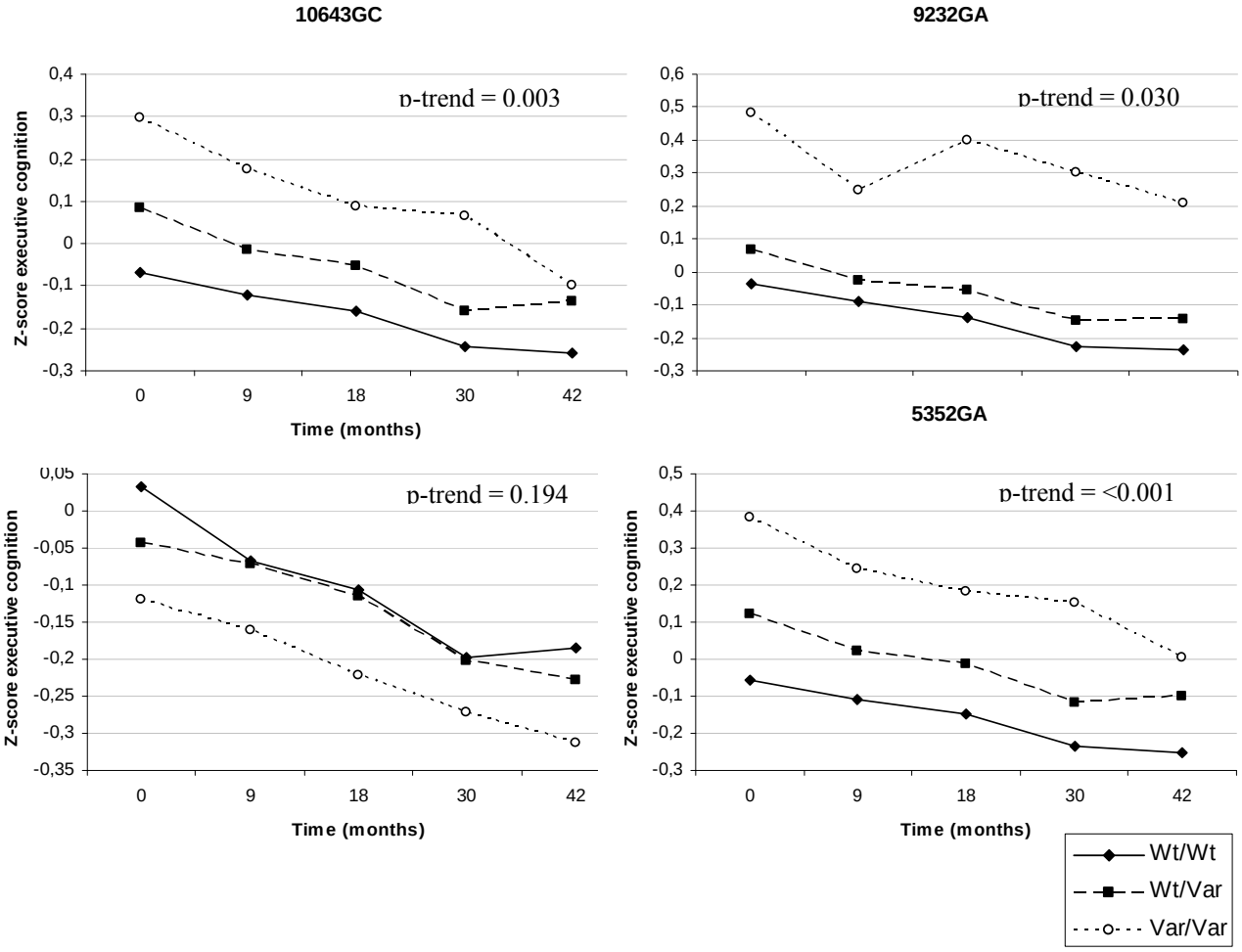


Figure 2: Representation of the longitudinal association between the four ICE polymorphisms and executive function.

P-values for trend represent the mean difference over time over the three genotypes. Black dots with a straight line indicates the homozygous wild-type carriers, the heterozygous carries are indicated by black dots and a dotted line and the homozygous carriers of the variant allele are indicated by white dots and a dotted line.

Table 4: Longitudinal association between ICE polymorphisms and cognitive function

	Time		10643GC		9323GA		8996AG		5352GA	
	Estimate (SE)	p	Estimate (SE)	p	Estimate (SE)	P	Estimate (SE)	p	Estimate (SE)	p
All subjects										
Attention, seconds	0.70 (0.07)	<0.001	**-1.81 (0.66)**	**0.006**	**-1.75 (0.82)**	**0.034**	**1.27 (0.56)**	**0.023**	**-2.31 (0.67)**	**0.001**
Processing speed*	-0.38 (0.02)	<0.001	**0.46 (0.18)**	**0.012**	0.38 (0.23)	0.090	-0.05 (0.15)	0.728	**0.54 (0.18)**	**0.003**
Immediate memory**	-0.01 (0.01)	0.038	0.06 (0.04)	0.149	0.04 (0.05)	0.494	-0.02 (0.04)	0.672	0.07 (0.04)	0.089
Delayed memory**	-0.06 (0.01)	<0.001	0.08 (0.06)	0.165	0.07 (0.07)	0.362	-0.04 (0.05)	0.433	0.10 (0.06)	0.087
Global cognition	-0.10 (0.01)	<0.001	**0.19 (0.07)**	**0.011**	0.16 (0.09)	0.088	-0.06 (0.06)	0.306	**0.23 (0.07)**	**0.002**
Executive function	-0.06 (0.01)	<0.001	**0.13 (0.04)**	**0.003**	**0.12 (0.05)**	**0.030**	-0.05 (0.04)	0.194	**0.16 (0.04)**	**<0.001**
Memory function	-0.04 (0.01)	<0.001	0.06 (0.04)	0.137	0.04 (0.05)	0.396	-0.02 (0.04)	0.539	0.07 (0.04)	0.081

Estimates and p-values were assessed with linear mixed models adjusted for sex, age, education, country, pravastatin use and where appropriate, version of test used. *assessed in number of digits, **assessed in number recalled

Table 5: Association between ICE haplotypes and cognitive function

	Time		H1111	H1121		H2212		H2112	
	Estimate (SE)	p	Estimate (SE)	Estimate (SE)	p	Estimate (SE)	p	Estimate (SE)	p
All subjects									
Attention, seconds	0.67 (0.07)	<0.001	ref	0.50 (0.72)	0.492	**-1.80 (0.90)**	**0.045**	**-2.53 (1.08)**	**0.019**
Processing speed*	-0.37 (0.01)	<0.001	ref	0.12 (0.20)	0.559	0.43 (0.25)	0.078	**0.76 (0.29)**	**0.010**
Immediate memory**	-0.02 (0.01)	0.038	ref	0.01 (0.05)	0.808	0.03 (0.06)	0.572	0.12 (0.07)	0.093
Delayed memory**	-0.07 (0.01)	<0.001	ref	-0.02 (0.06)	0.807	0.04 (0.08)	0.595	0.13 (0.10)	0.186
Global cognition	-0.10 (0.01)	<0.001	ref	0.02 (0.08)	0.848	0.15 (0.10)	0.119	**0.31 (0.12)**	**0.009**
Memory function	-0.04 (0.01)	<0.001	ref	0.00 (0.05)	0.975	**0.13 (0.06)**	**0.028**	**0.20 (0.07)**	**0.005**
Executive function	-0.06 (0.01)	<0.001	ref	0.00 (0.05)	0.930	0.03 (0.06)	0.560	0.11 (0.07)	0.117

Estimates and p-values were assessed with linear mixed models adjusted for sex, age, education, country, pravastatin use and where appropriate, version of test used. *assessed in number of digits, **assessed in number recalled

In table 5 the results of the association between ICE haplotypes and cognitive function during follow-up are shown. H1111 was used as reference. As in the SNP analysis, the term for time was significant for all domains of cognitive function, indicating that all domains declined over time. H2112 with the variant alleles of 10643C and 5352A was associated with better cognitive performance on global cognitive function and executive function compared to the reference haplotype (all $p<0.02$). With memory function no associations were found. There also was a significant association between H2212 and attention ($p=0.045$) and executive function ($p=0.028$). A comparable trend was also seen for the other cognitive domains, but did not reach statistical significance. There was no association with H1121 and cognition. Excluding subjects with a history of stroke and those who suffered a stroke during follow-up did not materially change our results. Again, the results remained similar when the analyses were done without adjusting for education.

Discussion

In this study we investigated the association between genetic variation in the ICE gene and cognitive function. We found that subjects carrying the variants 10643C and 5352A had significantly lower IL-1β production levels. Furthermore, we demonstrated that carriers of the 10643C and the 5352A allele performed better on all executive function tests at baseline and during follow-up compared to carriers of the wildtype allele (all $p<0.02$). The haplotype with these two variants present (10643C and 5352A) was also associated with better executive function (all $p<0.02$) compared to the reference haplotype without variant alleles. For memory function the same trend was observed, although not significant.

A previous study by Gemma *et al* demonstrated that inhibition of ICE in rats is associated with improved memory (7). They showed that the inhibition of ICE and improved memory coincides with a decrease in hippocampal IL-1β levels. In our study we showed that carriers of the 10643C and 5352A alleles in the ICE gene have lower IL-1β levels compared to carriers of the wild-type allele. This suggests that low levels of IL-1β might be protective for memory and learning deficits. IL-1β is a pro-inflammatory cytokine which has a key position in the innate immune and

inflammatory response by inducing a pro-inflammatory response (15). Binding to the IL-1 receptor evokes cytokines release of pro-inflammatory cytokines like Tumor Necrosis Factor alpha (TNFα), Interleukin-6 (IL-6) and Interferon gamma (IFNγ) (figure 2). It was originally described as a mediator in the periphery, however, IL-1β has also been reported to be synthesized in the brain (15). In addition, IL-1 receptors have been detected in different regions of the central nervous system with the highest density in the hippocampus (16).

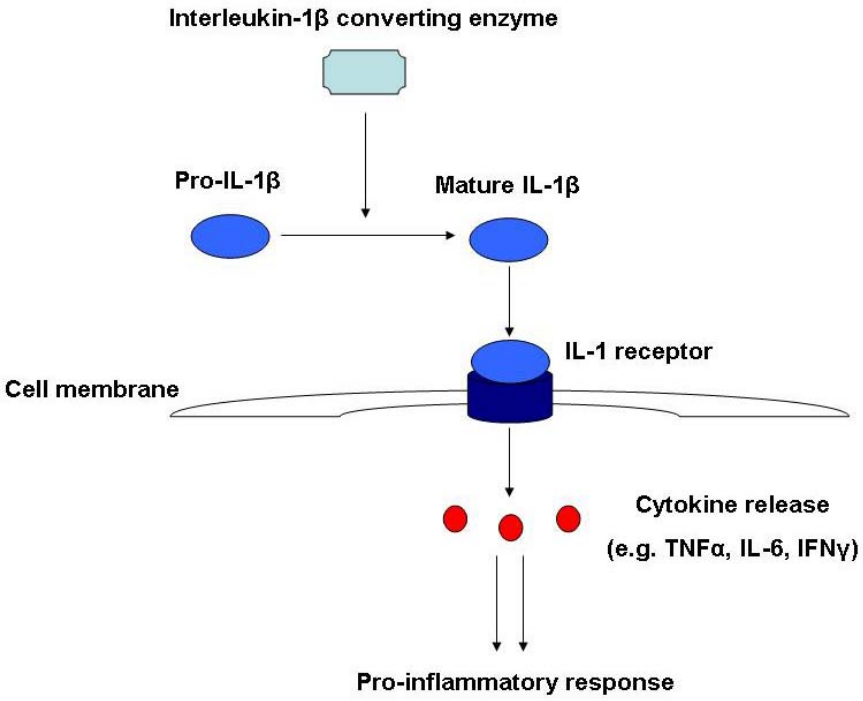


Figure 3: The interleukin-1β converting enzyme (ICE) pathway.

Pro-IL-1β is cleaved by interleukin-1β converting enzyme into mature IL-1β. The active form of IL-1β binds to the IL-1 receptor. This evokes a cytokine release of various pro-inflammatory cytokines like Tumor Necrosis Factor alpha (TNFα), Interleukin-6 (IL-6) and Interferon gamma (IFNγ).

The hypothesis that IL-1β associates with cognitive function was first proposed by Lynch in 1998 (17). She suggested that hippocampal concentration of IL-1β increases with age, while the cause of this increase remained unclear. *In vitro* studies have shown that increase in IL-1β in hippocampal tissue would increase lipid-peroxidation possibly by stimulating production of reactive oxygen species (18). IL-1β is thought to damage the neuronal membrane by lipid-peroxidation and is

thereby accompanied by impairment in expression of long-term potentation (16;19), an electrophysiological index of synaptic plasticity linked to memory and learning.

Peripheral administration of IL-1β induces diverse cognitive-behavioral effects. Gibertini *et al* demonstrated that IL-1β injections prior to training on the Morris water maze affects the learning ability of mice (2). Furthermore, when injecting the same mice afterwards with an anti-IL-1β antibody, the learning on the water maze was normalized. Subsequently, several other studies have reported that IL-1β administration induces cognitive defects like decreased exploratory behavior and decreased spatial learning (20;21). Moreover, increased expression of IL-1β is associated with neurodegenerative diseases like Alzheimer's disease and vascular dementia (5).

At baseline there were more subjects with a history of stroke within the group of homozygous carriers of the variant 10643C and 5352A allele compared to the carriers of the wild-type allele (data not shown). During follow-up the carriers of the variant 10643C and 5352A alleles did not develop more clinical strokes compared to wild-type carriers. This suggests that the unequal division of history of stroke at baseline is due to chance. To exclude the possibility that the better cognitive performance of subjects with the variants 10643C and 5352A was caused by a difference in prevalence and incidence of stroke, we repeated all analyses excluding subjects with a history of stroke or an incidence of stroke or TIA in follow-up. When we excluded subjects with clinical stroke in the cross-sectional and longitudinal associations, we still found that subjects carrying the 10643C and 5352A variant alleles had a better cognitive function compared to wild-type carriers. Therefore we think that the better cognitive function in subjects with these two variants is not caused by clinical strokes. This supports the hypothesis of Lynch et al (17) that IL-1β induces cognitive deficits by inflammation and hippocampal damage in stead of atherosclerosis.

By using TagSNPs in this study we can form the four main haplotypes within the ICE gene, and because of the strong linkage disequilibrium we know which SNPs are on which haplotype. H2112 with the two variants 10643C and 5352A has been found to have a beneficial effect on cognitive

function, but both variants may not be functional by themselves. The ICE 5352GA polymorphism we investigated is located in exon 5 but does not have an amino acid change as a result and the ICE 10643GC polymorphism is located in the intronic area of the gene. These variants might be in linkage disequilibrium with other polymorphisms in the gene at this haplotype (22). Although the functionality of the ICE polymorphisms is not well-known, we here demonstrated that carriers of the 10643C and 5352A variant alleles have a significantly lower IL-1β production capacity. Together with our finding that carriers of the 10643C and 5352A variants have better cognitive function, it is highly suggestive that low IL-1β levels might causally be related to a better cognitive function in old age.

We decided to adjust all analyses for education because education might affect the level of cognitive function. It might be argued that this is an overadjustment because prior cognitive ability might lead to more education. This might be the reason why education is related to later cognitive ability. However, we assessed our analyses also without adjustment for education and the results did not materially change.

One of the strengths of our study is our population size. We have prospective data of over 5000 subjects on cognitive function. Also the fact that we have a follow-up of 42 months with little lost to follow-up is a strong element of our study. We used the linear mixed models for our statistical analyses because this method can handle repeated measurement accurately. Furthermore, our population is appropriate to measure cognitive function because only subjects with a MMSE above 24 points could participate, which makes it a homogenous study group suitable for investigating cognitive function. We did not analyze the cognitive decline over time over the genotype groups because we did not expect that carriers will have an additional change per year. We expected that they have a difference in cognition developed earlier in life and that in this elderly population we could not find an additional decline.

Another strength of our study is that all subjects were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. Despite these inclusion criteria as well as the selection for subjects with MMSE scores above 24 we do not have an enrichment of the variant 10643C and 5352A alleles within our study population compared to the general population. Because the recruitment of subjects with pre-existing vascular disease, we could exclude subjects with a stroke in history and follow-up to exclude the possibility that the effects are attributable to clinical stroke.

In conclusion, we found an association between the ICE polymorphisms and cognitive function. Carriers of the variant 10643C and 5352A alleles performed better on all cognitive function tests compared to carriers of the wild-type allele, which was independent of clinical strokes. We also found that carriers of the variant alleles had significant lower IL-1β levels than homozygous wild-type carriers. This suggests that low levels of IL-1β are protective for memory and learning deficits. Inhibition of ICE will lower IL-1β levels and might thereby improve cognitive function. ICE inhibitors might therefore become an important therapeutic tool for subjects with cognitive decline.

Acknowledgements

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Reference List

(1) Wilson CJ, Finch CE, Cohen HJ. Cytokines and cognition--the case for a head-to-toe inflammatory paradigm. J Am Geriatr Soc 2002 Dec;50(12):2041-56.

(2) Gibertini M, Newton C, Friedman H, Klein TW. Spatial learning impairment in mice infected with Legionella pneumophila or administered exogenous interleukin-1-beta. Brain Behav Immun 1995 Jun;9(2):113-28.

(3) Schneider H, Pitossi F, Balschun D, Wagner A, del Rey A, Besedovsky HO. A neuromodulatory role of interleukin-1beta in the hippocampus. Proc Natl Acad Sci U S A 1998 Jun 23;95(13):7778-83.

(4) Thomson LM, Sutherland RJ. Systemic administration of lipopolysaccharide and interleukin-1beta have different effects on memory consolidation. Brain Res Bull 2005 Sep 30;67(1-2):24-9.

(5) Solfrizzi V, D'Introno A, Colacicco AM, Capurso C, Todarello O, Pellicani V, et al. Circulating biomarkers of cognitive decline and dementia. Clin Chim Acta 2006 Feb;364(1-2):91-112.

(6) de Craen AJ, Posthuma D, Remarque EJ, van den Biggelaar AH, Westendorp RG, Boomsma DI. Heritability estimates of innate immunity: an extended twin study. Genes Immun 2005 Mar;6(2):167-70.

(7) Gemma C, Fister M, Hudson C, Bickford PC. Improvement of memory for context by inhibition of caspase-1 in aged rats. Eur J Neurosci 2005 Oct;22(7):1751-6.

(8) Monraats PS, de Vries F, de Jong LW, Pons D, Sewgobind VD, Zwinderman AH, et al. Inflammation and apoptosis genes and the risk of restenosis after percutaneous coronary intervention. Pharmacogenet Genomics 2006 Oct;16(10):747-54.

(9) Shepherd J, Blauw GJ, Murphy MB, Cobbe SM, Bollen EL, Buckley BM, et al. The design of a prospective study of Pravastatin in the Elderly at Risk (PROSPER). PROSPER Study Group. PROspective Study of Pravastatin in the Elderly at Risk. Am J Cardiol 1999 Nov 15;84(10):1192-7.

(10) Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Cobbe SM, et al. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet 2002 Nov 23;360(9346):1623-30.

(11) Houx PJ, Shepherd J, Blauw GJ, Murphy MB, Ford I, Bollen EL, et al. Testing cognitive function in elderly populations: the PROSPER study. PROspective Study of Pravastatin in the Elderly at Risk. J Neurol Neurosurg Psychiatry 2002 Oct;73(4):385-9.

(12) Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005 Jan 15;21(2):263-5.

(13) Harel O, Zhou XH. Multiple imputation: review of theory, implementation and software. Stat Med 2007 Jan 29.

(14) Wallenstein S, Hodge SE, Weston A. Logistic regression model for analyzing extended haplotype data. Genet Epidemiol 1998;15(2):173-81.

(15) Dinarello CA, Savage N. Interleukin-1 and its receptor. Crit Rev Immunol 1989;9(1):1-20.

(16) Rothwell NJ, Hopkins SJ. Cytokines and the nervous system II: Actions and mechanisms of action. Trends Neurosci 1995 Mar;18(3):130-6.

(17) Lynch MA. Age-related impairment in long-term potentiation in hippocampus: a role for the cytokine, interleukin-1 beta? Prog Neurobiol 1998 Dec;56(5):571-89.

(18) Murray CA, Clements MP, Lynch MA. Interleukin-1 induces lipid peroxidation and membrane changes in rat hippocampus: An age-related study. Gerontology 1999 May;45(3):136-42.

(19) Griffin WS, Stanley LC, Ling C, White L, MacLeod V, Perrot LJ, et al. Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. Proc Natl Acad Sci U S A 1989 Oct;86(19):7611-5.

(20) Oitzl MS, van Oers H, Schobitz B, de Kloet ER. Interleukin-1 beta, but not interleukin-6, impairs spatial navigation learning. Brain Res 1993 Jun 4;613(1):160-3.

(21) Spadaro F, Dunn AJ. Intracerebroventricular administration of interleukin-1 to mice alters investigation of stimuli in a novel environment. Brain Behav Immun 1990 Dec;4(4):308-22.

(22) Blankenberg S, Godefroy T, Poirier O, Rupprecht HJ, Barbaux S, Bickel C, et al. Haplotypes of the caspase-1 gene, plasma caspase-1 levels, and cardiovascular risk. Circ Res 2006 Jul 7;99(1):102-8.

Chapter 5

Variation in the IL-10 gene is a marker for risk prediction of cognitive function

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Abstract

Inflammation contributes to the development of cognitive decline in old age in a cytokine-mediated manner. In contrast, circulating markers of inflammation can poorly be associated with cognitive impairment. We assessed the association between genetic variation in the promoter region of the interleukin-10 (IL-10) gene and cognitive function in the elderly. All 5804 participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) were genotyped for the 4259AG, -1082GA, -592CA and -2849GA promoter polymorphisms in the IL-10 gene. Four neuropsychological tests were used to measure cognitive function over a mean follow-up period of 42 months. All associations were assessed with linear mixed models adjusted for sex, age, education, country and pravastatin use. The estimates and p-values for the haplotype analysis were assessed with linear mixed models after multiple imputation analysis. We demonstrated that -2849A and 4259G variants were associated with worse cognitive function (all $p<0.05$). Similar trends were observed for the -1082A and -592A variants. The haplotype with three variants present (4259G, -1082A and -2849A) was associated with decreased cognitive function (all $p<0.03$). Genetic variation in the promoter region of the IL-10 gene is associated with decreased cognitive function in the elderly.

Introduction

Inflammation plays an important role in the development of cognitive decline and dementia in old age (1). There is abundant evidence that inflammatory mechanisms contribute to cognitive impairment via cytokine-mediated interactions (1). Animal models expressing high levels of pro-inflammatory cytokines in the brain suffer from neurodegeneration (2). Furthermore, up-regulation of pro-inflammatory cytokines in tissue cultures leads to microglial activation and neuronal damage (3) and moreover, several markers of inflammation have been found in and around plaques in the brain (4).

Various studies have reported only moderate associations between inflammatory markers and cognitive decline (5-7). Therefore, systemic markers are unlikely to be useful as risk predictors for

cognitive decline (8). On the contrary, genetic variation in inflammatory genes is more likely to be a good marker for risk prediction. Based on Mendel's law, that inheritance of one trait is independent of inheritance of other traits, associations between genetic variation and cognitive function are assumed to be unconfounded (9). Moreover, uncertainty exists whether levels of cytokines are risk factors for cognitive decline or whether they are a consequence of cognitive decline. Functional polymorphisms determine the level of cytokine plasma levels, therefore genetic variation can be used as useful marker to overcome this problem of reverse causality.

IL-10 production levels are under tight genetic control. An extended twin study found that approximately two-thirds of the variance in production level of IL-10 is genetically determined (10). Moreover, we have previously reported that genotypic variation in the IL-10 gene is associated with significantly lower IL-10 responsiveness upon stimulation with bacterial lipopolysaccharide (LPS) (11). Since genetic variation in the promoter region of the IL-10 gene influences the production levels of IL-10, we assessed the association between single nucleotide polymorphisms (SNPs) in the promoter region of the IL-10 gene and cognitive function in an elderly population.

Methods

A detailed description of the protocol of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) has been published elsewhere (12;13). A short summary is provided here.

Participants

PROSPER was a prospective multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in the elderly. Between December 1997 and May 1999, we screened and enrolled subjects in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. A total number of 5804 subjects were randomly assigned to pravastatin or placebo.

Cognitive function

The Mini-Mental State Examination (MMSE) was used to measure global cognitive function. MMSE scores range from zero points (very severe cognitive impairment) to 30 points (optimal cognitive function). Participants with poor cognitive function (MMSE< 24) were not eligible for inclusion in the study. Four neuropsychological performance tests were used to measure various cognitive domains. The Stroop-Colour-Word-test for attention and the Letter-Digit Coding Test (LDT) for processing speed were used to measure executive functioning. The outcome parameter for the Stroop test was the total number of seconds to complete the third Stroop card containing 40 items. The outcome variable for the LDT was the total number of correct entries in 60 seconds. Memory was assessed with the 15-Picture Learning test (PLT) testing immediate and delayed recall. The main outcome parameters were the accumulated number of recalled pictures over the three learning trials and the number of pictures recalled after 20 minutes. Reliability and sensitivity of these tests in an elderly population have been published elsewhere (14). Cognitive function was tested at six different time points during the study, before randomization, at baseline, after 9, 18, and 30 months, and at the end of the study. The time point of this last measurement was different for the participants (at 36-48 months) therefore we performed the analyses with their individually varying time-point but report the results for the mean of these time points (at 42 months). The pre-randomized measurement was discarded in the analysis to preclude possible learning effects. Since the MMSE is not suitable for longitudinal research because of learning and ceiling effects, MMSE scores are not reported here.

Genotyping

We selected four SNPs in the promoter region of the IL-10 gene, 4259AG (rs3024498), -1082GA (rs1800896), -592CA (rs1800872), and -2849GA (rs6703630) based on the frequency of the minor allele and possible functionality. All polymorphisms were genotyped by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS), using the Sequenom MassARRAYtm methodology (Sequenom Inc, San Diego, CA, USA). Amplification reactions and parameters were based on the manufacturer's instructions.

Statistical analysis

The program Haploview (15)was used to estimate the allele frequencies, test the consistency of the genotype frequencies at each SNP locus with Hardy-Weinberg equilibrium, and estimate and plot pairwise linkage disequilibrium (LD) between the SNPs examined. Haplotypes and haplotype frequencies were calculated using SNPHAP software (http://www-gene.cimr.cam.ac.uk/clayton/software). We used multiple imputation analysis to deal with incomplete data and to account for many haplotype probabilities per subject. This method has been described elsewhere in more detail (16). Haplotypes with a frequency of less than 5 % were combined and included in all analyses, without reporting the results. The haplotype analysis approach used in this study assumes an additive effect of the haplotypes, and details of this approach have been described elsewhere (17).

The associations between the four IL-10 SNPs, the IL-10 haplotypes and cognitive function during follow-up were assessed with a linear mixed model for repeated measurements. The estimate for time represents the cognitive decline per year. The results for the genotypes represent the mean difference over time between the genotypes. All longitudinal analyses were adjusted for sex, age, education, country, use of pravastatin, and where appropriate, version of test used. All statistical analyses were performed with SPSS software (version 12.0.1, SPSS Inc, Chicago, Ill). P-values lower than 0.05 were considered statistically significant.

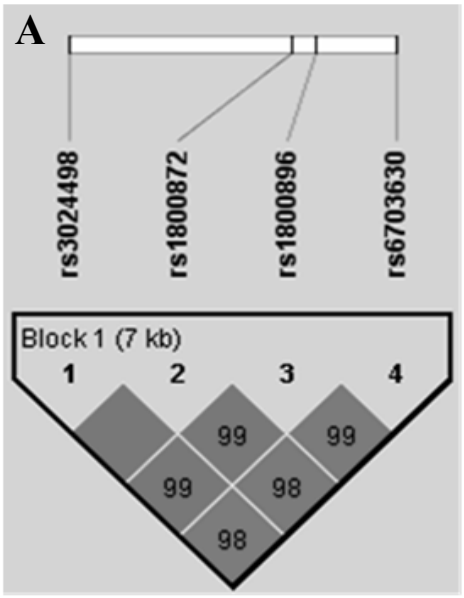
Results

The mean age of the participants was 75.3 years and approximately 50% were female (table 1). There were significant differences in minor allele frequencies between the countries (p-value Chi-square < 0.01). The variants 4259G, -1082A and -2849A were more common in the Irish subjects compared with the subjects from Scotland and the Netherlands. Therefore, all analyses were adjusted for country. Mean follow-up of study subjects was 42 months (range 36-48 months).

Table 1: Baseline characteristics of the participants of the PROSPER study per country.

	Scotland (N=2520)	Ireland (N=2184)	The Netherlands (N=1100)
Continous variates (mean, SD)			
Age (years)	75.3 (3.4)	75.5 (3.3)	75.1 (3.3)
Body Mass index, (kg/m2)	26.7 (4.2)	27.0 (4.4)	26.7 (3.8)
Total cholesterol, (mmol/L)	5.7 (1.0)	5.6 (0.9)	5.8 (0.9)
LDL cholesterol, (mmol/L)	3.8 (0.8)	3.7 (0.8)	3.9 (0.8)
HDL cholesterol, (mmol/L)	1.3 (0.4)	1.3 (0.4)	1.3 (0.3)
Categorical variates (n, %)			
Female	1283 (51)	1197 (55)	520 (47)
Current smoker	708 (28)	583 (27)	267 (24)
History of diabetes	213 (9)	225 (10)	185 (17)
History of hypertension	1446 (57)	1441 (66)	705 (64)
History of angina	811 (32)	523 (24)	225 (21)
History of claudication	229 (9)	114 (5)	47 (4)
History of myocardial infarction	379 (15)	258 (12)	139 (13)
History of vascular disease	1239 (49)	849 (39)	477 (43)
History of stroke or TIA	265 (11)	222 (10)	162 (15)
Genotype, minor allele frequency (%)			
IL-10 4259AG	30	33	28
IL-10 -1082GA	51	55	50
IL-10 -592CA	21	20	21
IL-10 -2849GA	30	33	29

Genotyping of the four IL-10 polymorphisms was complete for 5786 subjects. All four SNPs were in Hardy-Weinberg equilibrium (all $p>0.3$). The four SNPs were in strong linkage disequilibrium (LD) and occurred together in one haploblock (figure 1A). Six haplotypes were found in our study population (figure 1B). The four haplotypes with a frequency above 5% were included in analyses. We used H1111, with no variants present, as reference haplotype. H2122, the most frequent haplotype, had three variant alleles, 4259G, -1082A and -2849A. H1121 carried the -1082A variant, and H1211 the -592A variant.



B

	4259AG	-592CA	-1082GA	-2849GA	Haplotype frequency
H1	2	1	2	2	0.304
H2	1	1	1	1	0.269
H3	1	1	2	1	0.209
H4	1	2	1	1	0.207
H5	1	1	2	2	0.009
H6	2	1	2	1	0.003

Figure 1: Haplotype information.

Figure A shows the linkage disequilibrium (LD) between the single nucleotide polymorphisms (SNPs) examined. All SNPs are in LD and occur together in one haploblock. Figure B shows the haplotype frequencies. Only the first four haplotypes (frequency> 5%) were included in the analyses.

Table 2 and figure 2 represents the results of the association between the four IL-10 polymorphisms and cognitive function during follow-up. The term for time was significant for all domains of cognitive function, indicating that all domains declined over time (table 2). Subjects carrying the 4259G variant had significantly worse cognitive function compared to carriers of the wild-type variant (all $p<0.05$) as depicted in figure 2A in a gene-dose dependent manner. Also carriers of the 2849A variant performed worse on all cognitive domains compared to carriers of the wild-type variant (figure 2B). The same trend was seen with -1082A and -592A carriers but not significant compared to wild-type subjects. Excluding subjects with a history of stroke and those who suffered a stroke during follow-up did not materially change our results. There was no significant interaction between time and genotypes for all cognitive domains (all $p>0.05$).

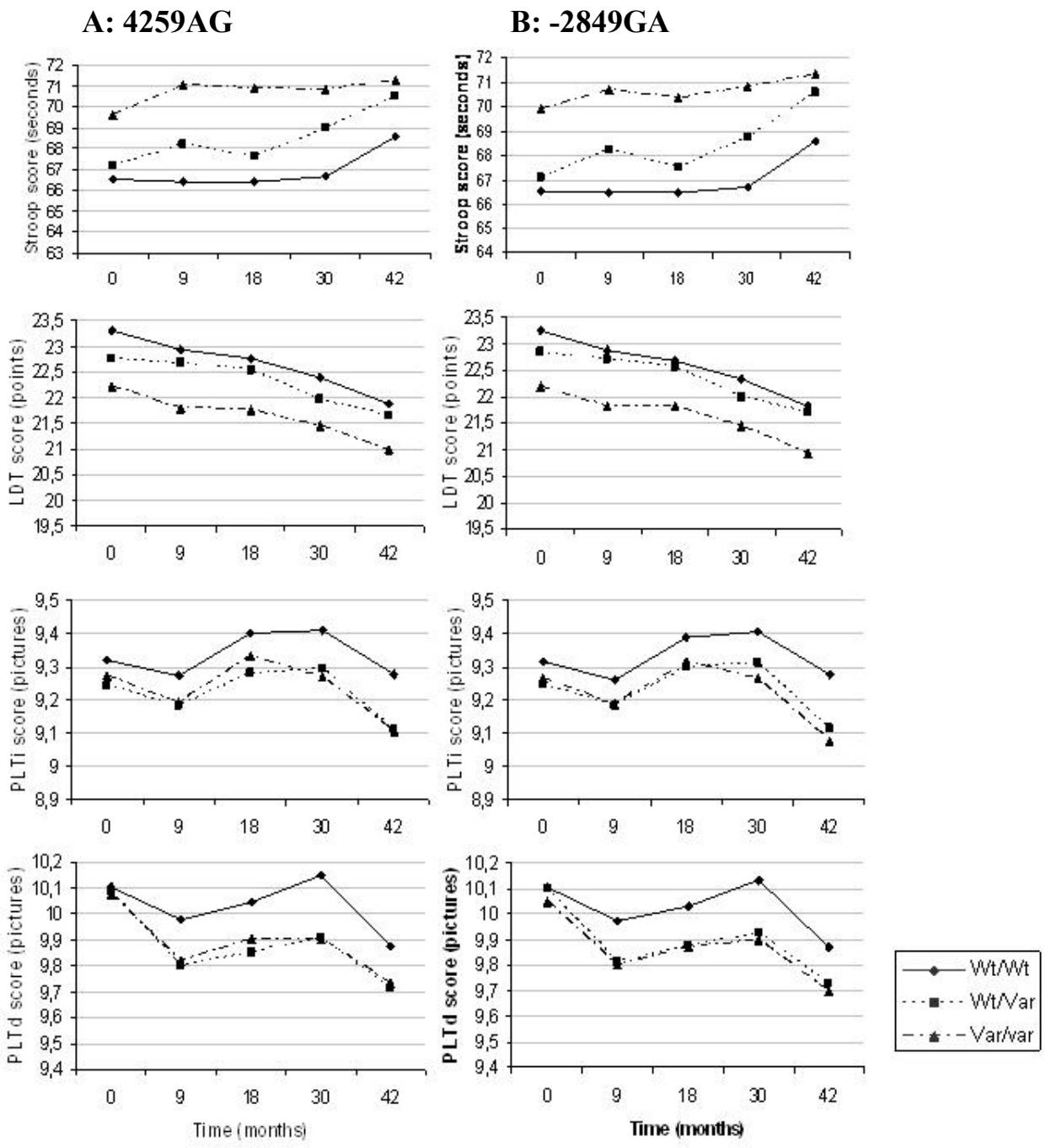


Figure 2: Representation of association between two IL-10 polymorphisms and cognition.
Figure2A represents the association between the 4259AG polymorphism with cognition and 2B the association between the -2849GA polymorphism with cognition. The straight line represents the homozygous wild-type carriers, the dotted line with the squares represents the heterozygous carriers, and the dotted line with the triangles represents the homozygous variant carriers

In table 3 the results of the association between IL-10 haplotypes and cognitive function during follow-up are shown. H1111 was used as reference. As in the SNP analysis, the term for time was

significant for all domains of cognitive function, indicating that all domains declined over time. H2122 with the variant alleles of 4259G, -1082A and -2849A was associated with worse cognitive function on all cognitive domains compared to the reference haplotype (all $p<0.03$). There also was a significant association between H1211 and attention ($p=0.01$). A comparable trend was also seen for the other cognitive domains, but did not reach statistical significance. There was no association with H1121 and cognition. Excluding subjects with a history of stroke and those who suffered a stroke during follow-up did not materially change our results. Again, no significant interaction between time and haplotypes was found for all cognitive domains (all $p>0.05$).

Discussion

Here we found consistent associations between genetic variation in the promoter region of the IL-10 gene and cognitive function in an elderly population. In the single SNP analysis we found that especially the -2849GA and 4259AG polymorphisms were associated with cognitive function over the follow-up period. Carriers of these variants performed significantly worse on all cognitive domains. For the other two SNPs, -1082GA and -592CA, the same trend was seen, but not significant. Also, the haplotype with three variants present (4259G, -1082A and -2849A) was associated with a decreased cognitive function on all cognitive domains. Excluding all subjects with a history of stroke and those who suffered a stroke during follow-up did not materially change our results.

We found four promoter polymorphisms within the IL-10 gene (4259AG, -1082GA, -592CA and -2849GA) to be associated with cognitive function. The haplotype most prominently associating with different cognitive variables (Table 3) is H2122, which is the only haplotype containing the minor allele of 4259AG. This allele on itself associated to cognition with the same significance (Table 2). It is very likely that our major haplotype association is driven by the 4295AG SNP. Various studies have investigated the association between IL-10 promoter polymorphisms and Alzheimer's disease before (18-23).

Table 2: Association between IL-10 polymorphisms and cognition

	Time		4259AG		-1082GA		-592CA		-2849GA	
All subjects	Estimate (SE)	P	Estimate (SE)	p	Estimate (SE)	p	Estimate (SE)	p	Estimate (SE)	p
Attention, seconds	0.68 (0.07)	<0.001	**1.73 (0.54)**	**0.001**	0.64 (0.50)	0.198	0.87 (0.62)	0.163	**1.66 (0.54)**	**0.002**
Processing speed*	-0.38 (0.02)	<0.001	**-0.45 (0.15)**	**0.003**	**-0.29 (0.14)**	**0.034**	0.02 (0.17)	0.921	**-0.40 (0.15)**	**0.008**
Immediate memory**	-0.01 (0.01)	0.086	**-0.07 (0.03)**	**0.036**	-0.03 (0.03)	0.340	-0.05 (0.04)	0.237	**-0.07 (0.03)**	**0.048**
Delayed memory**	-0.06 (0.01)	<0.001	**-0.10 (0.05)**	**0.043**	-0.04 (0.04)	0.361	-0.04 (0.06)	0.443	-0.09 (0.05)	0.053
Excluding subjects with stroke in history or follow-up										
Attention, seconds	0.51 (0.07)	<0.001	**1.54 (0.59)**	**0.008**	0.39 (0.54)	0.472	1.11 (0.66)	0.095	**1.48 (0.58)**	**0.011**
Processing speed*	-0.34 (0.02)	<0.001	**-0.45 (0.16)**	**0.006**	-0.26 (0.15)	0.085	0.04 (0.19)	0.834	**-0.42 (0.16)**	**0.011**
Immediate memory**	-0.01 (0.01)	0.086	-0.06 (0.04)	0.120	-0.00 (0.03)	0.964	**-0.09 (0.04)**	**0.028**	-0.06 (0.04)	0.099
Delayed memory**	-0.03 (0.01)	<0.001	-0.07 (0.05)	0.185	0.01 (0.05)	0.846	-0.10 (0.06)	0.086	-0.08 (0.05)	0.140

Estimates and p-values were assessed with linear mixed models adjusted for sex, age, education, country, pravastatin use and where appropriate, version of test used.

*assessed in number of digits, **assessed in number recalled

Table 3: Results of the association between IL-10 haplotypes and cognition

	Time		H1111	H2122		H1121		H1211	
All subjects	Estimate (se)	p-value	Estimate (se)	Estimate (se)	p-value	Estimate (se)	p-value	Estimate (se)	p-value
Attention, seconds	0.68 (0.07)	<0.001	Ref	**2.03 (0.66)**	**0.001**	0.12 (0.73)	0.571	**1.64 (0.74)**	**0.014**
Processing speed*	-0.36 (0.01)	<0.001	Ref	**-0.51 (0.18)**	**0.002**	-0.14 (0.20)	0.251	-0.23 (0.20)	0.131
Immediate memory**	-0.01 (0.01)	0.021	Ref	**-0.09 (0.04)**	**0.016**	-0.00 (0.05)	0.468	-0.06 (0.05)	0.117
Delayed memory**	-0.06 (0.01)	<0.001	Ref	**-0.11 (0.06)**	**0.033**	0.01 (0.06)	0.544	-0.06 (0.07)	0.200
Excluding subjects with stroke in history or follow-up									
Attention	0.53 (0.07)	<0.001	Ref	**1.78 (0.71)**	**0.006**	-0.16 (0.79)	0.579	**1.61 (0.78)**	**0.020**
Processing speed*	-0.34 (0.02)	<0.001	Ref	**-0.51 (0.19)**	**0.004**	-0.08 (0.22)	0.363	-0.23 (0.21)	0.136
Immediate memory**	-0.00 (0.01)	0.846	Ref	**-0.08 (0.05)**	**0.048**	0.01 (0.05)	0.599	**-0.09 (0.05)**	**0.040**
Delayed memory**	-0.04 (0.01)	<0.001	Ref	-0.09 (0.06)	0.078	0.05 (0.07)	0.813	-0.08 (0.07)	0.121

All estimates are assessed with linear mixed models adjusted for sex, age, education, country, pravastatin use, other haplotypes after 10 imputation analyses, and where appropriate, version of test used. *assessed in number of digits, **assessed in number recalled

The majority of the studies found that the prevalences of the variant alleles of the -1082GA and -592CA polymorphisms were increased in patients with Alzheimer's disease compared to healthy controls (18-20;23). To our knowledge, no previous studies have been performed so far with the other two SNPs, 4259AG and -2849GA. Moreover, to our knowledge we are also the first to investigate the association between the four promoter polymorphisms and cognitive function.

It has been shown that patients with Alzheimer's disease have lower IL-10 serum levels compared to healthy controls (18;24). Production of IL-10 is under tight genetic control, with heritability estimates between 50-70% (10). Part of this genetic variation comes from polymorphisms in its own promoter sequence. We have previously reported that carriers of the IL-10 -2849AA genotype have significantly lower IL-10 responsiveness upon stimulation with bacterial lipopolysaccharide (LPS) (11). Moreover, Koss *et al* have demonstrated that the variant -1082A allele is associated with a decreased IL-10 production (25). Also, the variant 4259G allele is associated with less IL-10 transcripts compared to the wild-type 4259A allele (26). Therefore, it is likely that subjects with one or more of these polymorphisms have lower IL-10 production capacity.

Because these four polymorphisms in the promoter region of the IL-10 gene are all associated with a decrease in IL-10 responsiveness, they are a good reflection of the systemic levels of IL-10. In observational studies it usually problematic to exclude the possibility of reverse causality, which means that lower levels of IL-10 might be a consequence of the disease rather than a risk factor for the disease. Therefore genetic variation is a very useful marker to overcome this problem of reverse causality. Furthermore, based on Mendel's law that inheritance of one trait is independent of inheritance of other traits (9), we assume that the association between genetic variation in inflammatory genes and cognitive function is unconfounded while the moderate associations found with systemic cytokine levels and cognitive function are confounded.

We have showed in a previous study that genetic variation in the promoter region of the IL-10 gene is associated with an increased risk for incident stroke (27). To exclude the possibility that the

decreased cognitive function in subjects with one or more variant alleles in the promoter region of the IL-10 gene was caused by a difference in prevalence and incidence of stroke, we repeated all analyses excluding subjects with a history of stroke or an incident stroke during follow-up. When we excluded subjects with stroke in all associations, we still found that subjects carrying the variant alleles had a decreased cognitive function compared to wild-type carriers. Therefore we think that genetic variation in the IL-10 gene decreases cognitive function in the elderly without overt evidence of a cerebrovascular event.

One of the strengths of our study is our population size. We have prospective data of over 5000 subjects on cognitive function in three different countries. Another strength of our study is that all subjects were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. Therefore we could exclude subjects with a stroke in history and follow-up to strengthen the hypothesis that genetic variation in the IL-10 gene decreases cognitive function in the elderly independent of stroke.

Furthermore, our population is appropriate to measure cognitive function because only subjects with a MMSE above 24 points could participate, which makes it a homogenous study group suitable for investigating cognitive function. Also the fact that we have a follow-up of 42 months with little lost to follow-up is a strong element of our study. No interaction between the genotypes and time was found, but prior to analysis we did not expect to find this interaction. We assumed that carriers of the polymorphisms would have developed a difference in cognition already early in life, therefore an additional decline in this elderly population was not expected.

In conclusion, genetic variation in the promoter region of the IL-10 gene is associated with decreased cognitive function in individuals without overt evidence of a cerebrovascular event. This provides evidence that genetic variation in the IL-10 gene is a good marker for risk prediction of cognitive function. If these findings are confirmed and adequately explained on the basis of independent studies, screening patients for the IL-10 promoter polymorphisms may contribute to a

better risk stratification of patients at increased risk for cognitive decline and may improve individual treatment.

Acknowledgements

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Reference List

(1) Wilson CJ, Finch CE, Cohen HJ. Cytokines and cognition--the case for a head-to-toe inflammatory paradigm. J Am Geriatr Soc 2002 Dec;50(12):2041-56.

(2) Campbell IL, Stalder AK, Chiang CS, Bellinger R, Heyser CJ, Steffensen S, et al. Transgenic models to assess the pathogenic actions of cytokines in the central nervous system. Mol Psychiatry 1997 Mar;2(2):125-9.

(3) Ho GJ, Drego R, Hakimian E, Masliah E. Mechanisms of cell signaling and inflammation in Alzheimer's disease. Curr Drug Targets Inflamm Allergy 2005 Apr;4(2):247-56.

(4) Eikelenboom P, Veerhuis R. The importance of inflammatory mechanisms for the development of Alzheimer's disease. Exp Gerontol 1999 Jun;34(3):453-61.

(5) Gibertini M, Newton C, Friedman H, Klein TW. Spatial learning impairment in mice infected with Legionella pneumophila or administered exogenous interleukin-1-beta. Brain Behav Immun 1995 Jun;9(2):113-28.

(6) Thomson LM, Sutherland RJ. Systemic administration of lipopolysaccharide and interleukin-1beta have different effects on memory consolidation. Brain Res Bull 2005 Sep 30;67(1-2):24-9.

(7) van Exel E, de Craen AJ, Remarque EJ, Gussekloo J, Houx P, Bootsma-van der Wiel A, et al. Interaction of atherosclerosis and inflammation in elderly subjects with poor cognitive function. Neurology 2003 Dec 23;61(12):1695-701.

(8) Schram MT, Euser SM, de Craen AJ, Witteman JC, Frolich M, Hofman A, et al. Systemic markers of inflammation and cognitive decline in old age. J Am Geriatr Soc 2007 May;55(5):708-16.

(9) Davey SG, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol 2003 Feb;32(1):1-22.

(10) de Craen AJ, Posthuma D, Remarque EJ, van den Biggelaar AH, Westendorp RG, Boomsma DI. Heritability estimates of innate immunity: an extended twin study. Genes Immun 2005 Mar;6(2):167-70.

(11) Westendorp RG, van Dunne FM, Kirkwood TB, Helmerhorst FM, Huizinga TW. Optimizing human fertility and survival. Nat Med 2001 Aug;7(8):873.

(12) Shepherd J, Blauw GJ, Murphy MB, Cobbe SM, Bollen EL, Buckley BM, et al. The design of a prospective study of Pravastatin in the Elderly at Risk (PROSPER). PROSPER Study Group. PROspective Study of Pravastatin in the Elderly at Risk. Am J Cardiol 1999 Nov 15;84(10):1192-7.

(13) Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Cobbe SM, et al. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet 2002 Nov 23;360(9346):1623-30.

(14) Houx PJ, Shepherd J, Blauw GJ, Murphy MB, Ford I, Bollen EL, et al. Testing cognitive function in elderly populations: the PROSPER study. PROspective Study of Pravastatin in the Elderly at Risk. J Neurol Neurosurg Psychiatry 2002 Oct;73(4):385-9.

(15) Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005 Jan 15;21(2):263-5.

(16) Harel O, Zhou XH. Multiple imputation: review of theory, implementation and software. Stat Med 2007 Jan 29.

(17) Wallenstein S, Hodge SE, Weston A. Logistic regression model for analyzing extended haplotype data. Genet Epidemiol 1998;15(2):173-81.

(18) Arosio B, Trabattoni D, Galimberti L, Bucciarelli P, Fasano F, Calabresi C, et al. Interleukin-10 and interleukin-6 gene polymorphisms as risk factors for Alzheimer's disease. Neurobiol Aging 2004 Sep;25(8):1009-15.

(19) Lio D, Licastro F, Scola L, Chiappelli M, Grimaldi LM, Crivello A, et al. Interleukin-10 promoter polymorphism in sporadic Alzheimer's disease. Genes Immun 2003 Apr;4(3):234-8.

(20) Ma SL, Tang NL, Lam LC, Chiu HF. The association between promoter polymorphism of the interleukin-10 gene and Alzheimer's disease. Neurobiol Aging 2005 Jul;26(7):1005-10.

(21) Peila R, Launer LJ. Inflammation and dementia: epidemiologic evidence. Acta Neurol Scand Suppl 2006;185:102-6.

(22) Ramos EM, Lin MT, Larson EB, Maezawa I, Tseng LH, Edwards KL, et al. Tumor necrosis factor alpha and interleukin 10 promoter region polymorphisms and risk of late-onset Alzheimer disease. Arch Neurol 2006 Aug;63(8):1165-9.

(23) Scassellati C, Zanardini R, Squitti R, Bocchio-Chiavetto L, Bonvicini C, Binetti G, et al. Promoter haplotypes of interleukin-10 gene and sporadic Alzheimer's disease. Neurosci Lett 2004 Feb 12;356(2):119-22.

(24) Remarque EJ, Bollen EL, Weverling-Rijnsburger AW, Laterveer JC, Blauw GJ, Westendorp RG. Patients with Alzheimer's disease display a pro-inflammatory phenotype. Exp Gerontol 2001 Jan;36(1):171-6.

(25) Koss K, Satsangi J, Fanning GC, Welsh KI, Jewell DP. Cytokine (TNF alpha, LT alpha and IL-10) polymorphisms in inflammatory bowel diseases and normal controls: differential effects on production and allele frequencies. Genes Immun 2000 Feb;1(3):185-90.

(26) Kurreeman FA, Schonkeren JJ, Heijmans BT, Toes RE, Huizinga TW. Transcription of the IL10 gene reveals allele-specific regulation at the mRNA level. Hum Mol Genet 2004 Aug 15;13(16):1755-62.

(27) Trompet S, Pons D, de Craen AJ, Slagboom P, Shepherd J, Blauw GJ, et al. Genetic variation in the interleukin-10 gene promoter and risk of coronary and cerebrovascular events: the PROSPER study. Ann N Y Acad Sci 2007 Apr;1100:189-98.

Chapter 6

High innate production capacity of pro-inflammatory cytokines increases risk of death from cancer. Results of the PROSPER study.

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Abstract

Various lines of evidence suggest that pro-inflammatory factors may play a role in tumor growth and metastasis, the leading cause of cancer-related mortality. However, most evidence originates from animal models, only few human studies reported an association between pro-inflammatory cytokines and death from cancer. Here, we investigated the association between circulating levels and innate production capacity of pro-inflammatory cytokines and cancer incidence and mortality in the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER). Circulating levels of IL-6 and CRP were measured in all 5804 participants. The innate production capacity of IL-6, IL1β and TNFα were measured in a random sample of 403 subjects. We showed that high circulating inflammatory markers were associated with an increased risk of cancer incidence and death from cancer during follow-up (all $p< 0.05$). Moreover, high innate pro-inflammatory cytokine production capacity is associated with an increased risk of death from cancer (all $p<0.04$) but not with higher cancer incidence during follow-up (all $p> 0.6$). In conclusion, high innate production capacity of pro-inflammatory cytokines is associated with an increased risk of death from cancer probably due to increased tumor growth and metastasis. As there was no association between innate production capacity and cancer incidence, the association between circulating levels and cancer incidence at least partially reflects reversed causality.

Introduction

Inflammation plays an important role in the development of various age-related diseases like atherosclerosis, stroke, cognitive decline, and dementia (4). Various studies support the hypothesis that inflammatory stimuli, like the pro-inflammatory cytokines interleukin-6 (IL-6), interleukin-1-beta (IL-1β), and tumor necrosis factor-alpha (TNFα) are involved in cancer pathogenesis (5-8). Moreover, elevated levels of various cytokines, like IL-1, IL-6, TNF, fibroblast growth factor (FGF), and transforming growth factor (TGF) have been found in blood, urine, and ascites of cancer patients, suggesting that these cytokines are involved in incidence and growth and spread of cancer (9).

Inflammatory responses are thought to be critical in many aspects of promoting the growth and spread of cancers. A recent study of Kim *et al* showed that cell lines of Lewis lung carcinoma had an increased production of the pro-inflammatory cytokines IL-6 and TNFα through activation of the Toll-like receptor (TLR) family members TLR2 and TLR6 (10). Moreover, pro-inflammatory cytokines are also involved in promoting tumor cell adhesion in metastatic sites which then activate local normal cells to produce tumor growth factors (9). Distant-site metastases are the leading cause of cancer-associated mortality. Furthermore, animal studies have suggested a role for pro-inflammatory cytokines in the generation of cancer-associated cachexia, which is the most important cause of morbidity among cancer patients (6;11-13).

These various lines of evidence suggest that pro-inflammatory factors may play a role in cancer metastasis eventually leading to death. However, most evidence originates from animal models, only a few human studies have reported an association between pro-inflammatory cytokines and death from cancer (14;15). Here, we investigated the association between circulating levels and innate production capacity of pro-inflammatory cytokines and cancer incidence and mortality in the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER).

Methods

A detailed description of the protocol of the PROSPER Study has been published elsewhere (16;17). A short summary is provided here.

Participants

PROSPER was a prospective multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in the elderly. Between December 1997 and May 1999, we screened and enrolled subjects in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. A total number of 5804 subjects were randomly assigned to pravastatin or placebo.

Inflammatory markers

In all subjects C-Reactive Protein (CRP) was measured on stored (at –80°C) and previously unthawed samples by automated particle-enhanced immunoturbidimetric assay (Roche UK, Welwyn Garden City, UK). The method has inter- and intra-assay coefficients of variation of <3%. The laboratory participates in the United Kingdom national external quality control for high-sensitivity CRP. Interleukin-6 was assayed using a high-sensitivity ELISA (R & D Systems, Oxford, UK) with inter and intra-assay coefficients of variation of <6%. All samples were processed by technicians blinded to the identity of samples.

Innate cytokine production capacity was measured in the final 30% of the Dutch participants at baseline, resulted in a random subsample of 403 subjects. Whole blood samples were stimulated with 10 ng/ml of lipopolysaccharide (LPS) to assess the innate production capacity of IL-1β, IL-6, and TNFα. Unstimulated baseline samples were obtained to serve as a control for contamination.

Cancer incidence and mortality

All subjects included into the PROSPER study did not have a history of malignancy within the 5 years prior to the start of the trial. Tertiary study endpoints of the PROSPER trial included cancer incidence and mortality. All study endpoints were adjudicated by a study endpoint committee. We extended the follow-up period for the 1100 Dutch participants of the PROSPER study. First, incident cancer was requested for the 1100 subjects at the Dutch cancer registry for the period December 1997 and May 1999 until September 2005 (censor date was 15 September 2005). For the same period the mortality status was checked for the 1100 Dutch participants. From the deceased participants the cause of death was obtained from the Dutch Central Bureau of Statistics. Only the primary cause of death on the death certificate was taken into account.

Statistical analysis

The circulating CRP and IL-6 measurements of all subjects and the innate cytokine production levels of the 403 participants were dichotomized in two groups based on the median cytokine production level. All associations between the two groups of cytokine production levels and cancer

incidence or death from cancer were assessed with a Cox-proportional hazard model adjusted for sex, age, current smoking, use of pravastatin and country where appropriate. These associations were visually depicted with Kaplan-Meier survival curves. The SPSS software (version 16.0.1, SPSS Inc, Chicago, Ill) was used for all statistical analyses. P-values lower than 0.05 were considered statistically significant.

Results

Baseline characteristics of the 5804 subjects of the PROSPER study are presented in table 1. The mean age of the subjects was 75.3 years and about half of them were female. The baseline characteristics of the random sample of the 403 subjects with additionally obtained innate cytokine production capacities are also shown in table 1. Both groups were similar in baseline characteristics.

Table 1: Baseline characteristics of the participants of the PROSPER study

	Total Group (N = 5804)	Random Sample (N = 403)
Demographics		
Age	75.3 (3.3)	75.1 (3.3)
Female, N(%)	3000 (52)	187 (46)
Education ≥ 13 years	15.1 (2.0)	15.0 (2.8)
Current smokers, N(%)	1558 (27)	96 (24)
Weight	73.4 (13.4)	77.7 (11.6)
Body Mass Index	26.8 (4.2)	26.9 (3.6)
Cancer, N(%)		
Cancer incidence	444 (8)*	45 (11)†
Cancer mortality	206 (4)*	26 (7)†

Data is presented as mean (SD) unless otherwise stated.

* Measured after 3 years of follow-up

† Measured after 7 years of follow-up

Cancer incidence and mortality was measured for the total group for a mean follow-up period of 3.2 years, for the random sample we extended the initial follow-up period with 3.5 years to 6.7 years.

The percentages of cancer incidence and cancer mortality are therefore higher in the random sample.

Table 2: Association between circulating levels of inflammatory markers and cancer risk

	Inflammatory marker			
	< Median n/N (%)	> Median n/N (%)	Hazard ratio (95% CI)	p-value
Cancer incidence				
CRP	197/2838 (7)	234/2842 (8)	1.20 (0.99-1.45)	0.063
Interleukin-6	179/2826 (6)	253/2827 (9)	1.35 (1.11-1.64)	**0.003**
Cancer mortality				
CRP	82/2838 (3)	119/2842 (4)	1.42 (1.07-1.89)	**0.014**
Interleukin-6	78/2826 (3)	123/2827 (4)	1.55 (1.16-2.07)	**0.003**

Hazard ratios are assessed with the Cox-proportional hazard model adjusted for sex, age, country, current smokers, and use of pravastatin.

The association between circulating inflammatory markers and cancer risk is shown in table 2. The hazard ratio for cancer incidence for subjects with high levels of CRP was 1.20 (p=0.06) compared to subjects with low CRP levels. Moreover, the hazard ratio for cancer incidence for subjects with high levels of interleukin-6 was 1.35 (p=0.003) compared to subjects with low IL-6 levels. High levels of both inflammatory markers were also significantly associated with an increased risk for death from cancer compared to low levels (HR=1.42, p=0.01 and HR=1.55, p=0.003 respectively).

In table 3 the association is shown between the innate production capacity and cancer risk in a random sample of 403 subjects. No associations were found for innate cytokine production capacity and cancer incidence. However, innate production capacity levels of the pro-inflammatory cytokines IL-1β, IL-6, and TNFα were significantly associated with death from cancer (all p-values below 0.04). Participants with high production capacity levels of these cytokines had a higher risk for death from cancer compared to participants with low cytokine production levels. There was no association between high IL-1β and TNFα cytokine production levels and other causes of death, whereas high IL-6 production capacity was also associated with an increased risk for all other deaths except cancer (HR 1.92, p=0.04).

Table 3: Association between innate inflammatory cytokine production capacity and cancer risk

	Cytokine level			
	< Median n/N (%)	> Median n/N (%)	Hazard ratio (95% CI)	p-value
Cancer incidence				
IL-1β	24/201 (12)	20/202 (10)	0.85 (0.47-1.55)	0.60
IL-6	21/198 (11)	22/199 (11)	1.11 (0.61-2.02)	0.74
TNFα	23/201 (11)	21/202 (10)	0.92 (0.51-1.67)	0.78
Cancer mortality				
IL-1β	8/201 (4)	18/202 (9)	2.67 (1.15-6.20)	**0.02**
IL-6	8/198 (4)	18/199 (9)	2.51 (1.09-5.81)	**0.03**
TNFα	7/201 (3)	19/202 (9)	3.14 (1.31-7.54)	**0.01**

Hazard ratios are assessed with the Cox-proportional hazard model adjusted for sex, age, current smokers, and use of pravastatin.

Discussion

We assessed the association between circulating levels and innate production capacity of pro-inflammatory cytokines in whole blood samples and cancer incidence and mortality. High levels of the circulating inflammatory markers were associated with an increased risk of cancer incidence and death from cancer. Furthermore, we showed that high innate pro-inflammatory cytokine production capacity was associated with an increased risk of death from cancer during follow-up, while high innate production capacity of pro-inflammatory cytokines was not associated with incident cancer.

We found that a high innate pro-inflammatory cytokine production capacity is a risk factor for cancer mortality but not for cancer incidence and also not for any other causes of death. This indicates that circulating markers of inflammation are increased in cancer patients, probably by autocrine production of the cancer cells themselves. There are two ways to explain the association between the innate production capacity of IL-1β, IL-6, and TNFα and death from cancer. First, pro-inflammatory cytokines play an important role in promoting the growth and spread of cancers.

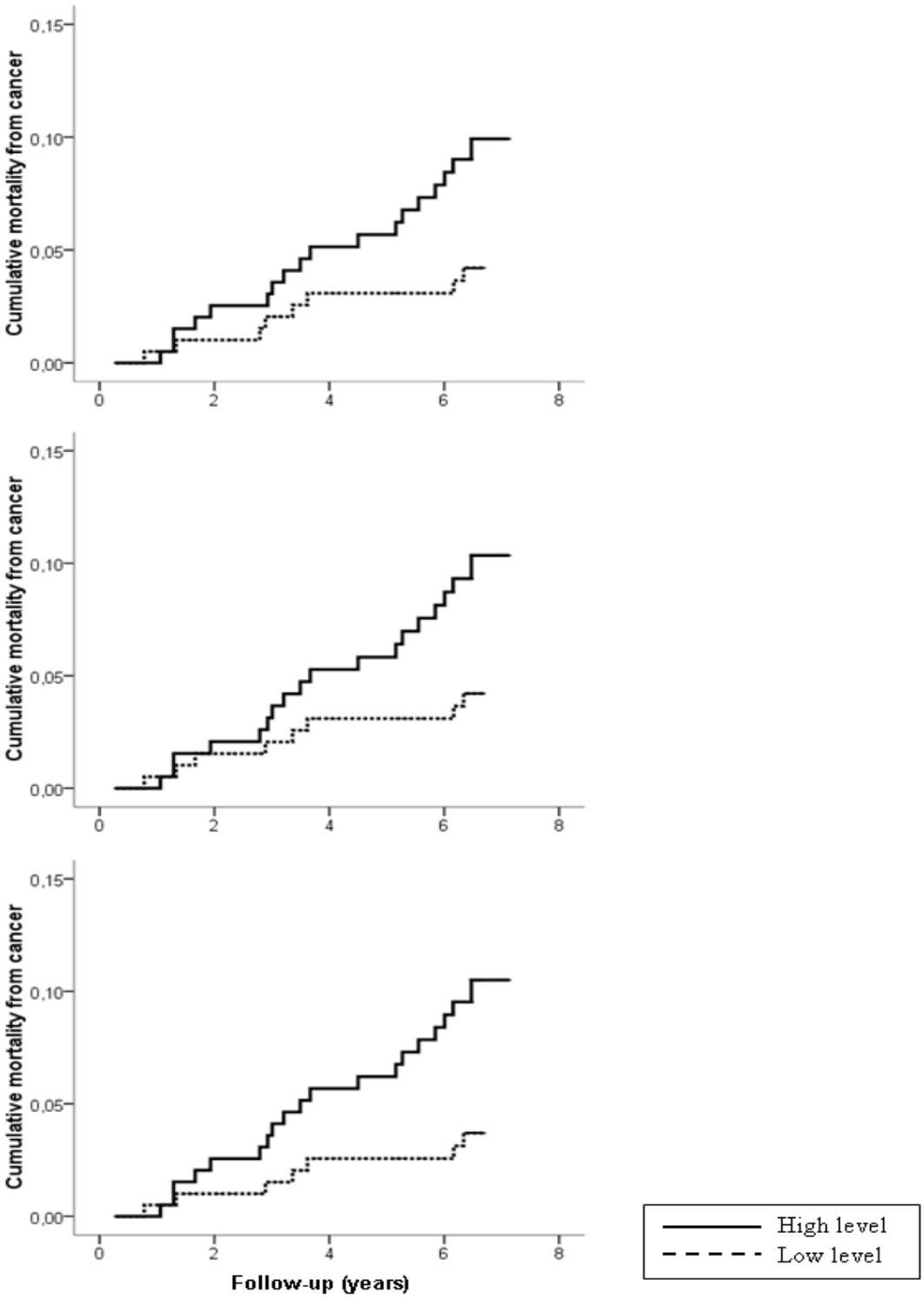

Figure 1: Kaplan-Meier curves

The dotted line indicates high innate cytokine production capacity; the straight line indicates low innate cytokine production capacity.

There are some examples of solid tumors proliferating in response to IL-1, IL-2 and IL-6 (9). Cytokines are also involved in promoting tumor cell adhesion in metastatic sites and then activate local normal cells to produce tumor growth factors (9). Furthermore, TNFα receptors have been associated with tumor cells, suggesting that TNFα could play a role in cancer growth (18-20). The most convincing evidence comes from the recent study of Kim *et al* who reported that cell lines of Lewis lung carcinoma had an increased production of the pro-inflammatory cytokines IL-6 and TNFα through activation of the Toll-like receptor (TLR) family members TLR2 and TLR6 (10). Moreover, both TNFα and TLR2 were found to be required for Lewis lung carcinoma metastases in mice (10).

Second, animal studies have suggested that pro-inflammatory cytokines may have a role in cancer related cachexia, which is an important cause of morbidity and mortality in cancer patients (6;11-13). In a tumor model used by Strassmann *et al* it was suggested that IL-1 and IL-6 are involved in mediating cachexia (21;22). Administrating IL-6 antibodies in a similar model partially reversed the weight loss. Mice with TNFα producing tumors also developed cachexia and administration of TNF neutralizing antibodies reversed the weight loss related to cachexia (19).

While we found an association between circulating inflammatory markers and cancer incidence, we found no association between innate production capacity and incident cancer. This might indicate that the association between circulating inflammatory markers and cancer incidence might be disturbed by reverse causality since it has been shown in various studies that tumor cells have autocrine production of pro-inflammatory cytokines (9). Although all participants of the PROSPER study had to be free of cancer in the 5 years prior to the study, underlying cancer which had not been diagnosed yet could have resulted in higher levels of circulating inflammatory markers. Alternatively, strong cancer risk factors may have contributed to an altered inflammatory milieu. By investigating the association between innate cytokine production capacity and cancer incidence we do not have the problem of reverse causality, since innate production capacity reflects the maximum response to LPS in an individual independent of cytokine production by tumor cells. Therefore we

suggest that in subjects with cancer a strong pro-inflammatory profile is associated with an increased risk of dying, but the increased innate production capacity does not lead to an increased risk for developing cancer.

A possible limitation to use the PROSPER study cohort for this research question is that subjects were selected to have a history of vascular disease or have an increased risk for such a disease, and the results can only be extrapolated with this in mind to the general population. One of the strengths of our study is our population size. We had prospective data of over 5000 subjects on various outcomes in three different countries. Because of the large population size, we had sufficient cases of incident cancer to reach a high power for statistical analyses. Furthermore, all subjects were included into the study when they did not have a history of malignancy within the 5 years prior to the start of the trial. Cancer incidence and mortality were main outcomes of our study and were accurately monitored. Also the fact that we had a follow-up of 3.2 years for all subjects with little lost to follow-up is a strong element of our study.

In conclusion, high innate production capacity of pro-inflammatory cytokines is associated with an increased risk of cancer mortality probably due to increased tumor growth and metastasis. No association was found between innate production capacity and cancer incidence, which indicates that the association between circulating levels and cancer incidence is probably disturbed by reversed causality. Anti-cytokine therapy for the IL-1b, IL-6, and TNFa cytokines might be of therapeutic interest for advanced cancer (1;2). Blocking the pro-inflammatory cytokines by anti-cytokine based therapies might reduce tumor growth and metastasis, the leading cause of cancer-associated mortality. Moreover it might reverse cachexia-induced weight loss (3). Hence, when tumor growth and progression and cancer-related cachexia can be delayed or reversed by administering antibodies against pro-inflammatory cytokines, the survival time for cancer patients might be extended.

Acknowledgements

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Reference List

(1) Yan L, Anderson GM, Dewitte M, Nakada MT. Therapeutic potential of cytokine and chemokine antagonists in cancer therapy. Eur J Cancer 2006 Apr;42(6):793-802.

(2) McMillan DC, Leen E, Smith J, Sturgeon C, Preston T, Cooke TG, et al. Effect of extended ibuprofen administration on the acute phase protein response in colorectal cancer patients. Eur J Surg Oncol 1995 Oct;21(5):531-4.

(3) McMillan DC, Wigmore SJ, Fearon KC, O'Gorman P, Wright CE, McArdle CS. A prospective randomized study of megestrol acetate and ibuprofen in gastrointestinal cancer patients with weight loss. Br J Cancer 1999 Feb;79(3-4):495-500.

(4) Caruso C, Lio D, Cavallone L, Franceschi C. Aging, longevity, inflammation, and cancer. Ann N Y Acad Sci 2004 Dec;1028:1-13.

(5) Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G. Inflammation and cancer: how hot is the link? Biochem Pharmacol 2006 Nov 30;72(11):1605-21.

(6) de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer 2006 Jan;6(1):24-37.

(7) Dinarello CA. The paradox of pro-inflammatory cytokines in cancer. Cancer Metastasis Rev 2006 Sep;25(3):307-13.

(8) Tan TT, Coussens LM. Humoral immunity, inflammation and cancer. Curr Opin Immunol 2007 Apr;19(2):209-16.

(9) Dunlop RJ, Campbell CW. Cytokines and advanced cancer. J Pain Symptom Manage 2000 Sep;20(3):214-32.

(10) Kim S, Takahashi H, Lin WW, Descargues P, Grivennikov S, Kim Y, et al. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. Nature 2009 Jan 1;457(7225):102-6.

(11) Deans C, Wigmore SJ. Systemic inflammation, cachexia and prognosis in patients with cancer. Curr Opin Clin Nutr Metab Care 2005 May;8(3):265-9.

(12) Fearon KC, Barber MD, Falconer JS, McMillan DC, Ross JA, Preston T. Pancreatic cancer as a model: inflammatory mediators, acute-phase response, and cancer cachexia. World J Surg 1999 Jun;23(6):584-8.

(13) McNamara MJ, Alexander HR, Norton JA. Cytokines and their role in the pathophysiology of cancer cachexia. JPEN J Parenter Enteral Nutr 1992 Nov;16(6 Suppl):50S-5S.

(14) Falconer JS, Fearon KC, Ross JA, Elton R, Wigmore SJ, Garden OJ, et al. Acute-phase protein response and survival duration of patients with pancreatic cancer. Cancer 1995 Apr 15;75(8):2077-82.

(15) Heikkila K, Ebrahim S, Lawlor DA. Systematic review of the association between circulating interleukin-6 (IL-6) and cancer. Eur J Cancer 2008 May;44(7):937-45.

(16) Shepherd J, Blauw GJ, Murphy MB, Cobbe SM, Bollen EL, Buckley BM, et al. The design of a prospective study of Pravastatin in the Elderly at Risk (PROSPER). PROSPER Study

Group. PROspective Study of Pravastatin in the Elderly at Risk. Am J Cardiol 1999 Nov 15;84(10):1192-7.

(17) Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Cobbe SM, et al. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet 2002 Nov 23;360(9346):1623-30.

(18) Naylor MS, Stamp GW, Foulkes WD, Eccles D, Balkwill FR. Tumor necrosis factor and its receptors in human ovarian cancer. Potential role in disease progression. J Clin Invest 1993 May;91(5):2194-206.

(19) Sharma R, Anker SD. Cytokines, apoptosis and cachexia: the potential for TNF antagonism. Int J Cardiol 2002 Sep;85(1):161-71.

(20) Yoneda T, Alsina MA, Chavez JB, Bonewald L, Nishimura R, Mundy GR. Evidence that tumor necrosis factor plays a pathogenetic role in the paraneoplastic syndromes of cachexia, hypercalcemia, and leukocytosis in a human tumor in nude mice. J Clin Invest 1991 Mar;87(3):977-85.

(21) Strassmann G, Jacob CO, Evans R, Beall D, Fong M. Mechanisms of experimental cancer cachexia. Interaction between mononuclear phagocytes and colon-26 carcinoma and its relevance to IL-6-mediated cancer cachexia. J Immunol 1992 Jun 1;148(11):3674-8.

(22) Strassmann G, Masui Y, Chizzonite R, Fong M. Mechanisms of experimental cancer cachexia. Local involvement of IL-1 in colon-26 tumor. J Immunol 1993 Mar 15;150(6):2341-5.

Chapter 7

Variation in the CBP gene involved in epigenetic control associates with cognitive function.

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Abstract

Research into the pathologic mechanisms of neurodegenerative diseases has revealed that CREB Binding protein (CBP) plays an important role in cognitive dysfunction. Loss of one copy of this gene leads to a syndrome with severe cognitive dysfunction. We investigated the association between four common variants in the CBP gene and cognitive function in 5804 participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER). Baseline associations between genetic variation and cognitive function were assessed with linear regression. Longitudinal associations were assessed with linear mixed models. All analyses were adjusted for sex, age, education, country, version of test, and pravastatin use. The intron 4CT and intron 3AC polymorphisms in the CBP gene were associated with better cognitive performance at baseline and during follow-up. Furthermore, the haplotype with the variant alleles of these two polymorphisms also showed a protective effect on cognitive function in all cognitive domains (all $p<0.03$). Genetic variation in the CBP gene is associated with better cognitive performance in an elderly population. Future research is necessary to investigate the effect of these polymorphisms on the expression of CBP levels and how these polymorphisms affect the gene expression mediated by CBP.

Introduction

Research into the pathologic mechanisms of complex neurodegenerative diseases has revealed that Cyclic AMP Response Element Binding (CREB)- binding protein (CBP) plays an important role in memory formation and cognitive dysfunction (1-4). CBP and its close relative p300 are vital components of the cellular machinery that regulate gene expression. CBP plays a dual role in gene expression (5). First, CBP is involved in epigenetic control as a histone acetyltransferase (HAT) by facilitating the binding of transcription complexes to DNA. Second, CBP acts as a co-activator interacting with CREB and other transcription factors (5). Therefore, alterations in CBP gene expression can ultimately affect the function of entire neuronal circuits such as memory formation (5).

One of the neurodegenerative diseases where CBP is found to be important for cognitive dysfunction is the Rubinstein-Taybi Syndrome (RTS) (6;7). RTS is an autosomal dominant disorder caused by mutations in the CBP gene. The syndrome is characterized by physical abnormalities including broad thumbs and toes, short stature, craniofacial anomalies, and mental retardation (8). Loss of one functional copy of the CBP gene underlies all abnormalities in RTS patients (6;7). Oike *et al* have developed a $CBP^{+/-}$ mutant mouse model with a truncated CBP protein (9). These mice show the same clinical features as RTS patients, including mental retardation. In the same mouse model it was shown that these mice had normal short term memory, but seemed to display deficiencies in long term memory, object recognition, and contextual memory tasks (9).

Since loss of one copy of the CBP gene results in severe cognitive dysfunction, we hypothesized that common genetic variation in the CBP gene might be associated with cognitive function in the general population. Therefore we investigated the association between four single nucleotide polymorphisms (SNPs) in the CBP gene and cognitive function in the participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER).

Methods

A detailed description of the protocol of the PROSPER study has been published elsewhere (10;11). A short summary is provided here.

Participants

PROSPER was a prospective multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in the elderly. Between December 1997 and May 1999, we screened and enrolled subjects in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. A total number of 5804 subjects were randomly assigned to pravastatin or placebo.

Cognitive function

The Mini-Mental State Examination (MMSE) was used to measure global cognitive function. MMSE scores range from zero points (very severe cognitive impairment) to 30 points (optimal cognitive function). Participants with poor cognitive function (MMSE< 24) were not eligible for inclusion in the study. Four neuropsychological performance tests were used to measure various cognitive domains. The Stroop-Colour-Word-test for attention and the Letter-Digit Coding Test (LDT) for processing speed were used to measure executive functioning. The outcome parameter for the Stroop test was the total number of seconds to complete the third Stroop card containing 40 items. The outcome variable for the LDT was the total number of correct entries in 60 seconds. Memory was assessed with the 15-Picture Learning test (PLT) testing immediate and delayed recall. The main outcome parameters were the accumulated number of recalled pictures over the three learning trials and the number of pictures recalled after 20 minutes. Reliability and sensitivity of these tests in an elderly population have been published elsewhere (12). Cognitive function was tested at six different time points during the study, before randomization, at baseline, after 9, 18, and 30 months, and at the end of the study. The time point of this last measurement was different for the participants (at 36-48 months) therefore we performed the analyses with their individually varying time-point but report the results for the mean of these time points (at 42 months). The pre-randomized measurement was discarded in the analysis to preclude possible learning effects. Since the MMSE is not suitable for longitudinal research because of learning and ceiling effects, MMSE scores are not reported here.

Genotyping

We selected four SNPs in the CBP gene (CREBBP), intron 10AG (rs130005), intron 4CT (rs11076787), intron 3AC (rs1296720), and intron 2CG (rs2239317). These polymorphisms were selected from the SNPper database (http://snpper.chip.org) based on the frequency of the minor allele (>5%) and to cover the genomic region of the CBP gene for haplotype analyses. All polymorphisms were genotyped by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS), using the Sequenom MassARRAYtm methodology

(Sequenom Inc, San Diego, CA, USA). Amplification reactions and parameters were based on the manufacturer's instructions. Each 384-wells plate contained at least 4 positive (CEPH DNA) and 4 negative controls, to check for assay performance and contaminations, respectively. Spectrocaller software supplied by the manufacturer was used to automatically call the genotypes. Clusters were checked and all doubtful calls were manually evaluated. Ten percent of the genotypes were performed in duplicate and the error rate was below 1%.

Statistical analysis

The program Haploview (13) was used to estimate the allele frequencies, test the consistency of the genotype frequencies at each SNP locus with Hardy-Weinberg equilibrium, and estimate and plot pairwise linkage disequilibrium (LD) between the SNPs examined. Haplotypes and haplotype frequencies were calculated using SNPHAP software (http://www-gene.cimr.cam.ac.uk/clayton/software). We used multiple imputation analysis to deal with incomplete data and to account for many haplotype probabilities per subject. This method has been described elsewhere in more detail (14;15). Haplotypes with a frequency of less than 5% were combined and included in all analyses, without reporting the results. The haplotype analysis approach used in this study assumes an additive effect of the haplotypes, and details of this approach have been described elsewhere (16). Cross-sectional associations were assessed using linear regression adjusted for sex, age, education, country, and where appropriate, version of test used. The associations between the four CBP SNPs and cognitive function during follow-up were assessed with a linear mixed model for repeated measurements without interaction with time. In the model we used to estimate the effect of the genotypes on cognitive function, we incorporated time as a factor and genotype as covariate. The estimates for the genotypes represent the mean difference over time between the genotypes. To estimate the cognitive decline over time for all participants we incorporated time as a covariate in the model. The estimate for time represents the cognitive decline per year. The associations between the CBP haplotypes and cognitive function over time were also assessed with linear mixed models. All longitudinal analyses were adjusted for sex, age, education, history of diabetes, hypertension, vascular disease, myocardial infarction, stroke or TIA, angina, and claudication, country, use of

pravastatin, and where appropriate, version of test used. All statistical analyses were performed with SPSS software (version 12.0.1, SPSS Inc, Chicago, Ill). In first instance, p-values lower than 0.05 were considered statistically significant. Secondly, we adjust our analyses for multiple testing with the Bonferroni correction, p-values lower than 0.012 ($\alpha = 0.05 / 4$ tests) were now considered statistically significant.

Results

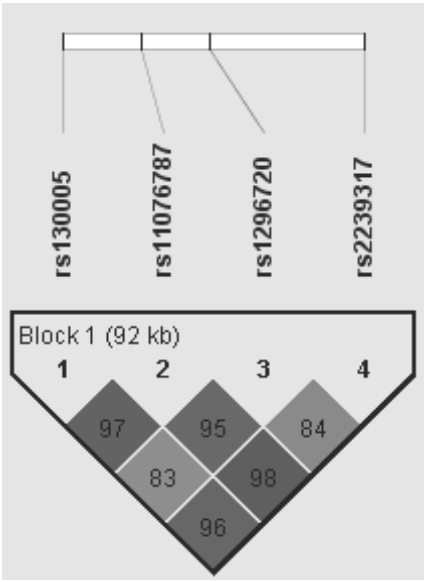
Table 1: Baseline characteristics of the participants of the PROSPER study per country.

	Scotland (N=2520)	Ireland (N=2184)	The Netherlands (N=1100)
Continous variates (mean, SD)			
Age (years)	75.3 (3.4)	75.5 (3.3)	75.1 (3.3)
Body Mass index, (kg/m2)	26.7 (4.2)	27.0 (4.4)	26.7 (3.8)
Total cholesterol, (mmol/L)	5.7 (1.0)	5.6 (0.9)	5.8 (0.9)
LDL cholesterol, (mmol/L)	3.8 (0.8)	3.7 (0.8)	3.9 (0.8)
HDL cholesterol, (mmol/L)	1.3 (0.4)	1.3 (0.4)	1.3 (0.3)
Categorical variates (n, %)			
Female	1283 (51)	1197 (55)	520 (47)
Current smoker	708 (28)	583 (27)	267 (24)
History of diabetes	213 (9)	225 (10)	185 (17)
History of hypertension	1446 (57)	1441 (66)	705 (64)
History of angina	811 (32)	523 (24)	225 (21)
History of claudication	229 (9)	114 (5)	47 (4)
History of myocardial infarction	379 (15)	258 (12)	139 (13)
History of vascular disease	1239 (49)	849 (39)	477 (43)
History of stroke or TIA	265 (11)	222 (10)	162 (15)
Genotype, minor allele frequency (%)			
Intron 10AG	21	21	23
Intron 4CT	20	18	22
Intron 3AC	9	10	11
Intron 2CG	9	10	11

The mean age of the participants was 75.3 years and approximately 50% were female (table 1). Genotyping of the four CBP SNPs was complete for 5653 subjects. The results of the remaining 151 subjects were missing because of insufficient DNA or incomplete genotyping. All four SNPs were

in Hardy-Weinberg equilibrium (all p>0.3). There was a significant difference in minor allele frequency between the countries for the polymorphism intron 3AC (p-value Chi-square = 0.02, data not shown). The variant allele C was more common in the Dutch subjects when compared with the subjects from Scotland and Ireland. Therefore, all analyses were adjusted for country. Mean follow-up of study subjects was 42 months (range 36-48 months).

The four SNPs were in strong linkage disequilibrium (LD) and occurred together in one haploblock (figure 1A). Four haplotypes were found in our study population (figure 1B). The three haplotypes with a frequency above 5% were included in analyses. We used H1111, with no variants present, as reference haplotype. H1221 had two variant alleles, intron 4T and intron 3C. H2112 carried the intron 10G variant and the intron 2G variant.



B

	Intron 10AG	Intron 4CT	Intron 3AC	Intron 2CG	Haplotype Frequency
H1111	1	1	1	1	0.677
H1221	1	2	2	1	0.192
H2112	2	1	1	2	0.091
H1211	1	2	1	1	0.021

Figure 1: Haplotype information.

Figure 1A shows the linkage disequilibrium (LD) between the single nucleotide polymorphisms (SNPs) examined. All SNPs are in LD and occur together in one haploblock. Figure 1B shows the haplotype frequencies. Only the first three haplotypes (frequency> 5%) were included in the analyses.

The results of the longitudinal association between the four CBP polymorphisms and cognitive function are presented in table 2.

Table 2: Longitudinal association between four single polymorphisms in the CBP gene and cognitive function.

	Time		Mean difference over time	
	Est (SE)	p-value	Est (SE)	p-value
Intron 10AG				
Attention	0.67 (0.07)	<0.001	0.66 (0.84)	0434
Processing speed	-0.36 (0.01)	<0.001	-0.07 (0.23)	0.754
Immediate memory	-0.01 (0.01)	0.020	0.01 (0.05)	0.816
Delayed memory	-0.06 (0.01)	<0.001	-0.00 (0.07)	0.984
Intron 4CT				
Attention	0.67 (0.07)	<0.001	-0.69 (0.61)	0.257
Processing speed	-0.36 (0.01)	<0.001	0.36 (0.17)	**0.028**
Immediate memory	-0.01 (0.01)	0.032	0.10 (0.04)	**0.010**
Delayed memory	-0.06 (0.01)	<0.001	0.12 (0.05)	**0.024**
Intron 3AC				
Attention	0.67 (0.07)	<0.001	-1.07 (0.64)	0.094
Processing speed	-0.36 (0.01)	<0.001	0.42 (0.17)	**0.015**
Immediate memory	-0.01 (0.01)	0.030	0.12 (0.04)	**0.004**
Delayed memory	-0.06 (0.01)	<0.001	0.17 (0.06)	**0.004**
Intron 2CG				
Attention	0.67 (0.07)	<0.001	0.17 (0.86)	0.838
Processing speed	-0.36 (0.01)	<0.001	-0.07 (0.23)	0.769
Immediate memory	-0.01 (0.01)	0.023	0.06 (0.05)	0.235
Delayed memory	-0.06 (0.01)	<0.001	0.05 (0.07)	0.518

All p-values are assessed with linear mixed models adjusted for sex, age, education, history of diabetes, hypertension, vascular disease, myocardial infarction, stroke or TIA, angina, and claudication, country, use of pravastatin, , and where appropriate, version of test used.

The term for time was significant for all domains of cognitive function, indicating that all domains declined over time. No associations were found with the intron 10AG and intron 2CG polymorphisms and cognitive function. Subjects carrying the intron 4T variant had significantly better cognitive performance compared to carriers of the wild-type variant on memory function and processing speed (all $p<0.03$). For attention a comparable trend was observed. Also carriers of the intron 3C variant performed better on all cognitive domains compared to carriers of the wild-type variant (all $p<0.01$ except for attention). No additional cognitive decline was found when we tested for the formal interaction between time and genotype.

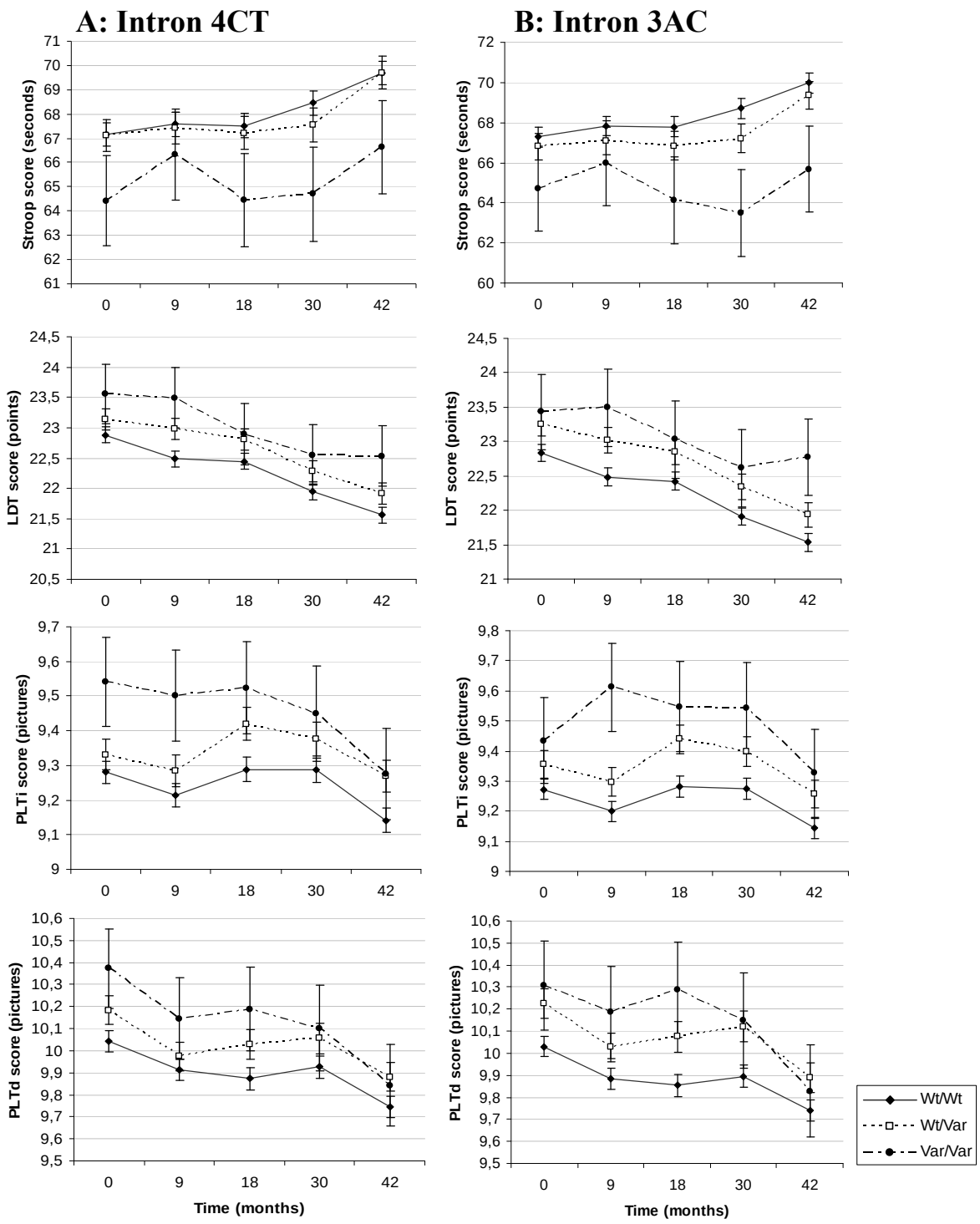


Figure 2: Representation of the longitudinal association between the two CBP polymorphisms and cognition. Figure 2A represents the association between intron 4CT and cognition and figure 2B the association between intron 3AC and cognition. In all graphs it is shown that carriers of the variant alleles performed better compared to carriers of the wild-type alleles.

Figure 2A represents the longitudinal association between the CBP intron 4CT polymorphism and different domains of cognitive function. Carriers of the variant allele had better performance compared to carriers of the wild-type allele in a gene-dose dependent relationship. The heterozygous subjects performed better compared to the homozygous wild-type carriers and the homozygous variant carriers performed better than the heterozygous subjects. In figure 2B the association between the CBP intron 3AC polymorphism and cognitive function is presented. Again, carriers of the variant allele performed better on all cognitive domains compared to wild-type carriers in a gene-dose dependent relationship.

In table 3 the results of the longitudinal association between CBP haplotypes and cognitive function are shown. H1111 was used as reference. Again, the term for time was significant for all domains of cognitive function, indicating that all domains declined over time. H1221 with the variant alleles intron 3T and intron 4C was associated with better memory function and processing speed compared to reference (all $p<0.03$). For attention a comparable trend was seen which was not significant. After the Bonferroni correction, H1221 was still significantly associated with better memory function (all $p<0.012$). There was no association between H2112 and cognitive function.

Table 3: Results of the longitudinal association between CBP haplotypes and cognitive function

	Time		H1111	H1221		H2112	
	Est (SE)	p-value	Est (SE)	Est (SE)	p-value	Est (SE)	p-value
Stroop	0.68 (0.07)	<0.001	Ref	-0.92 (0.65)	0.093	0.15 (0.88)	0.492
LDT	-0.36 (0.01)	<0.001	Ref	**0.37 (0.18)**	**0.022**	-0.03 (0.23)	0.453
PLTi	-0.01 (0.01)	0.016	Ref	**0.11 (0.04)**	**0.002**	0.06 (0.05)	0.128
PLTd	-0.06 (0.01)	<0.001	Ref	**0.15 (0.06)**	**0.008**	0.04 (0.08)	0.734

All p-values are assessed with linear mixed models after multiple imputations and adjusted for sex, age, education, history of diabetes, hypertension, vascular disease, myocardial infarction, stroke or TIA, angina, and claudication, country, use of pravastatin, and presence of other haplotypes.

Discussion

In this study we investigated the association between genetic variation in the CBP gene and cognitive function. We demonstrate that the intron 4CT and intron 3AC polymorphisms are associated with better cognitive performance in all cognitive domains during follow-up. Furthermore, the haplotype with the variant alleles of these two polymorphisms also shows a strong protective effect on cognitive function in all cognitive domains.

Many experimental studies have investigated the role of CBP in memory formation and cognitive dysfunction in animals (1-4). $CBP^{+/-}$ mice show the same clinical features as RTS patients, including mental retardation (9). In this mouse model the CBP protein was truncated by lacking the intrinsic HAT-domain (9). In the same mouse model it was shown that $CBP^{+/-}$ mutant mice had normal short-term memory, but deficiencies in long-term memory, object recognition, and contextual memory tasks (9). Moreover, three other mouse models with a defect in the CBP pathway (defect in CBP activation (17), truncated form of CBP protein (18) and CBP protein lacking HAT activity (19) show that CBP truncated mice have defects in learning and memory.

Loss of one functional copy of the CBP gene underlies all abnormalities in Rubinstein-Taybi Syndrome patients (6;7). The mutations found in RTS patients vary from large deletions, which can remove the entire gene, to point mutations, which can lead to a truncated protein (7). Therefore it is likely that polymorphisms in the CBP gene have an effect on cognitive function, but in a relatively milder form compared to RTS patients. Phenotypic effects as described in humans and mice models have so far been the result of loss of function mutations. Here we may have found a protective effect on cognition of a yet unknown functional variation which is likely to be due to a gain of function mutation in LD with haplotype H1221.

A possible weakness of our study is that we genotyped only four SNPs within the CBP gene. These polymorphisms were selected to cover the whole genomic region of the CBP gene for haplotype analyses. Based on HapMap and Haploview data additional SNPs should have been genotyped to

capture all common variation in the CBP gene. It could be a major problem for negative studies if not all common genetic variation in a gene is captured, however since our study is not negative, this is not a major problem. Moreover, when the additional polymorphisms would be genotyped, only three out of the possible 20 haplotypes would contain the two associated polymorphisms (intron 4CT and intron 3AC). To further refine these three haplotypes, two polymorphisms should be additionally genotyped. However these polymorphisms have no effect on the functionality of the gene since both polymorphisms are in non-coding areas.

We genotyped four intron polymorphisms with the CBP gene, which may not be functional themselves, but may be in linkage disequilibrium with other functional unknown polymorphisms in the gene. We have shown that the linkage disequilibrium within this gene is high, therefore the protective effect on cognitive function we found with the intron polymorphisms indeed may be caused by other polymorphisms within the gene. As a result we do not know the effect of the polymorphisms on protein expression levels and protein functionality. Future research is required to clarify the relation between the polymorphisms and CBP levels as well as the effect of these polymorphisms on gene expression mediated by CBP. However, loss of one copy of the CBP gene have been proven to lead to phenotypic effects in Rubinstein-Taybi Syndrome, like severe cognitive dysfunction (6;7). This shows that large alterations within this gene cause major defects in cognitive function. We have shown that also more frequent single nucleotide polymorphisms within this gene can lead to changes in cognitive function.

Moreover, this study met the four criteria described by Rosenthal and Schwartz to establish medically useful links between genetic variations and disease (20). First, the change in the gene must cause a relevant alteration in the function or level of the gene product (which is always a protein); this has been shown since loss of one cope of this gene leads to the Rubinstein-Taybi Syndrome with severe cognitive dysfunction. Second, the beneficial and harmful phenotypes must have apparent clinical differences; we showed that genetic variation within the CREB gene leads to better performance on four neuropsychological performance test in a prospective analyses. Third,

the hypothesis linking the genotype to disease must be convincing; this criterion has been met since it is known that alterations within the gene lead to this syndrome and fourth, the number of cases linking a genotype to disease must be sufficient; this study has been performed in a large study group of over 5000 subjects.

A major strength of our prospective study is our large cohort size, with data on several measures of cognitive function gathered serially in over 5000 subjects in three different countries. Over a follow-up period of 42 months, few subjects were lost to follow-up. As all subjects at study entry had an MMSE of 24 points or greater, our observations are highly relevant to comparisons of cognitive change in older people with good baseline cognitive functional status, enabling stratification of the risk of future decline. Moreover, in previous studies it has been shown that from 70 years onwards there is substantial cognitive decline. All subjects have significantly deteriorated in their cognitive function within the three years of follow-up. No interaction between the genotypes and time was found, but prior to analysis we did not expect to find this interaction. We assumed that life time exposure of the polymorphisms would have developed a difference in cognition already earlier in life, therefore an additional decline in this elderly population was not expected.

In conclusion, we have demonstrated an association between two polymorphisms in the CBP gene and cognitive function in an elderly population. The variant alleles of the intron 4CT and intron 3AC polymorphisms were associated with better cognitive performance in all cognitive domains at baseline and in follow-up. Furthermore, the haplotype with the variant alleles of these two polymorphisms also showed a protective effect on cognitive function in all cognitive domains. Future research is warranted to assess the functionality of these polymorphisms on the expression of CBP levels and how these polymorphisms affect the gene expression mediated by CBP. If these findings are confirmed and adequately explained on the basis of independent studies, screening patients for the CBP polymorphisms may contribute to a better risk stratification of patients at risk for cognitive decline and may improve individual treatment.

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Reference List

(1) Bito H, Deisseroth K, Tsien RW. CREB phosphorylation and dephosphorylation: a Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression. Cell 1996 Dec 27;87(7):1203-14.

(2) Lonze BE, Ginty DD. Function and regulation of CREB family transcription factors in the nervous system. Neuron 2002 Aug 15;35(4):605-23.

(3) Rouaux C, Loeffler JP, Boutillier AL. Targeting CREB-binding protein (CBP) loss of function as a therapeutic strategy in neurological disorders. Biochem Pharmacol 2004 Sep 15;68(6):1157-64.

(4) Weeber EJ, Sweatt JD. Molecular neurobiology of human cognition. Neuron 2002 Mar 14;33(6):845-8.

(5) Carlezon WA, Jr., Duman RS, Nestler EJ. The many faces of CREB. Trends Neurosci 2005 Aug;28(8):436-45.

(6) Hallam TM, Bourtchouladze R. Rubinstein-Taybi syndrome: molecular findings and therapeutic approaches to improve cognitive dysfunction. Cell Mol Life Sci 2006 Aug;63(15):1725-35.

(7) Roelfsema JH, White SJ, Ariyurek Y, Bartholdi D, Niedrist D, Papadia F, et al. Genetic heterogeneity in Rubinstein-Taybi syndrome: mutations in both the CBP and EP300 genes cause disease. Am J Hum Genet 2005 Apr;76(4):572-80.

(8) Hennekam RC. Rubinstein-Taybi syndrome. Eur J Hum Genet 2006 Sep;14(9):981-5.

(9) Oike Y, Hata A, Mamiya T, Kaname T, Noda Y, Suzuki M, et al. Truncated CBP protein leads to classical Rubinstein-Taybi syndrome phenotypes in mice: implications for a dominant-negative mechanism. Hum Mol Genet 1999 Mar;8(3):387-96.

(10) Shepherd J, Blauw GJ, Murphy MB, Cobbe SM, Bollen EL, Buckley BM, et al. The design of a prospective study of Pravastatin in the Elderly at Risk (PROSPER). PROSPER Study Group. PROspective Study of Pravastatin in the Elderly at Risk. Am J Cardiol 1999 Nov 15;84(10):1192-7.

(11) Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Cobbe SM, et al. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet 2002 Nov 23;360(9346):1623-30.

(12) Houx PJ, Shepherd J, Blauw GJ, Murphy MB, Ford I, Bollen EL, et al. Testing cognitive function in elderly populations: the PROSPER study. PROspective Study of Pravastatin in the Elderly at Risk. J Neurol Neurosurg Psychiatry 2002 Oct;73(4):385-9.

(13) Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005 Jan 15;21(2):263-5.

(14) Harel O, Zhou XH. Multiple imputation: review of theory, implementation and software. Stat Med 2007 Jan 29.

(15) Schafer JL. Multiple imputation: a primer. Stat Methods Med Res 1999 Mar;8(1):3-15.

(16) Wallenstein S, Hodge SE, Weston A. Logistic regression model for analyzing extended haplotype data. Genet Epidemiol 1998;15(2):173-81.

(17) Kobayashi M, Masaki T, Hori K, Masuo Y, Miyamoto M, Tsubokawa H, et al. Hippocalcin-deficient mice display a defect in cAMP response element-binding protein activation associated with impaired spatial and associative memory. Neuroscience 2005;133(2):471-84.

(18) Wood MA, Kaplan MP, Park A, Blanchard EJ, Oliveira AM, Lombardi TL, et al. Transgenic mice expressing a truncated form of CREB-binding protein (CBP) exhibit deficits in hippocampal synaptic plasticity and memory storage. Learn Mem 2005 Mar;12(2):111-9.

(19) Korzus E, Rosenfeld MG, Mayford M. CBP histone acetyltransferase activity is a critical component of memory consolidation. Neuron 2004 Jun 24;42(6):961-72.

(20) Rosenthal N, Schwartz RS. In search of perverse polymorphisms. N Engl J Med 1998 Jan 8;338(2):122-4.

Chapter 8

The -2481C variant allele of the lysine acetyltransferase PCAF is associated with reduced vascular mortality. Results from three independent prospective studies

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Abstract

This study was designed to investigate the influence of genetic variation in the promoter of the gene encoding P300/CBP associated factor (PCAF), a lysine acetyltransferase (KAT) on coronary heart disease (CHD) mortality. We investigated the association of genetic variation in the promoter region of the PCAF-gene on CHD mortality in two statin trials (PROSPER and WOSCOPS) available to us and on restenosis risk in a third study of percutaneous coronary intervention (GENDER). We combined the results from these cohorts to examine overall effects on CHD mortality and on restenosis risk and determined the contribution of PCAF in an animal model of reactive stenosis. Compared with the homozygous -2481G allele in the PCAF promoter, we observed a significant reduction in CHD mortality risk with the homozygous -2481C PCAF promoter allele in PROSPER (risk reduction 22%; 2%-37%) and in WOSCOPS (risk reduction 17%; 14%-39%), and of restenosis in GENDER (risk reduction 20%; 3%-33%). A combined risk reduction for CHD death/ restenosis for the three studies was 21% (15%-26%; p= 8.1 x 10^{-4}). Furthermore, this PCAF allele was significantly associated with all cause mortality in PROSPER (p=0.001). Functional analysis showed that the -2481 G/C polymorphism affected factor binding to this region of the PCAF promoter, and modulation of PCAF gene expression was detectable upon cuff-placement in an animal model of reactive stenosis. Our observations promote the concept that epigenetic processes are under genetic control and that, other than environment, genetic variation in genes encoding KATs may also determine susceptibility to CHD outcomes and mortality.

Introduction

Investigations into the pathogenetic mechanisms of human complex disease, such as cardiovascular disease and cancer, may lead to better risk prediction, treatment and new targets for future therapy. Cell proliferation regulatory pathways and pro-inflammatory transcription factors, such as NFκB, have been associated with progression of these diseases (1). In the past decade, research into cardiovascular diseases such as atherosclerosis and restenosis, has been focused on the identification of genetic factors that determine disease risk. Several genes involved in inflammation

and cell proliferation appeared to be common denominators of these diseases (2-6). It has become clear, however, that part of the gene-environmental interactions relevant for complex diseases is regulated by epigenetic mechanisms such as histone acetylation and DNA methylation (7). Epigenetic processes modulate gene expression patterns without modifying the actual DNA sequence and have profound effects on the cellular repertoire of expressed genes (8). Evidence is growing that epigenetic mechanisms also regulate the expression of genes in the inflammatory and cell proliferation pathways (9,10) and may therefore also play a role in cardiovascular disease (11-13) and in cancer (14).

A major influence on gene expression is attributed to the counterbalancing action of lysine acetyltransferases (KATs) and lysine deacetylases (KDACs) (15). KATs acetylate histones by transfer of an acetyl-group to the ε-portion of lysine residues, which results in an open modification of chromatin structure and in accessibility of DNA to transcription factors and recruitment of the basal transcription initiation machinery. Conversely, gene repression is mediated via KDACs, which remove acetyl groups and counteract the activity of KATs resulting in a closed chromatin structure. Thus far, the main focus has been to investigate the environmental influence on epigenetic processes. Research in this field has shown that epigenetic differences arise during the lifetime of monozygotic twins (8). Furthermore, oxidative stress has been shown to influence the balance between KATs and KDACs in favour of KATs, leading to an increase in inflammation (16). Notably, genetic variations in the genes encoding KATs and KDACs, which affect the activities of the enzymes they encode, have a bearing on the global and gene-specific levels of histone acetylation. As such, these genetic variations in the genes encoding KATs and KDACs could also be important determinants contributing to susceptibility to major human diseases.

P300/CBP associated factor (PCAF) is a transcriptional co-activator with intrinsic KAT-activity. Besides its role in lysine acetylation of histones at the site of NFκB-regulated genes and the resultant inflammatory gene activation (17,18), PCAF is also found to act as a factor acetyltransferase (FAT) that acetylates non-histone proteins, including several tumor-suppressor

proteins, such as p53 (19,20) and the phosphatase and tensin homolog (PTEN) (21). Because PCAF is involved in proliferation and inflammation, common denominators of the major diseases determining human mortality, with clear evidence for inflammatory factors predicting incident CVD events (22), incident cancers (23) and mortality, we hypothesized that the PCAF-gene could be of major importance in the development of cardiovascular disease and cancer, and death from such disease.

We investigated the impact of genetic variation in the promoter region of the PCAF-gene on all-cause mortality and mortality due to coronary heart disease and cancer in the PROSPER-study, a randomized controlled trial in which 5804 elderly patients (age 70-82) at risk for vascular disease were randomized to pravastatin or placebo (24). In order to validate the observed effects and to be able to extrapolate our findings to a younger population, we investigated the PCAF gene in the WOSCOPS study, a randomized controlled trial similar to the PROSPER-study, designed to determine the effect of pravastatin in middle-aged men with hypercholesterolemia without a history of cardiovascular disease. Finally, in order to further test the validity of results in the two statin trials, and to get insights in the mode of action, we investigated these variants in another large prospective study, the GENDER-study, a prospective follow-up study that included 3104 patients undergoing percutaneous coronary intervention (PCI) (25). The primary endpoint in this study was clinical restenosis, a process that is known to be mainly determined by inflammation and proliferation (4).

Besides the effect of genetic variants on KAT activity of PCAF, which would affect global and gene-specific levels of histone acetylation, the total quantity of acetylated histones can also change during life as a result of exposure to environmental factors. This is illustrated by studies with monozygotic twins showing that older monozygotic twins differ more from each other in histone H3- and H4-acetylation than young monozygotic twins (8). Therefore, as a result of genetic variants of PCAF that affect KAT activity in addition to exposure to environmental factors, cumulative effects on histone acetylation profiles could become more evident with increasing age. Because of

this, we investigated in these three large prospective follow-up studies with various age ranges whether the effect of the PCAF gene on mortality and vascular events is associated with increasing age.

In short, all patients were analyzed for 2 polymorphisms (SNPs) in the promoter region of the PCAF-gene. Of these polymorphisms, the -2481G/C SNP was significantly associated with CHD mortality risk in both PROSPER and WOSCOPS, and, in addition, with differential risk for restenosis in the GENDER study. The -2481G/C SNP in the PCAF promoter affected transcription factor binding as was demonstrated in an electrophoretic mobility shift assay (EMSA) analysis. Finally, we demonstrate in an animal model for reactive stenosis (26) that PCAF expression is indeed rapidly upregulated after vascular injury, which supports the notion that PCAF is involved in vascular remodeling.

Methods

Study Design and Follow-up of the PROSPER Study

The protocol of PROSPER has been described elsewhere (27). PROSPER is a prospective multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in elderly individuals. Between December 1997 and May 1999, we screened and enrolled subjects in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. A total number of 5804 subjects were randomly assigned to pravastatin or placebo. In this genetic sub-study, we evaluated the predefined endpoints all-cause mortality and mortality due to vascular events and cancer. Mean follow-up was 3.2 years (range 2.8-4.0) and 604 (10.4%) patients died during the study (24). Of these patients, 292 (48%) died from vascular disease and 206 (31%) from cancer.

Study Design and Follow-up of the WOSCOPS Study

The WOSCOPS study (the West of Scotland Coronary Prevention Study), a primary prevention trial, included 6595 men who had LDL cholesterol levels between 174 and 232 mg/dL (4.5 and 6.0 mmol/L), who had no history of myocardial infarction, but were considered to be at enhanced risk for developing CHD (28). All participants were randomly assigned to receive 40 mg of pravastatin or placebo daily. The present genetic study was performed in a previously described nested case-control cohort (29). In brief, the prospective nested case-control study included all of the 580 on-trial CHD events (death from CHD, nonfatal MI, or revascularization procedures) from the WOSCOPS cohort as case subjects and 1,160 control subjects matched to case subjects by age and smoking. In the present genetic study we used death from coronary heart disease as our primary endpoint.

Study Design and Follow-up of the GENDER Study

The present study sample has been described previously (25). In brief, the GENetic DEterminants of Restenosis project (GENDER) was a multicenter follow-up study designed to study the association between various gene polymorphisms and clinical restenosis. Patients eligible for inclusion in the GENDER-study were treated successfully for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia by PCI in four out of 13-referral centers for interventional cardiology in the Netherlands. Patients treated for acute ST-elevation myocardial infarction were excluded. Experienced operators, using a radial or femoral approach, performed standard angioplasty and stent placement. During the study, no drug-eluting stents were used. Follow-up lasted for at least nine months, except when a coronary event occurred. Clinical restenosis, defined as TVR, either by PCI or coronary artery bypass grafting (CABG), was the primary endpoint. Median follow-up duration was 9.6 months (interquartile range 3.9) and 304 (9.8%) patients underwent TVR during follow-up. A prespecified subpopulation of 478 patients was scheduled for re-angiography at 6 months, according to standard procedures as described previously (30). Identical projections were used before, during and 6 months after the PCI for all assessed angiograms. Quantitative Computer Analyses (QCA) were independently performed by Heartcore (Leiden, the Netherlands).

For all three studies, all endpoints were adjudicated by independent clinical events committees. The protocols meet the criteria of the Declaration of Helsinki and were approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from all participating patients.

Genotyping

Blood was collected in EDTA tubes at baseline and genomic DNA was extracted following standard procedures. As a first step to investigate this gene, we selected 2 validated polymorphisms in the PCAF-promoter. The -4556 C/T (rs2623074) and the -2481 G/C (rs2948080) polymorphisms were selected on the basis of their high minor allele frequency (>5%) and measured using the Sequenom Massarray genotyping platform. A multiplex assay was designed using Assay designer software (Sequenom). As quality controls, 5-10% of the samples were genotyped in duplo. No inconsistencies were observed. Cluster plots of the signals from the low and the high mass allele were drawn. Two independent researchers carried out scoring. Disagreements or vaguely positioned dots produced by Genotyper 4.0 (Sequenom Inc.) were left out of the results.

Gene Expression Micro-array

Gene expression micro-array data were generated in 50 unrelated nonagenarians from the Leiden Longevity Study, 50 of their offspring and 50 controls. The 150 samples that met the RNA quality criteria were hybridized on 54k CodeLink Human Whole Genome Bioarrays (GE Healthcare, currently of Applied Microarrays). cDNA synthesis, amplification and biotin labeling and hybridization onto the microarray were performed according to the manufacturer's instructions. Two probes on the microarray corresponded to the PCAF gene region; GE693780, corresponding to Unigene alignment Hs.668165 and GE81332, corresponding to an exon of the PCAF gene.

Cells and Cell Culture

The cell lines (HeLa, U251, Raji) used in this study were obtained through the ATCC (Rockville, MD, USA) and were cultured in Iscove's modified Dulbecco's medium (IMDM; BioWhittaker

Europe, Verviers, Belgium) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Greiner, Alphen a/d Rijn, The Netherlands), 100 IU/ml streptomycin and 100 IU/ml penicillin. For interferon-γ (IFN-γ) induction, cells were treated with 500U/ml of IFN-γ (Boehringer-Ingelheim, Alkmaar, The Netherlands) for 4 hours, hereafter nuclear extracts were prepared (see below). HUVECs were cultured in Medium 199 with Earl's salt and L-glutamine (Life Technologies, Breda, The Netherlands), supplemented with 20% (v/v) FCS (PAA, Pasching, Austria), 100 IU/ml streptomycin and 100 IU/ml penicillin, 10 IU/ml heparine (Leo Pharma, Breda, The Netherlands) and 25 mg bovine pituitary extract (BPE; Life Technologies).

Transcription Factor Binding Site Search

Potential transcription factor binding sites were identified using the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html), which searches the TRANSFAC database (31). Cutoff was set at 75% of the consensus TF binding site.

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts and EMSAs were performed as described previously (32). In brief, 2 µl of nuclear extracts (HeLa, HUVEC, U251, Raji) in binding buffer were incubated for 30 min on ice, with 2 ng of a [^{33}P]-labeled dsDNA probe. The probe sequences were similar to either the C or G promoter variants of the PCAF-gene (PCAF-C: 5'-GCAATAAGCCTC-CTCAATCCTTTGCCCTTG-3';PCAF-G: 5'-GCAATAAGCCTCCTGAATCCTTTGCCC-TTG-3'). Probe sequences for transcription factors MZF1 and GATA1-3 were similar to their previously described consensus sequence (MZF1 (zinc fingers 1-4): 5'- GATCTAAAAGTGGGGAGAAAA-3'; MZF1(zinc fingers 5-13): 5'- GATCCGGCTG-GTGAGGGGGAATCG-3'; GATA: 5'- GGACCTTGATCTTATCTT-3') (33,34).

For competition assays, nuclear extracts were incubated with unlabelled oligonucleotides in 100-fold excess for 30 min on ice, prior to incubation with the labeled probe. In case of IFN-γ treated samples, cells were stimulated with IFN-γ (500U/ml; Boehringer-Ingelheim) for 4-hours prior to

preparing nuclear extracts. Samples were run on a 6% polyacrylamide gel in 0.25x TBE-buffer. Gels were densitometrically analyzed using the ImageJ software (35).

Mouse Model for Reactive Stenosis

The institutional committee on animal welfare approved all animal experiments. For all experiments hyperlipidemic male ApoE*3-Leiden mice (36) were fed a high-cholesterol diet (ArieBlok, Woerden, The Netherlands). Blood samples to determine plasma cholesterol were collected at time of surgery. After 3 weeks on diet, a non-constrictive polyethylene cuff was placed loosely around one femoral artery and mice were sacrificed at several time points after surgery. After sacrifice, at t=0 hours (control, no cuff placement), 6 hours, 24 hours, 2 days, 3 days, 7 days and 14 days, both femoral arteries were isolated and snap-frozen in liquid nitrogen (n=6 mice for each timepoint).

RNA Isolation and cDNA Synthesis from Femoral Artery Tissue

Per time point, femoral arteries were pooled to n=4, allowing better isolation of RNA. After RNA isolation, cDNA was synthesized and RT-PCR analysis was performed as previously described (37).

PCAF mRNA Quantification

Expression levels of PCAF were measured by virtue of quantitative RT-PCR using TaqMan® gene expression assay (Mm00451387_m1). PCR runs were carried out in the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). HPRT was assayed as control gene and its cycle threshold (Ct) was substracted from the Ct of the gene of interest, yielding ΔCt. For each timepoint, ΔΔCt was determined by substracting the average ΔCt at timepoint 0 hours from the ΔCt at each other timepoint. This ΔΔCt was used to calculate the displayed fold increase for each gene (38).

Statistical Analysis

Allele frequencies were determined by gene counting. The Chi-squared test was used to test the consistency of the genotype frequencies at the SNP locus with Hardy-Weinberg equilibrium.

Hazard ratios (HR) with 95% confidence intervals (CI) were calculated using a Cox-proportional hazards model. All analyses were adjusted for sex and age. The analyses with PROSPER data were additionally adjusted for pravastatin use and country. In the GENDER-study, polymorphisms were included in a multivariable model containing clinical and procedural risk factors for restenosis, such as diabetes, smoking, hypertension, stenting, total occlusion and residual stenosis >20%. A combined-effect analysis was performed to pool the results of the effect of the -2481G/C polymorphism on study endpoints (all-cause mortality, CHD death and clinical restenosis) and coronary endpoints (CHD death in two studies and clinical restenosis in the third study) in the three separate studies at old age. The random-effects model was used to consider both the between-study and within-study variability. The pooled hazard ratio over the genotypes was assessed with ordinary logistic regression. The SPSS software (version 12.0.1, SPSS Inc, Chicago, IL) was used for all statistical analyses.

Results

PROSPER study

Participant characteristics are presented in table 1. Genotyping success rates were higher than 96% for all polymorphisms and there were no significant deviations from Hardy-Weinberg equilibrium.

Using a Cox proportional hazards model, which included several clinical variables such as sex, age, pravastatin use, and country, we found a significant association of the -2481 G/C promoter polymorphism with all-cause mortality in PROSPER. As presented in table 2 and figure 1, heterozygotes had a reduced mortality risk by 17% (2% to 30%, p=0.03), whereas individuals homozygous for the -2481C allele had a 39% lower mortality (16% to 56%, p=0.003). The effect of PCAF is more profound in the association with coronary heart disease (CHD) death (risk reduction 22% (2% to 37%), p=0.03) compared to the association with cancer mortality (risk reduction 13% (9% to 30%), p=0.22). Due to its proximity to the -2481 G/C polymorphism, we also present the data of the -4556 C/T polymorphism (table 2). It shows a small and non-significant trend towards a

decrease in all-cause mortality. The linkage disequilibrium (LD)-coefficient between these promoter polymorphisms was 0.79.

Table 1: Baseline characteristics of the PROSPER, the GENDER and the WOSCOPS study

	PROSPER N=5804	WOSCOPS N=1740	GENDER N=3104
Continuous variates (mean, SD)			
Age (years)	75.3 (3.3)	56.8 (5.2)	62.1 (10.7)
Body mass index, (kg/m^2)	26.8 (4.2)	26.0 (3.2)	27.0 (3.9)
Categorical variates (%)			
Male sex	48	100	71
Current smoker	27	54	25
History of diabetes	11	2	15
History of hypertension	62	18	41
History of myocardial infarction	13	0	40
History of stable angina	27	8	67
Genotype, minor allele frequency (%)			
PCAF -4556 C/T [a]	9	9	11
PCAF -2481 G/C [b]	33	33	33

All data are presented in number (%) unless otherwise stated.

[a] In PROSPER, WOSCOPS, and GENDER, measured in 5583, 1090 and 2850, participants, respectively.

[b] In PROSPER, WOSCOPS, and GENDER, measured in 5595, 1092, and 2852, participants, respectively.

WOSCOPS study

To validate the association of the -2481 G/C polymorphism with CHD mortality, and to investigate whether its effect is also present in a population largely without vascular disease, we aimed to replicate our findings in a previously described [29] nested case-control cohort of the WOSCOPS study. Baseline characteristics of the WOSCOPS study are presented in table 1.

In this case, the -2481 G/C polymorphism was associated with a non-significant trend towards protection against death from coronary heart disease (CHD) (risk reduction 17% (-14% to 39%), p=0.26) at all ages. Heterozygotes (risk reduction 11% (-39% to 42%), p=0.62) and homozygotes (risk reduction 37% (-36% to 71%), p=0.24) had a non-significantly lower mortality risk. Although these data did not reach statistical significance, the point estimate for CHD mortality in WOSCOPS was similar to that observed in the PROSPER study (figure 1). The -4556 C/T polymorphism was

not significantly associated with the risk of CHD death (risk reduction 20% (-36% to 53%), p=0.40).

GENDER study

To extent the observed results in the PROSPER study, we tested the relevance of the gene to clinical conditions in which cell proliferation and inflammation play a role in a large patient population included in the GENDER-study. In this study patients were followed for at least 9 months after a PCI to determine absence or presence of clinical restenosis, as defined by the need for target vessel revascularization (TVR). We considered TVR a suitable intermediate endpoint as it is a direct consequence of mainly proliferative and inflammatory processes.[4]

In agreement with the results from the PROSPER and WOSCOPS-study, the -4556 C/T polymorphism was not associated with the risk for TVR in the GENDER-study, whereas the -2481 G/C polymorphism showed a significant association with TVR (p-trend = 0.02) (table 3 and figure 1). Heterozygous patients were at lower risk for TVR (risk reduction 20% (-2% to 38%), p=0.07), and patients carrying two -2481C alleles had a greater protection against the development of restenosis (risk reduction 36% (0% to 59%), p=0.05). In analogy of the clinical restenosis (TVR) results, carriers of the -2481C allele also had less angiographic restenosis in a subpopulation of 478 patients undergoing angiography six months after the PCI (risk reduction 44% (4% to 67%), p=0.03) (table 3).

Table 2. Results of the association between two promoter polymorphisms in the PCAF gene and mortality endpoints within the PROSPER study.

	All cause mortality			CHD death			Cancer mortality		
	N (%)	HR (95%CI)	p-value	N (%)	HR (95%CI)	p-value	N (%)	HR (95%CI)	p-value
PCAF -4556 C/T									
C/C	491 (11)	1.0 (ref)		168 (4)	1.0 (ref)		163 (4)	1.0 (ref)	
C/T	81 (9)	0.81 (0.64-1.02)	0.09	32 (3)	0.94 (0.64-1.37)	0.75	33 (4)	1.00 (0.69-1.46)	0.99
T/T	4 (7)	0.63 (0.24-1.69)	0.38	3 (5)	1.31 (0.42-4.10)	0.65	1 (2)	0.50 (0.07-3.59)	0.49
Trend	576 (10)	**0.80 (0.65-0.99)**	**0.04**	203 (4)	0.99 (0.71-1.38)	0.95	197 (4)	0.94 (0.67-1.33)	0.74
PCAF -2481 G/C									
G/G	290 (12)	1.0 (ref)		103 (4)	1.0 (ref)		93 (4)	1.0 (ref)	
G/C	246 (10)	**0.83 (0.70-0.98)**	**0.03**	86 (3)	0.81 (0.61-1.08)	0.16	90 (4)	0.95 (0.71-1.26)	0.70
C/C	41 (7)	**0.61 (0.44-0.84)**	**0.003**	14 (3)	**0.58 (0.33-1.00)**	**0.05**	15 (3)	0.68 (0.40-1.18)	0.17
Trend	577 (10)	**0.80 (0.70-0.91)**	**0.001**	203 (4)	**0.78 (0.63-0.98)**	**0.03**	198 (4)	0.87 (0.70-1.09)	0.22

HR=Hazard ratio. CI=Confidence interval. All hazard ratios and p-values were assessed with a Cox-proportional hazards model and adjusted for sex, age, country, and use of pravastatin.

Table 3. Results of the association between two promoter polymorphisms in the PCAF gene and clinical and angiographic restenosis within the GENDER study.

	Clinical restenosis			Angiographic restenosis [a]			Clinical restenosis in the stented subpopulation [b]		
	N (%)	HR (95%CI)	p-value	N (%)	HR (95%CI)	p-value	N (%)	HR (95%CI)	p-value
PCAF -4556 C/T									
C/C	218 (10)	1.0 (ref)		68 (21)	1.0 (ref)		148 (9)	1.0 (ref)	
C/T	54 (10)	0.98 (0.73-1.32)	0.90	16 (19)	0.80 (0.41-1.53)	0.50	31 (8)	0.86 (0.58-1.26)	0.43
T/T	2 (5)	0.46 (0.11-1.86)	0.28	0 (0)			1 (3)	0.34 (0.05-2.45)	0.34
Trend	274 (10)	0.92 (0.70-1.20)	0.54	84 (21)	0.77 (0.41-1.45)	0.42	180 (9)	0.80 (0.561.15)	0.23
PCAF -2481 G/C									
G/G	140 (11)	1.0 (ref)		47 (25)	1.0 (ref)		98 (10)	1.0 (ref)	
G/C	114 (9)	0.80 (0.62-1.02)	0.07	29 (16)	0.56 (0.33-0.96)	0.03	68 (7)	0.70 (0.51-0.95)	0.02
C/C	22 (7)	0.64 (0.41-1.00)	0.05	8 (18)	0.64 (0.27-1.52)	0.31	16 (7)	0.65 (0.38-1.10)	0.11
Trend	276 (10)	0.80 (0.67-0.97)	0.02	84 (21)	0.69 (0.47-1.02)	0.07	182 (9)	0.76 (0.60-0.95)	0.02

[a] measured in a subgroup of 478 subjects. [b] measured in a subgroup of 2309 subjects.

HR=Hazard ratio. CI=Confidence interval. All hazard ratios and p-values were assessed with a Cox-proportional hazards model and adjusted for sex, age, and clinical and procedural risk factors for restenosis.

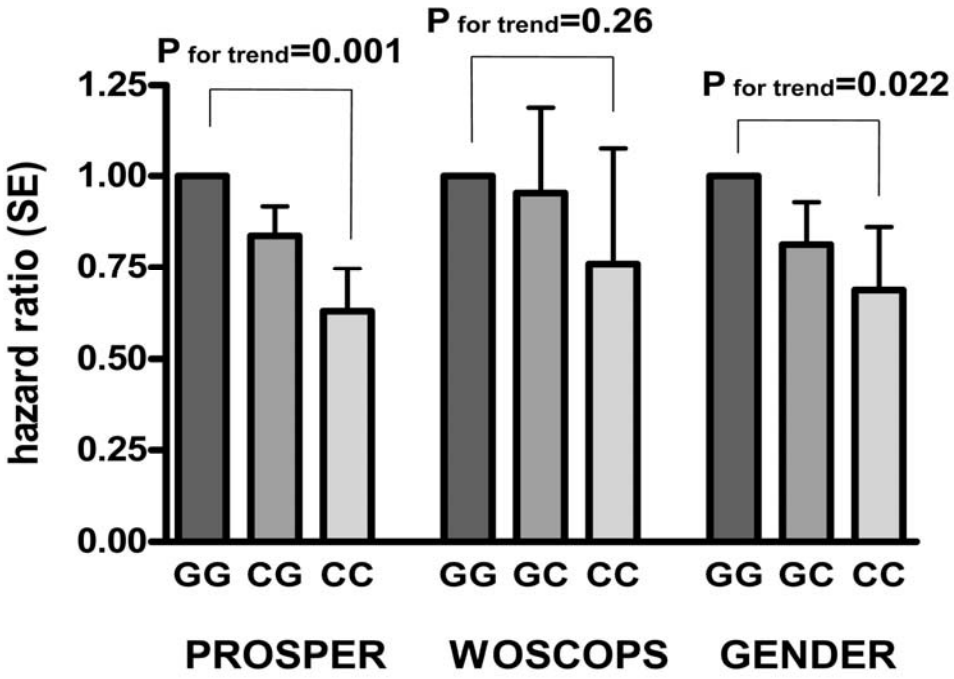

Figure 1: Hazard ratios for all-cause mortality in PROSPER, for coronary heart disease death in WOSCOPS, and for clinical restenosis in GENDER by PCAF -2481 G/C genotype at all ages.

The PCAF -2481 G/C polymorphism is associated with mortality in the PROSPER study, with coronary heart disease death in the WOSCOPS study, and with clinical restenosis in the GENDER study in all age groups

Combined Effect Analysis

Hazard ratios were almost remarkably equal in all three studies (figure 1). Therefore and since all three studies have comparable endpoints, we conducted a combined effect analysis to show the effect of the -2481C on the study endpoints (CHD death in PROSPER and WOSCOPS, and clinical restenosis in GENDER) at all ages (figure 2). The pooled hazard ratio for the- 2481C allele was 0.79 ((95%CI: 0.74-0.85), p =8.1 x 10^{-4}). Heterozygotes had a reduced risk (HR: 0.82, 95%CI: 0.68-0.98, p=0.03), and this risk was lower in subjects homozygous for the -2481C allele (HR: 0.61 95%CI: 0.44-0.84, p= 0.002).

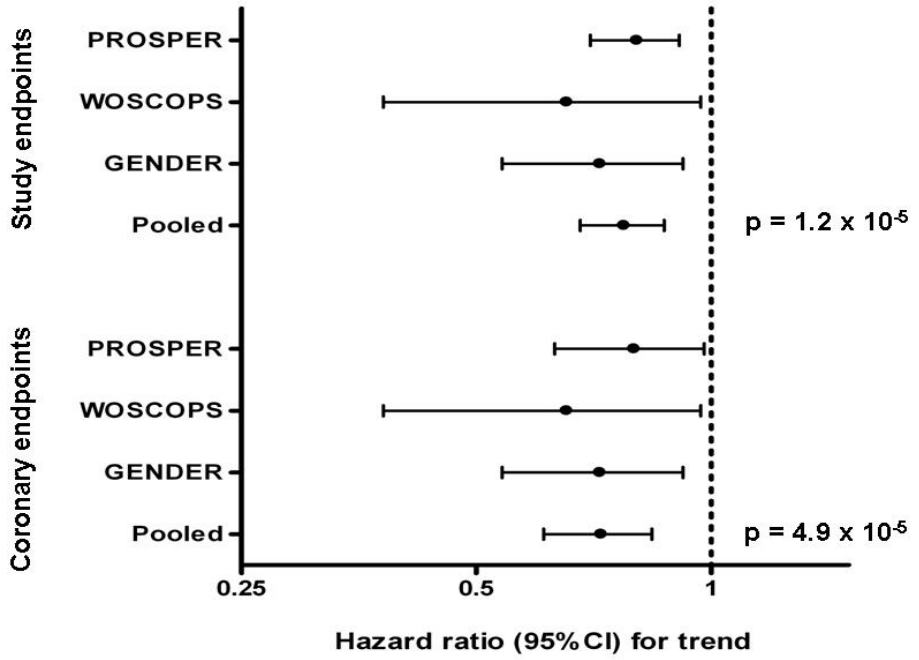

Figure 2: Combined effect estimate of the hazard ratios of the PROSPER, WOSCOPS, and GENDER studies for the PCAF -2481 G/C polymorphism.

This figure represents the hazard ratios for the additive model of the PCAF -2481 G/C polymorphism. Coronary endpoints consist of CHD death for the PROSPER and WOSCOPS study, and clinical restenosis for the GENDER study do in all and repeat. The top panel is the combined effect analysis at all ages, the bottom panel in subgroups with age > 58 years.

Examining for an Age Effect

From the work of Fraga *et al*. we know that as a result from exposure to environmental factors the total quantity of acetylated histones can change during life and that older monozygotic twins differ more from each other in total histone H3- and H4-acetylation than young monozygotic twins (8). This means that some (cumulative) effects of histone acetylation could become more evident with increasing age. In the PROSPER study we could not demonstrate an effect of the -2481C allele with increasing age, since everyone participating in this study was between 70-82 years of age. However, in both the WOSCOPS and the GENDER studies, with a considerably wider age range, we could indeed identify an effect of the -2481C allele with increasing age. In two age strata we investigated the effect of the PCAF -2481 G/C promoter polymorphism on CHD death in the WOSCOPS study.

Among participants ≥58 years old (median age), we found that the -2481C allele significantly reduced the risk of CHD death (risk reduction 39% (3% to 62%), p= 0.035), whereas there was no significant effect in the lower age group. We found similar results in the GENDER study. We again observed a strong protective effect of the C-allele in old patients (≥58 years, n=1800) (risk reduction 30% (12% to 45%), p=0.003) whereas there was no significant effect present in young patients (<58 years, n=1052).

We also conducted a combined effect analysis to show the effect of the -2481C allele at a high age only (median age >58). The pooled hazard ratio for the -2481C allele on coronary events (CHD death or TVR) in this high age group was 0.72 ((95%CI: 0.61-0.84), p=4.9 x 10^{-5}), which is somewhat stronger than the effect at all ages (figure 2).

Gene Expression Microarray

To find an explanation for the age-related difference in effect of the -2841 genotype we compared the gene expression levels of PCAF between nonagenarians from the Leiden Longevity Study and middle-aged controls. For the probe corresponding to an exon of the PCAF gene a lower expression level was observed in the nonagenarians compared to the middle-aged controls (FC 0.84, p=0.004). No differential expression of PCAF was observed for a probe corresponding to an intronic region of the PCAF gene (FC 1.01, p-value 0.90), indicating that the observed expression difference with age is specific for the PCAF gene and not for an alternative splice variant.

Functional Involvement: The -2481 Region Encompassing the C/G PCAF Variants

Using the EMSA technique we tested whether the observed polymorphism would lead to differential protein binding in vitro. Using nuclear extracts of different cell types, we studied complex formation at the -2481 region of PCAF. We could detect constitutive protein binding to both PCAF-C and G-variants (figure 3). In both human umbilical vein endothelial cells (HUVECs) and U251 cells nuclear factor binding is enhanced by IFN-γ stimulation. Densitometric analysis

revealed that the PCAF G-variant exhibits slightly stronger protein binding than the C-variant. The affinity difference was confirmed by competition assays (data not shown).

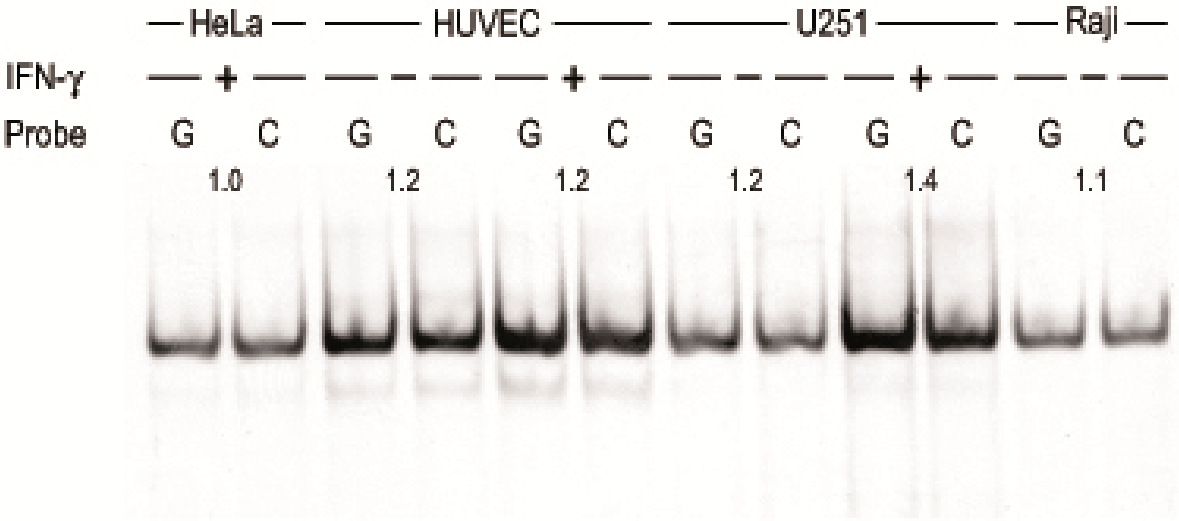

Figure 3: Protein binding to the C- and G-variant of the -2481 region.

EMSA showing binding of protein to the C- and G-variant of the -2481 region using nuclear extracts of various cell-lines. EMSA analysis revealed slightly stronger binding of protein to the G-variant of the -2481 region. Stimulation of HUVECs with interferon-γ increases nuclear factor binding to the labeled probe slightly. In IFN-γ stimulated U251 cells the difference between stimulated and unstimulated cells is much more pronounced. Numbers indicate the ratios of optical density ($^{G}/_{C}$).

Using the TFSearch program, we identified possible binding sites for MZF1 and GATA1-3 in the PCAF C-variant promoter. However, ds-oligonucleotides representing binding sites for MZF1 and GATA1-3 did not compete with factor binding to the PCAF C-promoter variant (data not shown).

PCAF mRNA Expression in a Mouse Model of Reactive Stenosis

Placement of a small non-constrictive cuff around the femoral artery in hyper-cholesterolemic (13.9 ± 3.6 mmol/L) ApoE3Leiden mice results in induction of neointima formation. In the developing neointima a rapid upregulation of the PCAF expression was observed after cuff placement in a time dependent manner (figure 4). PCAF expression showed a peak two days after vascular injury (~1.5 fold increase) and reduces to baseline levels of expression after 7 days.

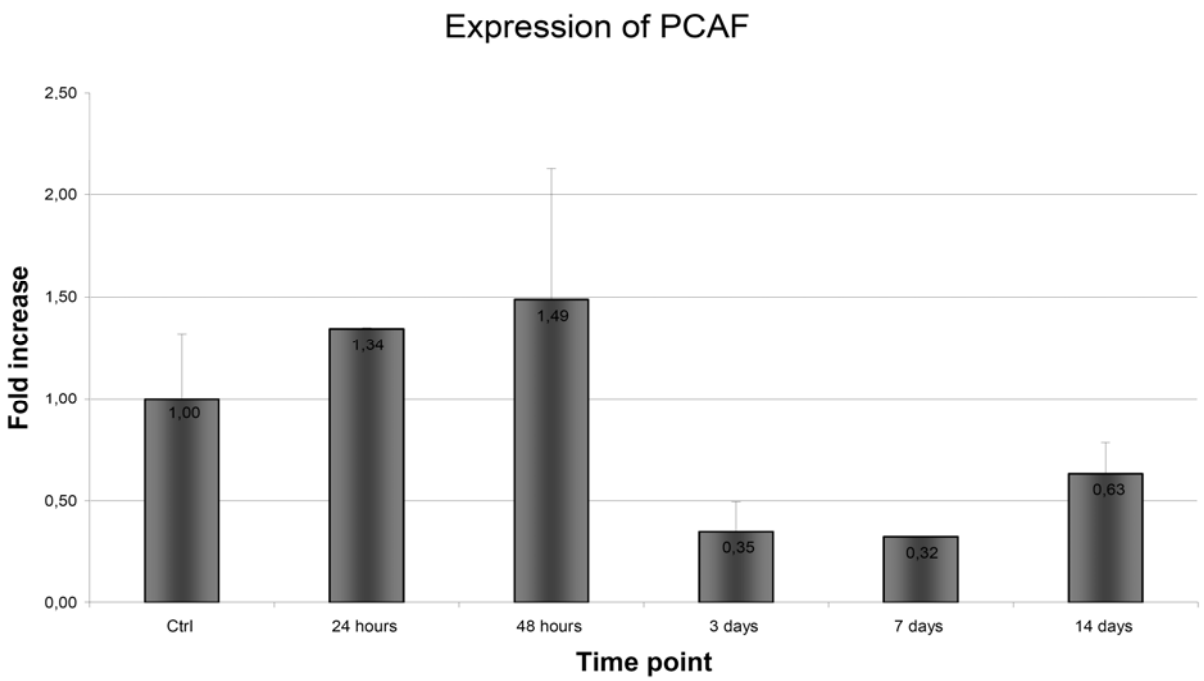

Figure 4: Expression of PCAF mRNA in a cuff-induced reactive stenosis mouse model.

The figure shows PCAF differentially expressed upon activation of the vessel wall in a time dependent manner, with an expression peak 48 hours after vascular injury. The data is presented as fold increase compared to the control arteries, using HPRT as an internal control for cDNA input.

Discussion

Our data indicate that the -2481C allele in the gene encoding PCAF, a protein which has been shown to acetylate histones at the site of NFkB regulated genes and several non-histone proteins, such as the p53 tumor-suppressor proteins, is associated with a significant survival advantage in three independent studies. We found in the PROSPER-study an advantage in survival mainly due to a significant risk reduction in CHD death. In line with this observation we also observed that the -2481C allele was associated with a lower death by CHD in the WOSCOPS study in an age dependent manner. Furthermore, this allele also protects against clinical and angiographic restenosis in the GENDER-study. The effects of the -2481C allele on mortality, CHD death and clinical restenosis were more profound at older age (>58 years), which was also corroborated in a gene expression array where PCAF expression was found to be reduced in nonagenarians compared to middle-aged controls. The -2481G/C polymorphism in the PCAF promoter affects transcription

factor binding, as was demonstrated by an EMSA band shift analysis. A role for PCAF in vascular disease was further confirmed in a mouse model for reactive stenosis, in which modulation of PCAF expression was detected during vascular remodeling.

Our observation in the PROSPER-study that this promoter variant associates to lower mortality from cardiovascular disease and cancer as well as clinical restenosis after PCI in GENDER, may indicate a role of PCAF in inhibiting cell proliferation also in more general terms. PCAF has been shown, for example, to activate p53-responsive enhancer elements within the p21waf1 promoter (20) and activity of p21waf1 is known to induce cell-cycle arrest in vascular smooth muscle cells (39-42). Furthermore, A20, a NFκB-dependent gene that has been shown to inhibit proliferation of VSMCs via increased expression of p21waf1, was able to prevent neointima formation after balloon angioplasty in a rat model of carotid artery stenosis (43).

Apart from its well-described role in cell-cycle regulation, PCAF is also known to be required to co-activate p65-dependent transcription and has been shown to directly activate the transcription of several NFκB-regulated genes known to be involved in cardiovascular disease (44). Miao *et al.* has shown that PCAF could enhance the p65 mediated increase in TNF-α promoter activity and that high glucose increased the recruitment of PCAF to the TNF-α and COX-2 promoters (17). Furthermore, they demonstrated concomitant acetylation of specific lysine residues of histone H3 and H4 at these promoters. Since TNF-α and COX-2 have been implicated in the development of atherosclerosis (45,46), and restenosis (30,47), , and also cancer (48,49), our data suggest that PCAF may also play a role in the development of these diseases.

Our finding in the WOSCOPS and GENDER-study that the strong protective effect of the -2481C allele was more profound in patients older than 58 years old, whereas it seemed not present in young patients (<58 years old) is of particular interest. It could reveal the combined effect of a life-time dysregulation of PCAF expression resulting from the -2481C genetic variant and the accumulating effect of exposure to environmental factors which affect histone acetylation profiles

during life as observed by Fraga *et al* (8). In the PROSPER population such an age dependent effect was not observed as this trial included only patients >70 years old. As expected, here the effects on cardiovascular and cancer mortality associated with the PCAF -2481 G/C polymorphism were evident for the entire population. After observing an age-dependent effect in the WOSCOPS and GENDER study, we suggest that this polymorphism in PCAF is associated with an altered tendency to acetylate histones and non-histone proteins (such as the tumor-suppressor p53 whose function relies on acetylation, reviewed in Spange *et al*.(50)) and may therefore become important especially in elderly patients, who may have been under the influence of altered PCAF activity for many years.

Although our findings in the GENDER-study do not directly replicate the effect of the -2481 G/C polymorphism on CHD mortality in the elderly, they are of much value as this study has a mechanistically linked and better defined concise endpoint. Thus in this way patho-physiologic insights would be obtained and not just replication only. Restenosis after a PCI is very well investigated and is now known to be mainly the consequence of inflammatory and proliferative processes, which is underscored by the fact that stents eluting drugs that suppress these processes are highly efficacious in the prevention of restenosis. Therefore, we believe that our finding that the -2481C allele protects against restenosis in the GENDER-study does not only confirm its functional significance, but also provides mechanistic insights in its beneficial role in survival in the PROSPER and WOSCOPS studies.

Although we show the association between this locus involved in epigenetic control and clinical conditions in three large follow-up studies with a mechanistically linked endpoint, our studies warrant further investigation into the influence of the -2481 G/C polymorphism on the activity of the PCAF promoter or expression levels of NFκB-regulated genes. Our data that PCAF expression is reduced in nonagenarians when compared to middle-aged controls confirms a role for this gene in survival and suggests that the -2481C variant leads to reduced transcription of the PCAF gene. Further research has to be performed to test this hypothesis. In a first analysis we were able to demonstrate binding of nuclear protein extracts to the specific region flanking the -2481 G/C

polymorphism in the PCAF promoter. EMSA analysis showed that the G-variant exhibits a higher affinity for nuclear factor binding than the C-variant, albeit a relatively small difference. The difference observed, however, might lead to a dramatic different outcome if the effects accumulate over years. In initial experiments we were unable to elucidate the nuclear factor binding to the -2481 region. Thus the protein(s) leading to the different outcome in disease remain(s) to be elucidated. It should be noted that IFN-γ stimulation increases nuclear factor binding in HUVECs and U251 cells, suggesting a role for IFN-γ induced nuclear factors. To further explore the role of this gene in vascular disease, we quantified PCAF-transcripts in the stenotic vessel wall in a mouse model of cuff induced reactive stenosis in the femoral artery. During the stenotic process, the PCAF gene expression was rapidly upregulated, indicating that PCAF gene expression is activated upon vascular injury and suggesting that this transcriptional coactivator is involved in the development of reactive stenosis, at least in the early stages.

In conclusion, we showed in three large prospective studies that the -2481C allele in the PCAF promoter is associated with a significant survival advantage in elderly patients while also protecting against clinical and angiographic restenosis after PCI. Although the exact mechanisms of these actions are thus far unknown, we suggest that the effect of this allele on these endpoints may be due to the well-known involvement of PCAF in inflammatory and proliferative processes. Our results indicate that this locus may be involved in the modulation of cardiovascular event or mortality, probably by epigenetic histone modifications, which may be a target for future therapy.

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ReferenceList

(1) Ross JS, Stagliano NE, Donovan MJ, Breitbart RE, Ginsburg GS. Atherosclerosis and cancer: common molecular pathways of disease development and progression. Ann N Y Acad Sci 2001;947:271-292.

(2) Ponder BA. Cancer genetics. Nature 2001;411:336-341.

(3) Auer J, Weber T, Berent R, Lassnig E, Lamm G, Eber B. Genetic polymorphisms in cytokine and adhesion molecule genes in coronary artery disease. Am J Pharmacogenomics 2003;3:317-328.

(4) Agema WR, Jukema JW, Pimstone SN, Kastelein JJ. Genetic aspects of restenosis after percutaneous coronary interventions: towards more tailored therapy. Eur Heart J 2001;22:2058-2074.

(5) Kathiresan S, Melander O, Anevski D, Guiducci C, Burtt NP, Roos C, Hirschhorn JN, Berglund G, Hedblad B, Groop L, Altshuler DM, Newton-Cheh C, Orho-Melander M. Polymorphisms associated with cholesterol and risk of cardiovascular events. N Engl J Med 2008;358:1240-1249.

(6) Monraats PS, Pires NM, Agema WR, Zwinderman AH, Schepers A, de Maat MP, Doevendans PA, de Winter RJ, Tio RA, Waltenberger J, Frants RR, Quax PH, van Vlijmen BJ, Atsma DE, van der LA, van der Wall EE, Jukema JW. Genetic inflammatory factors predict restenosis after percutaneous coronary interventions. Circulation 2005;112:2417-2425.

(7) Gosden RG, Feinberg AP. Genetics and epigenetics--nature's pen-and-pencil set. N Engl J Med 2007;356:731-733.

(8) Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suner D, Cigudosa JC, Urioste M, Benitez J, Boix-Chornet M, Sanchez-Aguilera A, Ling C, Carlsson E, Poulsen P, Vaag A, Stephan Z, Spector TD, Wu YZ, Plass C, Esteller M. Epigenetic differences arise during the lifetime of monozygotic twins. Proc Natl Acad Sci U S A 2005;102:10604-10609.

(9) Ito K, Ito M, Elliott WM, Cosio B, Caramori G, Kon OM, Barczyk A, Hayashi S, Adcock IM, Hogg JC, Barnes PJ. Decreased histone deacetylase activity in chronic obstructive pulmonary disease. N Engl J Med 2005;352:1967-1976.

(10) Lill NL, Grossman SR, Ginsberg D, DeCaprio J, Livingston DM. Binding and modulation of p53 by p300/CBP coactivators. Nature 1997;387:823-827.

(11) Pons D, de Vries FR, van den Elsen PJ, Heijmans BT, Quax PH, Jukema JW. Epigenetic histone acetylation modifiers in vascular remodelling: new targets for therapy in cardiovascular disease. Eur Heart J 2009;30:266-277.

(12) Turunen MP, Aavik E, Yla-Herttuala S. Epigenetics and atherosclerosis. Biochim Biophys Acta 2009.

(13) Wei JQ, Shehadeh LA, Mitrani JM, Pessanha M, Slepak TI, Webster KA, Bishopric NH. Quantitative control of adaptive cardiac hypertrophy by acetyltransferase p300. Circulation 2008;118:934-946.

(14) Tycko B. Epigenetic gene silencing in cancer. J Clin Invest 2000;105:401-407.

(15) Grunstein M. Histone acetylation in chromatin structure and transcription. Nature 1997;389:349-352.

(16) Moodie FM, Marwick JA, Anderson CS, Szulakowski P, Biswas SK, Bauter MR, Kilty I, Rahman I. Oxidative stress and cigarette smoke alter chromatin remodeling but differentially regulate NF-kappaB activation and proinflammatory cytokine release in alveolar epithelial cells. FASEB J 2004;18:1897-1899.

(17) Miao F, Gonzalo IG, Lanting L, Natarajan R. In vivo chromatin remodeling events leading to inflammatory gene transcription under diabetic conditions. J Biol Chem 2004;279:18091-18097.

(18) Vogel NL, Boeke M, Ashburner BP. Spermidine/Spermine N1-Acetyltransferase 2 (SSAT2) functions as a coactivator for NF-kappaB and cooperates with CBP and P/CAF to enhance NF-kappaB-dependent transcription. Biochim Biophys Acta 2006;1759:470-477.

(19) Liu L, Scolnick DM, Trievel RC, Zhang HB, Marmorstein R, Halazonetis TD, Berger SL. p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage. Mol Cell Biol 1999;19:1202-1209.

(20) Di Stefano V, Soddu S, Sacchi A, D'Orazi G. HIPK2 contributes to PCAF-mediated p53 acetylation and selective transactivation of p21Waf1 after nonapoptotic DNA damage. Oncogene 2005;24:5431-5442.

(21) Okumura K, Mendoza M, Bachoo RM, DePinho RA, Cavenee WK, Furnari FB. PCAF modulates PTEN activity. J Biol Chem 2006;281:26562-26568.

(22) Lowe GD. Circulating inflammatory markers and risks of cardiovascular and non-cardiovascular disease. J Thromb Haemost 2005;3:1618-1627.

(23) Allin KH, Bojesen SE, Nordestgaard BG. Baseline C-reactive protein is associated with incident cancer and survival in patients with cancer. J Clin Oncol 2009;27:2217-2224.

(24) Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Cobbe SM, Ford I, Gaw A, Hyland M, Jukema JW, Kamper AM, Macfarlane PW, Meinders AE, Norrie J, Packard CJ, Perry IJ, Stott DJ, Sweeney BJ, Twomey C, Westendorp RG. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet 2002;360:1623-1630.

(25) Agema WR, Monraats PS, Zwinderman AH, de Winter RJ, Tio RA, Doevendans PA, Waltenberger J, de Maat MP, Frants RR, Atsma DE, van der Laarse A, van der Wall EE, Jukema JW. Current PTCA practice and clinical outcomes in The Netherlands: the real world in the pre-drug-eluting stent era. Eur Heart J 2004;25:1163-1170.

(26) Lardenoye JH, Delsing DJ, de Vries MR, Deckers MM, Princen HM, Havekes LM, van Hinsbergh VW, van Bockel JH, Quax PH. Accelerated atherosclerosis by placement of a perivascular cuff and a cholesterol-rich diet in ApoE*3Leiden transgenic mice. Circ Res 2000;87:248-253.

(27) Shepherd J, Blauw GJ, Murphy MB, Cobbe SM, Bollen EL, Buckley BM, Ford I, Jukema JW, Hyland M, Gaw A, Lagaay AM, Perry IJ, Macfarlane PW, Meinders AE, Sweeney BJ, Packard CJ, Westendorp RG, Twomey C, Stott DJ. The design of a prospective study of Pravastatin in the Elderly at Risk (PROSPER). PROSPER Study Group. PROspective Study of Pravastatin in the Elderly at Risk. Am J Cardiol 1999;84:1192-1197.

(28) Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, Macfarlane PW, McKillop JH, Packard CJ. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. N Engl J Med 1995;333:1301-1307.

(29) Packard CJ, O'Reilly DS, Caslake MJ, McMahon AD, Ford I, Cooney J, Macphee CH, Suckling KE, Krishna M, Wilkinson FE, Rumley A, Lowe GD. Lipoprotein-associated phospholipase A2 as an independent predictor of coronary heart disease. West of Scotland Coronary Prevention Study Group. N Engl J Med 2000;343:1148-1155.

(30) Monraats PS, Pires NM, Schepers A, Agema WR, Boesten LS, de Vries MR, Zwinderman AH, de Maat MP, Doevendans PA, de Winter RJ, Tio RA, Waltenberger J, 't Hart LM, Frants RR, Quax PH, van Vlijmen BJ, Havekes LM, van der LA, van der Wall EE, Jukema JW. Tumor necrosis factor-alpha plays an important role in restenosis development. FASEB J 2005;19:1998-2004.

(31) Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel OV, Ignatieva EV, Ananko EA, Podkolodnaya OA, Kolpakov FA, Podkolodny NL, Kolchanov NA. Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Res 1998;26:362-367.

(32) Gobin SJ, Peijnenburg A, Keijsers V, van den Elsen PJ. Site alpha is crucial for two routes of IFN gamma-induced MHC class I transactivation: the ISRE-mediated route and a novel pathway involving CIITA. Immunity 1997;6:601-611.

(33) Ko LJ, Engel JD. DNA-binding specificities of the GATA transcription factor family. Mol Cell Biol 1993;13:4011-4022.

(34) Morris JF, Hromas R, Rauscher FJ, III. Characterization of the DNA-binding properties of the myeloid zinc finger protein MZF1: two independent DNA-binding domains recognize two DNA consensus sequences with a common G-rich core. Mol Cell Biol 1994;14:1786-1795.

(35) Abramoff MD, Magelhaes PJ, Ram SJ. Image Processing with ImageJ. Biophotonics International 2004;11:36-42.

(36) van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de B, I, van Vlijmen B, van der BH, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. J Biol Chem 1993;268:10540-10545.

(37) Pires NM, Eefting D, de Vries MR, Quax PH, Jukema JW. Sirolimus and paclitaxel provoke different vascular pathological responses after local delivery in a murine model for restenosis on underlying atherosclerotic arteries. Heart 2007;93:922-927.

(38) Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.

(39) Mnjoyan ZH, Dutta R, Zhang D, Teng BB, Fujise K. Paradoxical upregulation of tumor suppressor protein p53 in serum-stimulated vascular smooth muscle cells: a novel negative-feedback regulatory mechanism. Circulation 2003;108:464-471.

(40) Kusama H, Kikuchi S, Tazawa S, Katsuno K, Baba Y, Zhai YL, Nikaido T, Fujii S. Tranilast inhibits the proliferation of human coronary smooth muscle cell through the activation of p21waf1. Atherosclerosis 1999;143:307-313.

(41) Lee B, Kim CH, Moon SK. Honokiol causes the p21WAF1-mediated G(1)-phase arrest of the cell cycle through inducing p38 mitogen activated protein kinase in vascular smooth muscle cells. FEBS Lett 2006;580:5177-5184.

(42) Kato S, Ueda S, Yamaguchi M, Miyamoto T, Fujii T, Izumaru S, Kume S, Toyozumi Y, Arima N, Morimatsu M. Overexpression of p21Waf1 induces apoptosis in immortalized human vascular smooth muscle cells. J Atheroscler Thromb 2003;10:239-245.

(43) Patel VI, Daniel S, Longo CR, Shrikhande GV, Scali ST, Czismadia E, Groft CM, Shukri T, Motley-Dore C, Ramsey HE, Fisher MD, Grey ST, Arvelo MB, Ferran C. A20, a modulator of smooth muscle cell proliferation and apoptosis, prevents and induces regression of neointimal hyperplasia. FASEB J 2006;20:1418-1430.

(44) Kiernan R, Bres V, Ng RW, Coudart MP, El Messaoudi S, Sardet C, Jin DY, Emiliani S, Benkirane M. Post-activation turn-off of NF-kappa B-dependent transcription is regulated by acetylation of p65. J Biol Chem 2003;278:2758-2766.

(45) Sarzi-Puttini P, Atzeni F, Doria A, Iaccarino L, Turiel M. Tumor necrosis factor-alpha, biologic agents and cardiovascular risk. Lupus 2005;14:780-784.

(46) Cipollone F, Prontera C, Pini B, Marini M, Fazia M, De Cesare D, Iezzi A, Ucchino S, Boccoli G, Saba V, Chiarelli F, Cuccurullo F, Mezzetti A. Overexpression of functionally coupled cyclooxygenase-2 and prostaglandin E synthase in symptomatic atherosclerotic plaques as a basis of prostaglandin E(2)-dependent plaque instability. Circulation 2001;104:921-927.

(47) Wang K, Tarakji K, Zhou Z, Zhang M, Forudi F, Zhou X, Koki AT, Smith ME, Keller BT, Topol EJ, Lincoff AM, Penn MS. Celecoxib, a selective cyclooxygenase-2 inhibitor, decreases monocyte chemoattractant protein-1 expression and neointimal hyperplasia in the rabbit atherosclerotic balloon injury model. J Cardiovasc Pharmacol 2005;45:61-67.

(48) Mocellin S, Rossi CR, Pilati P, Nitti D. Tumor necrosis factor, cancer and anticancer therapy. Cytokine Growth Factor Rev 2005;16:35-53.

(49) Williams CS, Mann M, DuBois RN. The role of cyclooxygenases in inflammation, cancer, and development. Oncogene 1999;18:7908-7916.

(50) Spange S, Wagner T, Heinzel T, Kramer OH. Acetylation of non-histone proteins modulates cellular signalling at multiple levels. Int J Biochem Cell Biol 2009;41:185-198.

Chapter 9

ApoE genotype, plasma cholesterol, and cancer: A Mendelian randomization study.

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Abstract

Observational studies have shown an association between low plasma cholesterol levels and an increased risk of cancer whereas most randomized clinical trials with cholesterol lowering medications have not shown this association. The authors assessed the association between plasma cholesterol levels and cancer risk, free from confounding and reverse causality, by a Mendelian randomization study design using the ApoE genotype. ApoE genotype, plasma cholesterol levels, and cancer incidence and mortality were measured during three years in 2,913 participants in the PROspective Study of Pravastatin in the Elderly at Risk. Subjects within the lowest third of plasma cholesterol level at baseline had an increased risk of cancer incidence (HR (95% CI) = 1.90 (1.34, 2.70)) and cancer mortality (HR (95% CI) = 2.03 (1.23, 3.34)) relative to subjects within the highest third of plasma cholesterol. However, carriers of the ApoE2 genotype (n=332), who had 9% lower levels of plasma cholesterol than carriers of the ApoE4 genotype (n=635), did not have in increased risk of incident cancer incidence (HR (95% CI) = 0.86 (0.50, 1.47)) or cancer mortality (HR (95% CI) = 0.70 (0.30, 1.60)) compared to ApoE4 carriers. Our findings suggest that low levels of cholesterol are not causally related to an increased risk of cancer.

Introduction

Numerous observational studies have reported an association between low plasma cholesterol levels and increased risk of cancer (1-6). This has led to concerns that treatment or lifestyle changes that lower cholesterol might increase cancer risk. However, these observed associations between low plasma cholesterol and increased risk of cancer might originate from reverse causality or confounding. For example, low plasma cholesterol levels might be caused by a hypocholesterolemic effect of cancer in preclinical stages of cancer (7). In this case, subjects with cancer have an abnormal low cholesterol level because of the cancer and not vice versa (reverse causality). Furthermore, confounding factors such as age, smoking, and alcohol use might also explain some of the observed associations. Most randomized clinical trials have shown that cholesterol lowering medications (statins) have no effect on cancer risk (8-12), although some exceptions have been

reported (13;14). However, the length of these trials was limited and the answer to the question whether a lifelong low plasma cholesterol level increases cancer risk has remained elusive.

An alternative epidemiological method, Mendelian randomization, overcomes the problem of reverse causality and confounding since it is based on Mendel's law that inheritance of one trait is independent of inheritance of other traits (15). This means that the association between a genetically determined phenotypic trait and a disease is unlikely to be caused by reverse causality or confounding, provided that the presence of the genotype that causes the trait does not influence the subject's lifestyle or environment. This condition will usually be fulfilled as long as subjects are unaware of their genotype.

In 1986, one of us (MBK) first suggested to investigate the causality of the relation between plasma cholesterol and cancer by investigating the relationship between ApoE genotype and cancer risk (16). ApoE is involved in the clearance of lipoproteins from plasma and differences in its aminoacid sequence produce differences in plasma cholesterol levels within a population. Three independent alleles of the ApoE gene occur frequently. They give rise to the isoforms E2, E3, and E4, with one cysteine residue being replaced by arginine from E2 to E3 and another one from E3 to E4. Plasma cholesterol levels rise from E2 to E3 to E4. Therefore, if low cholesterol levels promote tumour growth, then subjects with the E2/E2 or E2/E3 phenotype should have the highest risk of cancer. Our way of analysis (16) constituted the first instance of what would later be named Mendelian randomization (17). In the cholesterol and cancer debate it has never been put to the test. We here report the association between the ApoE genotype, plasma cholesterol levels, and cancer risk in an elderly cohort.

Methods

Participants

Study participants came from the placebo group of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) trial. A detailed description of the protocol and results of the study have been published elsewhere (13;18). Here a short outline is provided.

PROSPER was a multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin decreases the risk of major vascular events in elderly individuals. Between December 1997 and May 1999, we screened and enrolled subjects in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or had increased risk of such disease because of smoking, hypertension, or diabetes. Subjects with a history of malignancy within 5 years prior to the trial were not eligible to participate. A total number of 5804 subjects were randomly assigned to pravastatin (N=2,891) or placebo (N=2,913). In our study, all analyses were performed in subjects with placebo allocation (N=2,913) so that a possible effect of pravastatin on cancer could not affect our results. The primary endpoint was the combined endpoint of fatal coronary heart disease (CHD), non-fatal myocardial infarct (MI), and occurrence of clinical stroke, either fatal or non-fatal. Other study endpoints were occurrence of transient ischemic attack, disability, cognitive function, and cancer incidence and mortality. Information on all deaths were received by post-mortem reports, death certificates, hospital records, general practitioners' records, and/or interviews of family members or witnesses. All endpoints were adjudicated by a study endpoint committee. Mean follow-up duration was 3.2 years (range 2.8-4.0).

Measurements

Plasma cholesterol levels were measured twice at fasting visits during the placebo run-in phase according to the Lipid Research Clinics protocol (19) in a central laboratory which was standardized through the Center for Disease Control network. The second measurement during the placebo run-in

phase was used as the baseline measurement. During the follow-up of the PROSPER study, lipid and lipoprotein measurements were again performed after 3, 6, 12, 24, and 36 months.

Apolipoprotein E phenotype was determined on plasma samples by western blotting following the method of Havekes *et al*.(20). Subjects were classified according to the presence of the E2, E3, or E4 bands on gel blots. The gel phenotyping method shows very high concordance (>95%) with genotype testing by allele specific oligonucleotide assay, therefore we consider this measurement as a measurement of the ApoE genotype (21).

Statistical analysis

All statistical analyses were performed in subjects with placebo allocation so that a possible effect of pravastatin on cancer could not affect our results. For the association with ApoE genotype, participants were divided into three categories, E2+ (genotypes E2/2, E2/3), E3/3 (the most frequent genotype) and E4+ (genotypes E3/4, and E4/4). Subjects with the ApoE2/4 (n=59) were excluded from all analyses. The plasma cholesterol levels measured at baseline were divided in three equal strata representing low (<5.22 mmol/L), intermediate (5.22-6.02 mmol/L) and high plasma cholesterol levels (>6.02 mmol/L). The association between ApoE genotype and plasma cholesterol level was assessed by linear regression. The cross-sectional associations between ApoE genotypes, plasma cholesterol levels, and potential confounders were assessed using the linear by linear association test for categorical variables and by linear regression for continuous variables. Hazard ratios (HR) with 95% confidence intervals (CI) for cancer incidence and cancer mortality were calculated using Cox-proportional hazards models. Subjects who died of other causes of death than cancer, subjects who withdrew consent, and subjects who were lost to follow-up were censored at the date of death or at the last date of follow-up. In all adjusted analyses we corrected for the potential confounders of gender, age, current smoking, alcohol use, history of hypertension, diabetes, and myocardial infarction. All analyses were performed with SPSS statistical software (version 12.0.1, SPSS Inc, Chicago, Ill). *P*-values lower than 0.05 were considered statistically significant.

Results

The mean age of the participants was 75.3 years and approximately 50% were female (Table 1). Mean follow-up of study subjects was 3.2 years (range 2.8-4.0) for participants who did not die or withdraw consent. Of the 2913 subjects allocated to placebo, apolipoprotein E phenotyping was available for 2794 (95.9%). The category E2+ consisted of 332 (12%) subjects, E3/E3 of 1768 (63%) subjects, and E4+ of 694 (25%) subjects. Translated into genotypes, the frequencies were in Hardy-Weinberg equilibrium. The genotype frequencies between the three countries were not significantly different (*P*=0.15).

Table 1: Baseline Characteristics of the Participants in the Placebo Arm of the PROSPER Study

	All participants (n=2,913)
Continuous variates (mean, SD)	
Age (years)	75.3 (3.4)
Body Mass index, (kg/m2)	26.8 (4.3)
Total cholesterol, (mmol/L)	5.7 (0.9)
LDL cholesterol, (mmol/L)	3.8 (0.8)
HDL cholesterol, (mmol/L)	1.3 (0.4)
Categorical variates (n, %)	
Female	1,505 (52)
Current smoker	805 (28)
Diabetes	320 (11)
Hypertension	1,793 (62)
ApoE genotype (n, %)*	
E2+	332 (11)
E3/E3	1,768 (61)
E4+	635 (23)

* ApoE genotype was measured in 2,735 participants

The association between the ApoE genotypes and plasma lipoprotein levels is depicted in Figure 1. As expected, ApoE2/E2 carriers had lowest plasma cholesterol levels (mean 5.26 (SE 0.25)), ApoE3/3 subjects had intermediate plasma cholesterol levels (mean 5.66 (SE 0.02)) and subjects with the ApoE4/E4 genotype had the highest plasma cholesterol levels (mean 5.97 (SE 0.12)). The p-value for trend over the five categories was statistically significant (*P*<0.01).

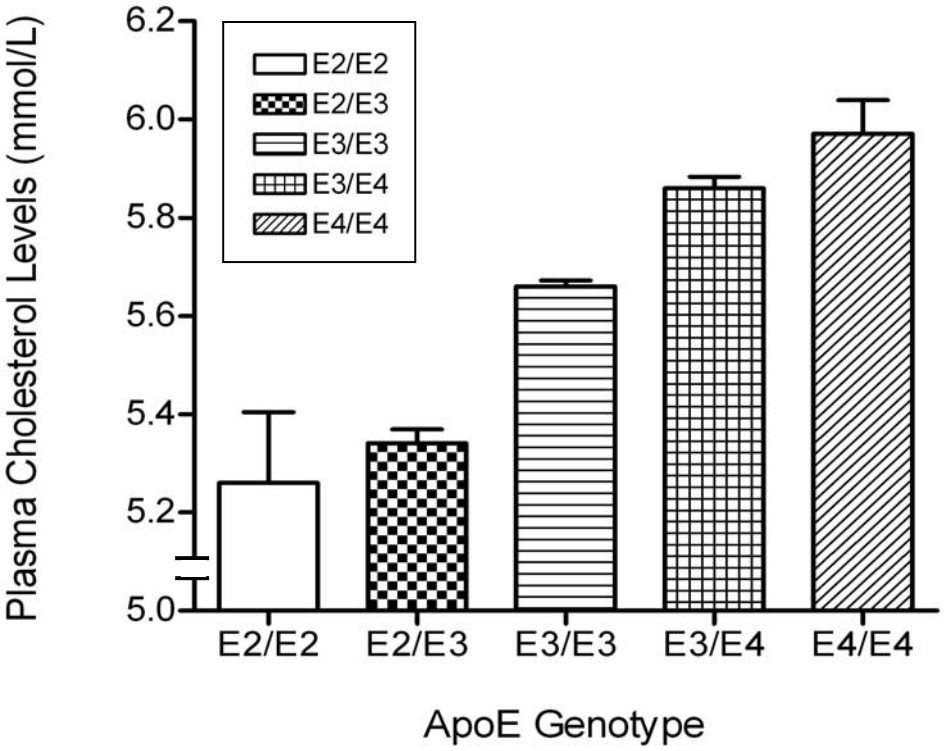

Figure 1: Association between ApoE genotype and plasma cholesterol levels.

Plasma cholesterol levels (mmol/L) are presented in mean (se).

We divided participants in three equal strata representing low, intermediate and high plasma cholesterol levels, and compared various characteristics between the subjects within these three groups. Gender, alcohol use, current smoking, history of diabetes, history of myocardial infarction and history of hypertension were all significantly different over strata of cholesterol (Table 2, all $P=$ or <0.01). As expected, when we divided the subjects in the three ApoE genotype groups, cholesterol level significantly increased over strata of ApoE genotype ($P<0.01$). We found the same trend for LDL cholesterol levels with ApoE2 carriers having the lowest LDL level and ApoE4 carriers the highest levels ($P<0.01$). For HDL cholesterol levels the trend was reversed, ApoE2+ carriers had the highest levels and ApoE4+ the lowest HDL levels ($P=0.01$). However, no other characteristic was significantly different between subjects with different ApoE genotypes (all $P>0.07$).

During follow-up there were 199 subjects who developed cancer and 91 subjects who died of it. The results of the association between plasma cholesterol levels and ApoE genotype on the one hand and cancer incidence and cancer death on the other hand are shown in Table 3. The group with low cholesterol levels had an increased risk of cancer incidence compared to the group with intermediate cholesterol levels (HR (95%CI) = 1.45 (1.05-2.01), *P*=0.02) or high cholesterol levels (HR (95%CI) = 1.90 (1.34-2.70), *P*<0.01). After adjustment for potential confounders, subjects with low cholesterol levels still had an increased risk for cancer incidence compared to subjects with intermediate cholesterol levels (HR (95%CI) =1.35 (0.97-1.89), *P*=0.08) or compared to subjects with high cholesterol levels (HR (95%CI) = 1.70 (1.16-2.50), *P*<0.01). Results were similar for LDL cholesterol levels, subjects with low LDL cholesterol levels had an increased risk for cancer incidence compared to subjects with higher LDL cholesterol levels. Moreover, subjects with incident cancer decreased significantly more in cholesterol levels prior cancer diagnosis compared to subjects without incident cancer (mean (se) = -0.23 (0.05) vs -0.13 (0.01) respectively, *P*=0.05), this remained significant after adjustment for gender, age, and country (*P*=0.04). The association between ApoE genotype and cancer incidence presented a different picture (Table 3). E2+ carriers, with the lowest cholesterol levels, had no increased risk for cancer incidence compared to the E3/E3 subject (HR (95%CI) = 0.90 (0.56-1.45), *P*=0.67) or to E4+ carriers (HR (95%CI) = 0.91 (0.53-1.54), *P*=0.72).

A similar trend was seen for cancer mortality as with cancer incidence (table 3). Subjects with low levels of plasma cholesterol had an increased risk of cancer mortality compared to intermediate levels of cholesterol (HR (95%CI) = 2.10 (1.27-3.50), *P*=<0.01) and high levels of cholesterol (HR (95%CI) = 2.03 (0.95-3.02), *P*<0.01). When we adjusted this association for the potential confounders, the results did not change. In the association with ApoE genotype and cancer mortality we found that ApoE2 carriers, who had the lowest plasma cholesterol levels, had a similar risk for cancer mortality compared to ApoE3/E3 carriers (HR (95%CI) = 0.88 (0.55-1.41), *P*=0.59) and compared to ApoE4 carriers (HR (95%CI) = 0.86 (0.50-1.47), *P*=0.59).

Table 2: Association between ApoE, Plasma Cholesterol and Various Characteristics in Subjects Treated With Placebo[a].

	Plasma cholesterol levels[b]				ApoE genotype			
	Low (n=978)	Intermediate (n=967)	High (n=968)	P-value[c]	E2+ (n=332)	E3/E3 (n=1,768)	E4+ (n=635)	P-value[c]
Lipoprotein profile								
Total cholesterol, mmol/L (mean, SE)	4.72 (0.01)	5.62 (0.01)	6.67 (0.02)	NA	5.34 (0.05)	5.66 (0.02)	5.87 (0.04)	**<0.01**
LDL cholesterol, mmol/L (mean, SE)	3.01 (0.01)	3.75 (0.01)	4.59 (0.02)	NA	3.33 (0.04)	3.80 (0.02)	4.00 (0.03)	**<0.01**
HDL cholesterol, mmol/L (mean, SE)	1.19 (0.01)	1.29 (0.01)	1.35 (0.01)	NA	1.31 (0.02)	1.28 (0.01)	1.24 (0.01)	**0.01**
ApoE genotype								
ApoE4 carriers	151 (17)	229 (25)	255 (28)	**<0.01**	NA	NA	NA	NA
Demographics								
Age, years (mean, SE)	75.2 (0.10)	75.2 (0.11)	75.5 (0.11)	0.10	75.3 (0.19)	75.4 (0.08)	75.0 (0.13)	0.07
Education, years (mean, SE)	15.1 (0.07)	15.1 (0.06)	15.1 (0.06)	0.77	15.1 (0.11)	15.1 (0.05)	15.0 (0.07)	0.78
BMI, kg/m2 (mean, SE)	26.9 (0.14)	26.7 (0.14)	26.9 (0.14)	0.78	27.0 (0.22)	26.9 (0.10)	26.7 (0.17)	0.57
Alcohol use, units/week (mean, SE)	5.5 (0.29)	5.6 (0.32)	4.1 (0.24)	**<0.01**	5.3 (0.47)	5.2 (0.21)	4.4 (0.30)	0.10
Female	300 (31)	512 (53)	693 (72)	**<0.01**	163 (49)	928 (53)	325 (51)	0.74
Current Smoker	310 (32)	266 (28)	229 (24)	**<0.01**	94 (28)	484 (27)	162 (26)	0.30
History of vascular disease	431 (44)	424 (44)	404 (42)	0.30	134 (40)	784 (44)	273 (43)	0.64
History of hypertension	559 (57)	594 (61)	640 (66)	**<0.01**	197 (59)	1,101 (62)	399 (63)	0.35
History of diabetes	149 (15)	101 (10)	70 (7)	**<0.01**	45 (14)	187 (11)	62 (10)	0.10
History of Stroke or TIA	103 (11)	109 (11)	109 (11)	0.61	34 (10)	193 (11)	77 (12)	0.33
History of Myocardial infarction	167 (17)	136 (14)	96 (10)	**<0.01**	54 (16)	231 (13)	89 (14)	0.54

Abbreviations: BMI, Body Mass Index; NA, Not applicable; TIA, transient ischemic attack

[a]All data are presented as number (%), unless otherwise stated.

[b]Plasma cholesterol levels are divided in three equal strata representing low, intermediate, and high plasma cholesterol levels.

[c]P-values for categorical variables are assessed with the linear-by linear association test, p-values for continuous variables are assessed with linear regression.

Table 3: Association Between ApoE, Cholesterol and Cancer Risk.

	Plasma cholesterol levels[a]						ApoE genotype					
	Low vs intermediate[c]			Low vs high[c]			E2+ vs E3/E3[c]			E2+ vs E4+[c]		
	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
Crude Model												
Cancer incidence	1.45	1.05, 2.01	**0.02**	1.90	1.34, 2.70	**<0.01**	0.90	0.41, 1.81	0.67	0.91	0.53, 1.54	0.72
Cancer mortality	2.10	1.27, 3.50	**<0.01**	2.03	1.23, 3.34	**0.01**	0.86	0.56, 1.45	0.69	0.74	0.33, 1.68	0.47
Adjusted model[b]												
Cancer incidence	1.35	0.97, 1.89	0.08	1.70	1.16, 2.50	**0.01**	0.88	0.55, 1.41	0.59	0.86	0.50, 1.47	0.59
Cancer mortality	2.16	1.28, 3.64	**<0.01**	1.93	1.12, 3.34	**0.02**	0.85	0.40, 1.79	0.67	0.70	0.30, 1.60	0.39

Abbreviations: CI, confidence interval; HR, hazard ratio

[a]Plasma cholesterol levels are divided in three equal strata representing low, intermediate, and high plasma cholesterol levels.

[b]Adjusted model is additionally adjusted for sex, age, smokers, alcohol use, and history of hypertension, diabetes, and myocardial infarction.

[c]The last group indicates the reference category.

Discussion

In this study we assessed the association between plasma cholesterol levels and cancer risk, free of confounding and reverse causality by using the method of Mendelian randomization.

We found that subjects, when grouped by their baseline levels of cholesterol, had an increased cancer risk if the cholesterol levels were lower. This risk remained even after adjustment for potential confounders. However, when we categorized subjects according to their ApoE genotype, which also resulted in groups with significantly different cholesterol levels, no increased risk for cancer risk was observed between groups. These findings suggest that low levels of cholesterol are not causally related to an increased risk of cancer.

If cholesterol is causally related to an increased risk of cancer, we would have found similar results for the association between plasma cholesterol levels and cancer risk as for the ApoE genotype and cancer risk. When we grouped subjects based on their cholesterol level, those in the low cholesterol group had an increased risk of cancer. However, when we grouped according to ApoE genotype, subjects in the ApoE2+ group had no increased risk of cancer, despite their significantly lower level of cholesterol. We were planning to formally test with statistical software using Mendelian randomization, whether the two different methods gave different results. However, to our knowledge, this is only possible for continuous outcome data. Since we have dichotomous outcome data, we were unable to formally test whether the two methods actually yielded different results. When we adjusted the association between plasma cholesterol levels and cancer for a wide range of potential confounders, including age, sex, current smoking, alcohol use, diabetes, myocardial infarction, and history of hypertension we still found a significant association between low cholesterol level and higher risk of cancer. Therefore, we think that that the association between low plasma cholesterol levels and increased risk for cancer is more likely to be due to reverse causality, and less so by confounding.

Substantial evidence indicates that cancer can reduce plasma cholesterol levels prior to cancer diagnosis. This phenomenon is known as the preclinical cancer effect (7). The mechanism by which

cancer can lower plasma cholesterol is unclear. However, research into this mechanism has revealed that tumor cells need cholesterol for their growth and proliferation. Therefore there is an increased uptake of cholesterol from the blood by tumor cells (22;23). This might lead to lower plasma cholesterol levels prior to cancer diagnosis. Moreover, alterations in plasma lipids and lipoprotein fractions have been demonstrated in patients with acute leukemia and non-Hodgkin's lymphoma (24;25). Similarly, there is ample evidence, as recently reviewed (26) for an inverse relationship between the magnitude of inflammatory response and lipid levels in a variety of conditions such as sepsis, rheumatoid arthritis and other cancers: cholesterol levels are lowered in these illnesses but can increase dramatically and spontaneously with resolution of sepsis or with treatments which potently suppress the inflammatory response.

In 1986 one of us (MBK) proposed to investigate the causality of cholesterol in cancer risk by making use of the ApoE genotype (16). He reasoned that if naturally low cholesterol favours tumour growth, then carriers of the ApoE2+ genotype, who have lower levels of plasma cholesterol, should have an increased risk of cancer. Until 2004 no one had taken up his idea (27). Now more than 20 years after this initial idea we finally address the causality of cholesterol in the risk of cancer.

There are some limitations to the use of the PROSPER study cohort. Subjects were selected to have a history of vascular disease or have an increased risk for such a disease. Although the frequencies of the ApoE genotypes in our study are similar to those in the general population, when extrapolating the results to the general population, the enrichment of cardiovascular disease in our study population should be kept in mind. Furthermore, we think that the association between plasma cholesterol and cancer risk is mostly affected by reverse causality, and less by confounding, because adjustment for potential confounders did not change the results. However, the number of confounders we have adjusted for might not be sufficient; it could be that there are still confounders that we do not know of. Therefore we cannot completely exclude the possibility that the association between cholesterol and cancer is due to confounding rather than disturbed by reverse causality.

Moreover, although our study was adequately powered to find a hazard ratio of 1.5 between cholesterol groups, it was relatively small to demonstrate equivalence between genotype groups. Given a 9% difference in cholesterol level between most extreme ApoE groups, the estimated difference in cancer risk would also be small. Therefore our study has a relatively low power, which is an important drawback of Mendelian randomization studies (28). Therefore we cannot state with absolute certainty that low cholesterol does not cause cancer. But given the fact that all hazard ratios are below unity, it is unlikely that low levels of cholesterol have a substantial impact on cancer risk.

One strength of our study is that we have a follow-up period of 3.2 years and were able to track more than 95% of all participants over this time. Moreover, cancer incidence and mortality were main outcomes of our study and were precisely monitored, which increases the accuracy of this study accordingly.

In conclusion, we have used the Mendelian randomization concept to suggest that the association between low plasma cholesterol and risk of cancer does not derive from a cause effect. Carriers of the ApoE2+ genotype, associated with low plasma cholesterol levels, had no increased risk of cancer. We therefore believe that subjects with low plasma cholesterol levels are not at an increased risk for cancer and that treatment with cholesterol lowering medications does not increase cancer risk by itself.

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Reference List

(1) Cowan LD, O'Connell DL, Criqui MH, Barrett-Connor E, Bush TL, Wallace RB. Cancer mortality and lipid and lipoprotein levels. Lipid Research Clinics Program Mortality Follow-up Study. Am J Epidemiol 1990 Mar;131(3):468-82.

(2) Iribarren C, Reed DM, Chen R, Yano K, Dwyer JH. Low serum cholesterol and mortality. Which is the cause and which is the effect? Circulation 1995 Nov 1;92(9):2396-403.

(3) Keys A, Aravanis C, Blackburn H, Buzina R, Dontas AS, Fidanza F, et al. Serum cholesterol and cancer mortality in the Seven Countries Study. Am J Epidemiol 1985 Jun;121(6):870-83.

(4) Knekt P, Reunanen A, Aromaa A, Heliovaara M, Hakulinen T, Hakama M. Serum cholesterol and risk of cancer in a cohort of 39,000 men and women. J Clin Epidemiol 1988;41(6):519-30.

(5) Schuit AJ, Van Dijk CE, Dekker JM, Schouten EG, Kok FJ. Inverse association between serum total cholesterol and cancer mortality in Dutch civil servants. Am J Epidemiol 1993 May 1;137(9):966-76.

(6) Weverling-Rijnsburger AW, Blauw GJ, Lagaay AM, Knook DL, Meinders AE, Westendorp RG. Total cholesterol and risk of mortality in the oldest old. Lancet 1997 Oct 18;350(9085):1119-23.

(7) Kritchevsky SB, Kritchevsky D. Serum cholesterol and cancer risk: an epidemiologic perspective. Annu Rev Nutr 1992;12:391-416.

(8) Bonovas S, Filioussi K, Flordellis CS, Sitaras NM. Statins and the risk of colorectal cancer: a meta-analysis of 18 studies involving more than 1.5 million patients. J Clin Oncol 2007 Aug 10;25(23):3462-8.

(9) Browning DR, Martin RM. Statins and risk of cancer: a systematic review and metaanalysis. Int J Cancer 2007 Feb 15;120(4):833-43.

(10) Alsheikh-Ali AA, Trikalinos T.A., Kent D.M., Karas R.H. Statins, low-density lipoprotein cholesterol, and risk of cancer. J Am Coll Cardiol 2008 Aug 20.

(11) Kuoppala J, Lamminpaa A, Pukkala E. Statins and cancer: A systematic review and meta-analysis. Eur J Cancer 2008 Aug 14.

(12) Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM, Jr., Kastelein JJ, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med 2008 Nov 20;359(21):2195-207.

(13) Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Cobbe SM, et al. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet 2002 Nov 23;360(9346):1623-30.

(14) Peto R, Emberson J, Landray M, Baigent C, Collins R, Clare R, et al. Analyses of cancer data from three ezetimibe trials. N Engl J Med 2008 Sep 25;359(13):1357-66.

(15) Davey SG, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol 2003 Feb;32(1):1-22.

(16) Katan MB. Apolipoprotein E isoforms, serum cholesterol, and cancer. Lancet 1986 Mar 1;1(8479):507-8.

(17) Keavney B. Commentary: Katan's remarkable foresight: genes and causality 18 years on. Int J Epidemiol 2004 Feb;33(1):11-4.

(18) Shepherd J, Blauw GJ, Murphy MB, Cobbe SM, Bollen EL, Buckley BM, et al. The design of a prospective study of Pravastatin in the Elderly at Risk (PROSPER). PROSPER Study Group. PROspective Study of Pravastatin in the Elderly at Risk. Am J Cardiol 1999 Nov 15;84(10):1192-7.

(19) Plasma lipid distributions in selected North American populations: the Lipid Research Clinics Program Prevalence Study. The Lipid Research Clinics Program Epidemiology Committee. Circulation 1979 Aug;60(2):427-39.

(20) Havekes LM, de KP, Beisiegel U, Havinga J, Smit M, Klasen E. A rapid micromethod for apolipoprotein E phenotyping directly in serum. J Lipid Res 1987 Apr;28(4):455-63.

(21) Packard CJ, Westendorp RG, Stott DJ, Caslake MJ, Murray HM, Shepherd J, et al. Association between apolipoprotein E4 and cognitive decline in elderly adults. J Am Geriatr Soc 2007 Nov;55(11):1777-85.

(22) Ueyama Y, Matsuzawa Y, Yamashita S, Funahashi T, Sakai N, Nakamura T, et al. Hypocholesterolaemic factor from gallbladder cancer cells. Lancet 1990 Sep 22;336(8717):707-9.

(23) Vitols S, Gahrton G, Bjorkholm M, Peterson C. Hypocholesterolaemia in malignancy due to elevated low-density-lipoprotein-receptor activity in tumour cells: evidence from studies in patients with leukaemia. Lancet 1985 Nov 23;2(8465):1150-4.

(24) Budd D, Ginsberg H. Hypocholesterolemia and acute myelogenous leukemia. Association between disease activity and plasma low-density lipoprotein cholesterol concentrations. Cancer 1986 Sep 15;58(6):1361-5.

(25) Spiegel RJ, Schaefer EJ, Magrath IT, Edwards BK. Plasma lipid alterations in leukemia and lymphoma. Am J Med 1982 May;72(5):775-82.

(26) Choy E, Sattar N. Interpreting lipid levels in the context of high-grade inflammatory states with a focus on rheumatoid arthritis: a challenge to conventional cardiovascular risk actions. Ann Rheum Dis 2009 Apr;68(4):460-9.

(27) Katan MB. Commentary: Mendelian Randomization, 18 years on. Int J Epidemiol 2004 Feb;33(1):10-1.

(28) Schatzkin A, Abnet CC, Cross AJ, Gunter M, Pfeiffer R, Gail M, et al. Mendelian randomization: how it can--and cannot--help confirm causal relations between nutrition and cancer. Cancer Prev Res (Phila Pa) 2009 Feb;2(2):104-13.

Chapter 10

General discussion

Main findings

The general objective of this thesis was to investigate associations between genetic variants involved in inflammation and epigenetics and age-related diseases. First we analyzed genetic variation in genes related to inflammatory processes, since inflammation is known to play an important role in age-related diseases like cardiovascular disease, cognitive decline and cancer. Analysis of polymorphisms of the genes that are key for regulation of the immune response might clarify the patho-physiology of age-related diseases like atherosclerosis. Therefore, we analyzed the association between genetic variation in pro- and anti-inflammatory genes and cardiovascular disease (chapter 2 & 3), cognitive function (chapter 4 & 5), and cancer (chapter 6)

In chapter 2 we investigated the relation between the C804A polymorphism in the pro-inflammatory gene Lymphotoxin-alpha (LTA) also known as Tumor Necrosis Factor-beta (TNF-β) and coronary and cerebrovascular events. The C804A polymorphism causes an amino-acid change from threonine (T) to asparagine (N) at codon 26 (1). The variant protein 26N is associated with a two-fold increase in the induction of cell-adhesion molecules in vascular smooth muscle cells (1). These adhesion molecules are implicated in cardiovascular disease which might explain the association of the polymorphisms in the LTA gene and the increased risk for incident stroke (2;3). Our results indicate that carriers of the 804A allele have an increased risk for the primary study endpoint consisting of coronary events and clinical strokes, primarily in men. Furthermore, we found that the association between the C804A polymorphism and the primary endpoint in males was mainly attributable to incident strokes.

In chapter 3 we assessed the relation between four promoter polymorphisms in the interleukin-10 (IL-10) gene and vascular events at old age. Carriers of the -2849AA genetype within the IL-10 promoter region have an increased risk for vascular disease, coronary and cerebrovascular. In IL-10 knock-out mice the absence of IL-10 leads to an increased susceptibility of atherosclerosis (4). Furthermore, a study using an overexpressing transgenic mice model and IL-10 null mice showed a marked difference in lesion size between the groups, the IL-10 null mice having far more lesion

formation when compared to the overexpressing mice model (5). We found that two of the main IL-10 haplotypes showed significant associations with vascular diseases compared to the reference haplotype with no variants present. This provides evidence that not only pro-inflammatory processes contribute to atherosclerosis but that also anti-inflammatory cytokines are implicated in vascular disease. These findings support the hypothesis that genetic programming of the inflammatory response may be relevant to the pathogenesis of atherosclerosis.

The relation between genetic variation in inflammatory genes and cognitive function was investigated in chapter 4 and 5. In chapter 4, we assessed the association between four polymorphisms within the ICE gene and cognitive function in an elderly population, since genetic variation in the gene coding for ICE influences expression and function of IL-1β. We found that two variants in the ICE gene significantly lowered IL-1beta levels, and that carriers of these variants performed better on all cognitive function tests. This indicates that low levels of the pro-inflammatory IL-1beta might be protective for memory and learning deficits. These results are consistent with earlier findings on inhibition of ICE coinciding with lower IL-1beta levels in the hippocampus and improved memory (6). ICE inhibitors might therefore become a therapeutic target for subjects to prevent cognitive decline.

In chapter 5, we found that genetic variation in the promoter region of the IL-10 gene is associated with decreased cognitive function in individuals without clinical evidence of a cerebrovascular event. This provides evidence that genetic variation in the IL-10 gene is a good marker for risk prediction of cognitive function. If these findings are confirmed and adequately explained on the basis of independent studies, screening patients for the IL-10 promoter polymorphisms may contribute to a better risk stratification of patients at increased risk for cognitive decline and additional preventive therapy may be warranted.

We have assessed the association between circulating levels and innate production capacity of pro-inflammatory cytokines in whole blood samples and cancer incidence and mortality in chapter 6.

High levels of the circulating inflammatory markers were associated with an increased risk of cancer incidence and death from cancer. However, high innate pro-inflammatory cytokine production capacity was only associated with an increased risk of death from cancer during follow-up. This indicates that circulating markers of inflammation are increased in cancer patients, probably by autocrine production of the cancer cells themselves, and that therefore the relation between circulating inflammatory markers and incident cancer might be disturbed by reverse causality. However our main finding was that subjects with a pro-inflammatory trait, i.e. subjects with a high innate cytokine production level, had an increased risk of dying of the consequences of cancer when they had developed a tumor. Hence, it may be hypothesized that by administration of antibodies that bind pro-inflammatory cytokines, survival time of cancer patients might be extended.

Furthermore, we studied genetic variation in genes related to epigenetics, to give additional insights into mechanisms that underlie age-related diseases. Since, epigenetics is a fairly new concept in the relation with age-related diseases, we have provided first evidence that this process can indeed play an important role in the patho-physiology of age-related diseases.

In chapter 7, we investigated the association between genetic variation in the CREB Binding Protein (CBP) gene and cognitive function. Many experimental studies have investigated the role of CBP in memory formation and cognitive dysfunction in animals (7-10). $CBP^{+/-}$ mutant mice have normal short-term memory, but deficiencies in long-term memory, object recognition, and contextual memory tasks (11). Moreover, loss of one functional copy of the CBP gene in humans underlies all abnormalities in Rubinstein-Taybi Syndrome patients, including mental retardation (12;13). Therefore it is likely that polymorphisms in the CBP gene have an effect on cognitive function. We have demonstrated an association between two polymorphisms in the CBP gene and cognitive function in an elderly population. The variant alleles of the intron 4CT and intron 3AC polymorphisms were associated with better cognitive performance in all cognitive domains at baseline and in follow-up. Furthermore, the haplotype with the variant alleles of these two

polymorphisms also showed a protective effect on cognitive function in all cognitive domains. Future research is warranted to assess the functionality of these polymorphisms on the expression of CBP levels and how these polymorphisms affect the gene expression mediated by CBP. When confirmed, screening patients for the CBP polymorphisms may contribute to a better risk stratification of patients at risk for cognitive decline and may improve individual treatment.

In chapter 8, we investigated the impact of genetic variation in the PCAF-gene on all-cause mortality, and mortality due to vascular events and cancer in the PROSPER-study and validated our findings in the WOSCOPS and GENDER study. We showed in these three large prospective studies that the -2481C allele in the PCAF promoter is associated with a significant survival advantage in elderly patients while carriers were protected against clinical and angiographic restenosis after percutaneous coronary intervention (PCI). Functional analysis showed that the -2481 G/C polymorphism is located in a functional region of the PCAF promoter, and modulation of PCAF gene expression was detectable in an animal model of reactive stenosis. Our observations promote the concept that epigenetic processes are under genetic control and that, other than environment, genetic variation in genes encoding HATs may determine susceptibility to coronary heart disease outcomes and mortality.

In chapter 9, we used an innovative concept in genetic epidemiology. We assessed the association between plasma cholesterol levels and cancer risk, free from confounding and reverse causality, by a Mendelian randomization study design using the ApoE genotype. We found that subjects, when grouped by their baseline levels of cholesterol, had an increased cancer risk when the cholesterol levels were lower. This risk remained even after adjustment for potential confounders. However, when we categorized subjects according to their ApoE genotype, which also resulted in groups with significantly different cholesterol levels, no increased risk for cancer risk was observed between groups. These findings suggest that low levels of cholesterol are not causally related to an increased risk of cancer and that treatment with cholesterol lowering agents does not increase cancer risk.

Clinical implications

We have shown that subjects carrying genetic variants coding for a high pro-inflammatory profile or a low anti-inflammatory profile have an increased risk to develop several age-related diseases. In the process of atherosclerosis, inflammatory mechanisms operate largely through cytokine secretion and their activation can cause plaque rupture, thrombosis, and acute ischemic symptoms (14-18). Anti-inflammatory and immunosuppressive mechanisms inhibit atherosclerosis and may be attractive targets for disease prevention and/or treatment (19). They include amongst others anti-inflammatory cytokines, protective antibodies, and regulatory T cells, and may be induced by immunization. These therapies could also play an important role in delaying the process of cognitive decline in old age (20). For example, inhibition of ICE with ICE inhibitors have shown to reduce the IL-1β production in the brain (21), indicating a potential therapeutic role for ICE inhibitors in subjects with cognitive decline.

Moreover, we have shown that besides inflammatory processes, epigenetic mechanisms could also play a role in age-related diseases. Epigenetic modifications, like histone acetylation, accumulate with ageing (22). It can be envisioned that when this accumulation comes to a halt, the development of age-related diseases might be delayed (23). Histone deacetylases (HDAC) inhibitors have been successfully introduced in clinical trials as anti-tumour agents. Recent findings identified HDACs as possible targets for therapy in cardiovascular disease (24). The investigators found that HDAC inhibition could reduce the size of myocardial infarction by ~50% in mice.

A better understanding on the role of epigenetic processes in age-related diseases might provide novel opportunities to understand disease pathology. This would provide the necessary knowledge platform for design of alternative treatment strategies aimed at interfering in these epigenetic processes.

Genetic epidemiology can also contribute to establishing the causal nature of environmentally modifiable risk factors, through the application of Mendelian randomization approaches. By making use of this Mendelian randomization approach we found that low cholesterol levels were not

causally associated with an increase in cancer risk. Low cholesterol levels are probably a result of an increased uptake of the cancer cells themselves. Cancer cells need cholesterol for their growth and proliferation (25;26). A sudden drop in plasma cholesterol levels can therefore be a predictive factor of an underlying tumour. Hence, in clinical practice, more attention could be paid to sudden drops in cholesterol levels as a prediction of cancer.

Future research

Mendelian randomization is a fairly new concept in genetic epidemiology. It has already proven to be a valuable tool, since Mendelian randomization provides an alternative way of dealing with the problems of causal inference in observational studies. Inferring causality from observational data is problematic as it is not always clear which of two associated variables is the cause and which is the effect, or whether both are common effects of a third unobserved variable, a confounder. In some instances the problem of causality and confounding can be resolved with randomized clinical trials, however this is not possible for a whole range of exposures like toxins and physical activity. For these questions Mendelian randomization can provide a solution.

The Mendelian randomization approach has developed rapidly over the past 5 years, and proof of principle is now abundantly available. For example, the MTHFR C677T polymorphism, that determines homocysteine levels, shows a consistent causal relation with stroke as was also shown in observational studies (27). In the relation with CRP levels and metabolic syndrome, where several CRP polymorphisms were used as a proxy for CRP levels, no causal relation was found (28). Furthermore, genome wide association studies will provide us with new genetic variants as proxies for intermediate phenotypes for Mendelian Randomization studies. Therefore, Mendelian randomization should be applied more in future research (29).

Genetic epidemiology has merely been based on investigating associations with genetic variation in genes related to the investigated mechanisms. This is called a candidate gene approach, which we also used in all studies reported in this thesis. However, most age-related diseases are not the result

of only one disturbance in one pathway. Age-related diseases are complex diseases where many pathways and mechanisms could play a role in the patho-physiology. To determine all mechanisms involved in age-related diseases, the candidate-gene approach is not sufficient. Nowadays, the genetic technologies are much more advanced in a way that we can screen the whole genome. These genome wide associations studies (GWA) are used to discover more genetic variants in various, mostly unknown, pathways associated with complex age-related diseases. When these genetic variants are being discovered, we can make a better risk profile per individual, and eventually implement personalized therapy based on the individual genetic risk profile.

One example of a new genetic study is the PHASE project (PHArmacogenomic study of Statins in the Elderly at risk for cardiovascular disease), which started in January 2009. In this project we perform a genome-wide scan in all 5804 participants of the PROSPER study in order to discover the genetic variation responsible for variation in lowering low-density lipoprotein levels, variation in clinical outcome, like cardiovascular disease, cancer and cognitive decline, and for the occurrence of adverse effects. By increasing the knowledge of which genetic variation is responsible for the variation in drug response, we can develop personalized cholesterol lowering drug therapy based on an individual's genetic make-up to obtain optimal cardiovascular event reduction, minimal side effects, and major cost reduction for society.

Reference List

(1) Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T, et al. Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. Nat Genet 2002 Dec;32(4):650-4.

(2) Belch JJ, Shaw JW, Kirk G, McLaren M, Robb R, Maple C, et al. The white blood cell adhesion molecule E-selectin predicts restenosis in patients with intermittent claudication undergoing percutaneous transluminal angioplasty. Circulation 1997 Apr 15;95(8):2027-31.

(3) Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM, Jr., et al. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. Circulation 1997 Dec 16;96(12):4219-25.

(4) Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F, Bureau MF, et al. Protective role of interleukin-10 in atherosclerosis. Circ Res 1999 Oct 15;85(8):e17-e24.

(5) Pinderski Oslund LJ, Hedrick CC, Olvera T, Hagenbaugh A, Territo M, Berliner JA, et al. Interleukin-10 blocks atherosclerotic events in vitro and in vivo. Arterioscler Thromb Vasc Biol 1999 Dec;19(12):2847-53.

(6) Gemma C, Fister M, Hudson C, Bickford PC. Improvement of memory for context by inhibition of caspase-1 in aged rats. Eur J Neurosci 2005 Oct;22(7):1751-6.

(7) Bito H, Deisseroth K, Tsien RW. CREB phosphorylation and dephosphorylation: a Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression. Cell 1996 Dec 27;87(7):1203-14.

(8) Lonze BE, Ginty DD. Function and regulation of CREB family transcription factors in the nervous system. Neuron 2002 Aug 15;35(4):605-23.

(9) Rouaux C, Loeffler JP, Boutillier AL. Targeting CREB-binding protein (CBP) loss of function as a therapeutic strategy in neurological disorders. Biochem Pharmacol 2004 Sep 15;68(6):1157-64.

(10) Weeber EJ, Sweatt JD. Molecular neurobiology of human cognition. Neuron 2002 Mar 14;33(6):845-8.

(11) Oike Y, Hata A, Mamiya T, Kaname T, Noda Y, Suzuki M, et al. Truncated CBP protein leads to classical Rubinstein-Taybi syndrome phenotypes in mice: implications for a dominant-negative mechanism. Hum Mol Genet 1999 Mar;8(3):387-96.

(12) Hallam TM, Bourtchouladze R. Rubinstein-Taybi syndrome: molecular findings and therapeutic approaches to improve cognitive dysfunction. Cell Mol Life Sci 2006 Aug;63(15):1725-35.

(13) Roelfsema JH, White SJ, Ariyurek Y, Bartholdi D, Niedrist D, Papadia F, et al. Genetic heterogeneity in Rubinstein-Taybi syndrome: mutations in both the CBP and EP300 genes cause disease. Am J Hum Genet 2005 Apr;76(4):572-80.

(14) Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005 Apr 21;352(16):1685-95.

(15) Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002 Mar 5;105(9):1135-43.

(16) Libby P. Inflammation in atherosclerosis. Nature 2002 Dec 19;420(6917):868-74.

(17) Packard RR, Libby P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. Clin Chem 2008 Jan;54(1):24-38.

(18) Vasto S, Candore G, Balistreri CR, Caruso M, Colonna-Romano G, Grimaldi MP, et al. Inflammatory networks in ageing, age-related diseases and longevity. Mech Ageing Dev 2007 Jan;128(1):83-91.

(19) Blankenberg S, Yusuf S. The inflammatory hypothesis: any progress in risk stratification and therapeutic targets? Circulation 2006 Oct 10;114(15):1557-60.

(20) Wilson CJ, Finch CE, Cohen HJ. Cytokines and cognition--the case for a head-to-toe inflammatory paradigm. J Am Geriatr Soc 2002 Dec;50(12):2041-56.

(21) Ravizza T, Lucas SM, Balosso S, Bernardino L, Ku G, Noe F, et al. Inactivation of caspase-1 in rodent brain: a novel anticonvulsive strategy. Epilepsia 2006 Jul;47(7):1160-8.

(22) Fraga MF, Esteller M. Epigenetics and aging: the targets and the marks. Trends Genet 2007 Aug;23(8):413-8.

(23) Pons D, de Vries FR, van den Elsen PJ, Heijmans BT, Quax PH, Jukema JW. Epigenetic histone acetylation modifiers in vascular remodelling: new targets for therapy in cardiovascular disease. Eur Heart J 2009 Feb;30(3):266-77.

(24) Granger A, Abdullah I, Huebner F, Stout A, Wang T, Huebner T, et al. Histone deacetylase inhibition reduces myocardial ischemia-reperfusion injury in mice. FASEB J 2008 Oct;22(10):3549-60.

(25) Ueyama Y, Matsuzawa Y, Yamashita S, Funahashi T, Sakai N, Nakamura T, et al. Hypocholesterolaemic factor from gallbladder cancer cells. Lancet 1990 Sep 22;336(8717):707-9.

(26) Vitols S, Gahrton G, Bjorkholm M, Peterson C. Hypocholesterolaemia in malignancy due to elevated low-density-lipoprotein-receptor activity in tumour cells: evidence from studies in patients with leukaemia. Lancet 1985 Nov 23;2(8465):1150-4.

(27) Casas JP, Bautista LE, Smeeth L, Sharma P, Hingorani AD. Homocysteine and stroke: evidence on a causal link from mendelian randomisation. Lancet 2005 Jan 15;365(9455):224-32.

(28) Timpson NJ, Lawlor DA, Harbord RM, Gaunt TR, Day IN, Palmer LJ, et al. C-reactive protein and its role in metabolic syndrome: mendelian randomisation study. Lancet 2005 Dec 3;366(9501):1954-9.

(29) Davey SG, Ebrahim S, Lewis S, Hansell AL, Palmer LJ, Burton PR. Genetic epidemiology and public health: hope, hype, and future prospects. Lancet 2005 Oct 22;366(9495):1484-98.

Nederlandse samenvatting

Het aantal ouderen boven de 70 jaar is de laatste jaren toegenomen. Dit komt door een significante reductie van sterfte op alle leeftijden waardoor een toename van de gemiddelde en maximale levensverwachting heeft plaatsgevonden. Een hoge leeftijd is geassocieerd met een verhoogde kans op ziekte en sterfte. De verhoogde sterfte bij ouderen aan verschillende ouderdomsziekten zoals infectieziekten, hart- en vaatziekten en kanker kan onder andere herleid worden naar een verstoorde respons van het immuunsysteem. Andere mechanismes die mogelijk veranderen met leeftijd en daardoor leiden tot ouderdomsziekten zijn nog niet bekend. Epigenetica is een dergelijk mechanisme dat wel kan veranderen met de leeftijd, maar waarvan nog niet bekend is of deze veranderingen ook leiden tot verschillende ouderdomsziekten. Het doel van deze thesis was om associaties te vinden tussen genetische variaties in genen betrokken bij het immuunsysteem en genen betrokken bij epigenetica en de verschillende ouderdomsziekten om meer inzicht te verkrijgen in de patho-fysiologische mechanismes van deze ziekten. Voor alle onderzoeken hebben we data gebruikt van de deelnemers aan de PROSpective Study of Pravastatin in the Elderly at Risk (PROSPER).

In hoofdstuk 2 onderzochten we de associatie tussen het C804A polymorfisme in het gen Lymfotoxin-alpha en coronaire ziekten en beroertes. Hieruit is gebleken dat dragers van dit polymorfisme een verhoogd risico hebben op het krijgen van een hartaanval of beroerte. Na stratificatie bleek dit effect het sterkst voor beroertes bij mannen. Aangezien Lymfotoxin-alpha een pro-inflammatoir eiwit is, betrokken bij de inductie van celadhesie moleculen in vasculaire gladde spiercellen, toonden we hiermee aan dat pro-inflammatoire markers risicofactoren kunnen zijn voor hart- en vaatziekten.

De relatie tussen 4 polymorfismes in het interleukine-10 (IL-10) gen en cardiovasculaire incidenten op oude leeftijd wordt beschreven in hoofdstuk 3. IL-10 is anti-inflammatoir omdat het de synthese

van pro-inflammatoire eiwitten remt. In IL-10 knock-out muizen is aangetoond dat de afwezigheid van IL-10 leidt tot een verhoogd risico op atherosclerose, een van de belangrijkste oorzaken van hart- en vaatziekten. Wij hebben aangetoond dat dragers van genetische varianten in het IL-10 gen, die een lagere expressie hebben van IL-10 eiwitten, een verhoogd risico hebben op het krijgen van hart- en vaatziekten. Hiermee lieten we zien dat niet alleen pro-inflammatoire eiwitten betrokken zijn bij hart- en vaatziekten maar dat ook een verlaagde expressie van een anti-inflammatoir eiwit een verhoogd risico geeft op hart- en vaatziekten.

In hoofdstuk 4 en 5 bekeken we de relatie tussen genen betrokken bij inflammatoire processen en cognitieve functie. De associatie tussen 4 polymorfismes in het interleukine-1-beta converting enzyme (ICE) gen en cognitieve achteruitgang is onderzocht in hoofdstuk 4. ICE is een eiwit dat de expressie en functie van het pro-inflammatoire cytokine interleukin-1-beta (IL-1β) beïnvloedt. Wij hebben gevonden dat twee variaties in het ICE gen leiden tot significant lagere IL-1β levels in het bloed. Dragers van deze varianten presteerden significant beter op alle cognitieve functietesten tijdens de 3,5 jaar follow-up. Deze resultaten suggereren dat lage levels van het pro-inflammatoire cytokine IL-1β beschermend kunnen zijn tegen cognitieve achteruitgang. Onze bevindingen zijn consistent met eerdere bevindingen in de literatuur. In muismodellen is gevonden dat wanneer het eiwit ICE geremd wordt, dit resulteert in lagere levels van IL-1β en een verbetering van het geheugen. ICE-remmers kunnen daarom gezien worden als een potentieel belangrijke target om cognitieve achteruitgang bij ouderen tegen te gaan.

In hoofdstuk 5 vonden we dat die 4 polymorfismes in het IL-10 gen niet alleen geassocieerd zijn met hart- en vaatziekten maar ook met een verslechterde cognitieve functie, onafhankelijk van het wel of niet krijgen van een beroerte. Deze studie laat zien dat genetische variatie in het IL-10 gen gebruikt kan worden als een risico marker om de mate van cognitieve functie in te schatten. Wanneer deze bevindingen gerepliceerd worden en er voldoende informatie over bekend is, zal screening van de IL-10 polymorfismes kunnen leiden tot een juiste risicostratificatie van patiënten

met een verhoogd risico op cognitieve achteruitgang. Hierdoor kan er naar een passende individuele therapie worden gezocht.

De relatie tussen circulerende levels en de van nature aanwezige productiecapaciteit van verschillende pro-inflammatoire cytokines en kanker incidentie en mortaliteit wordt beschreven in hoofdstuk 6. Hoge circulerende levels van deze pro-inflammatoire markers waren geassocieerd met een verhoogd risico op kanker incidentie en mortaliteit. Echter, een verhoogde productiecapaciteit was alleen geassocieerd met een verhoogd risico op kanker mortaliteit. Deze bevinding suggereert dat circulerende inflammatoire markers verhoogd zijn in patiënten met kanker, waarschijnlijk door autocriene productie van de kankercellen, en dat de relatie tussen circulerende markers en kanker incidentie waarschijnlijk verstoord is door omgekeerde oorzakelijkheid. Onze belangrijkste bevinding is dat mensen met een verhoogd van nature aanwezige productiecapaciteit van pro-inflammatoire cytokines, dus mensen met sterk pro-inflammatoir profiel, een verhoogd risico hebben om te sterven wanneer zij een tumor hebben ontwikkeld. Derhalve suggereren wij dat de overlevingsduur van kanker patiënten waarschijnlijk verlengd zou kunnen worden met middelen gericht tegen pro-inflammatoire cytokines.

Hierboven zijn de studies beschreven die de relatie tussen het immuunsysteem en ouderdomsziekten onderzochten. In hoofdstuk 7 en 8 beschrijven we twee studies die de relatie onderzoeken tussen genetische variatie in genen betrokken bij epigenetica en ouderdomsziekten om zo meer inzicht te krijgen in de patho-fysiologie van deze ziekten. Omdat epigenetica pas recent gelinkt wordt aan ouderdomsziekten, zijn wij de eersten die met deze studies bewijs leveren dat epigenetica inderdaad een belangrijke rol speelt in de patho-fysiologie van ouderdomsziekten.

In hoofdstuk 7 beschrijven we de associatie tussen genetische variatie in het CREB binding protein (CBP) gen en cognitieve functie. Meerdere studies in diermodellen hebben deze relatie al onderzocht. Hieruit bleek dat $CBP^{+/-}$ gemuteerde muizen gebreken hadden in hun lange termijn geheugen, object herkenning en geheugentaken. In mensen leidt het verlies van 1 kopie van het

CBP gen tot alle abnormaliteiten die bij het Rubinstein-Taybi syndroom voorkomen, waaronder mentale retardatie. Dit maakt het aannemelijk dat polymorfismes in het CBP gen een effect hebben op cognitieve functie bij oudere mensen. We hebben aangetoond dat 2 polymorfismes (intron 4CT en intron 3AC) associëren met betere cognitieve functie in alle domeinen. Verder hebben we ook laten zien dat dragers van het haplotype met deze varianten beschermd waren tegen cognitieve achteruitgang. Dit is een eerste aanwijzing dat het CBP gen betrokken is bij cognitieve functie bij ouderen. Verder onderzoek moet nog uitgevoerd worden naar de effecten van de twee polymorfismes op de expressie van CBP levels en hoe zij de gen expressie, gemediëerd door CBP, veranderen.

In hoofdstuk 8 hebben we het effect van genetische variatie in het PCAF gen op algemene en vasculaire mortaliteit onderzocht in 3 onafhankelijke studies, de PROSPER studie, de GENDER studie en de WOSCOPS studie. In deze drie studies vonden we dat mensen met het -2481C allel in de PCAF promoter een significant overlevingsvoordeel hebben en dat ze beschermd zijn tegen het ontstaan van klinische restenose. Uit een functionele studie bleek verder dat dit polymorfisme in een functioneel gebied van de PCAF promoter ligt en dat in een diermodel van restenose de mate van PCAF expressie was veranderd. Al deze bevindingen laten zien dat ook epigenetische processen een rol kunnen spelen bij hart- en vaatziekten en overleving.

In hoofdstuk 9 hebben we een vrij nieuwe methode gebruikt in de genetische epidemiologie. We hebben de associatie tussen plasma cholesterol levels and kanker risico onderzocht vrij van confounding en omgekeerde oorzakelijkheid door middel van een Mendeliaanse randomisatie methode gebruikmakend van het APOE genotype. We vonden dat mensen, gegroepeerd naar hun cholesterol waarden, een hoger risico hadden voor kanker wanneer de cholesterol waarden het laagst waren. Deze associatie bleef bestaan na correctie voor verschillende potentiële confounders. Daarentegen, wanneer we mensen groepeerden naar aanleiding van hun APOE genotype, dat resulteert in groepen met een verschillend cholesterol niveau, vonden we geen risicoverschil voor kanker in de verschillende groepen. Deze resultaten suggereren dat lage cholesterol niveaus niet

causaal geassocieerd zijn met kanker risico en dat therapie met cholesterol verlagende middelen het risico op kanker niet verhoogt.

Conclusie

In deze thesis hebben we studies beschreven die de relatie tussen inflammatie, epigenetica en ouderdomsziekten hebben onderzocht. We vonden dat pro- en anti-inflammatoire markers belangrijke risicofactoren kunnen zijn voor hart- en vaatziekten, voor cognitieve achteruitgang en voor kanker. Verder hebben we ook laten zien dat genetische variaties betrokken bij epigenetica een rol spelen in hart- en vaatziekten en cognitieve achteruitgang, wat impliceert dat ook epigenetische processen belangrijk zijn bij de patho-fysiologische processen van ouderdomsziekten. Echter het precieze biologische mechanisme hierachter is nog niet bekend.

In deze thesis hebben we gebruikt gemaakt van een kandidaat-gen methode om de associaties tussen genetica en ouderdomsziekten te onderzoeken. Daarbij hebben we biologische mechanismes onderzocht waarvan het vermoeden al bestond dat ze betrokken kunnen zijn bij de patho-fysiologie van de ouderdomsziekten. Maar ouderdomsziekten zijn complexe ziektebeelden waar meerdere mechanismes tegelijkertijd een belangrijke rol kunnen spelen. Om ook de nog onbekende mechanismes te ontrafelen, wordt er tegenwoordig gebruik gemaakt van een methode om het hele genoom in één keer te screenen, namelijk de '*Genome Wide Associations (GWA) studies'*. In januari 2009 is ook een GWA analyse gestart binnen de PROSPER studie, namelijk het '*PHASE'* project. Met dit project willen we nieuwe genen en mechanismes ontdekken betrokken bij onder andere hart- en vaatziekten, cognitieve achteruitgang en kanker, zodat we nog meer inzicht krijgen in de patho-fysiologie achter deze ziekten op hoge leeftijd.

List of publications

Trompet S, Pons D, DE Craen AJ, Slagboom P, Shepherd J, Blauw GJ, Murphy MB, Cobbe SM, Bollen EL, Buckley BM, Ford I, Hyland M, Gaw A, Macfarlane PW, Packard CJ, Norrie J, Perry IJ, Stott DJ, Sweeney BJ, Twomey C, Westendorp RG, Jukema JW. Genetic variation in the interleukin-10 gene promoter and risk of coronary and cerebrovascular events: the PROSPER study. Ann N Y Acad Sci. 2007 Apr;1100:189-98.

Schram MT, **Trompet S**, Kamper AM, de Craen AJ, Hofman A, Euser SM, Breteler MM, Westendorp RG. Serum calcium and cognitive function in old age. J Am Geriatr Soc. 2007 Nov;55(11):1786-92.

Trompet S, Westendorp RG, Kamper AM, de Craen AJ. Use of calcium antagonists and cognitive decline in old age. The Leiden 85-plus study. Neurobiol Aging. 2008 Feb;29(2):306-8.

Trompet S, de Craen AJ, Slagboom P, Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Ford I, Gaw A, Macfarlane PW, Packard CJ, Stott DJ, Jukema JW, Westendorp RG; PROSPER Group. Genetic variation in the interleukin-1 beta-converting enzyme associates with cognitive function. The PROSPER study. Brain. 2008 Apr;131(Pt 4):1069-77.

Trompet S, de Craen AJ, Slagboom P, Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Ford I, Gaw A, Macfarlane PW, Packard CJ, Stott DJ, Westendorp RG, Jukema JW; on behalf of the PROSPER Group. Lymphotoxin-alpha C804A polymorphism is a risk factor for stroke. The PROSPER study. Exp Gerontol. 2008 Aug;43(8):801-805.

Polisecki E, Peter I, Robertson M, McMahon AD, Ford I, Packard C, Shepherd J, Jukema JW, Blauw GJ, Westendorp RG, de Craen AJ, **Trompet S**, Buckley BM, Murphy MB, Ordovas JM, Schaefer EJ; for the PROSPER Study Group. Genetic variation at the PCSK9 locus moderately lowers low-density lipoprotein cholesterol levels, but does not significantly lower vascular disease risk in an elderly population. Atherosclerosis. 2008 Sep;200(1):95-101.

Willems JM, **Trompet S**, Slagboom PE, de Craen AJ, Westendorp RG. Hematopoietic capacity and exceptional survival. The Leiden Longevity Study. J Am Geriatr Soc. 2008 Nov;56(11):2009-13.

Trompet S, Jukema JW, Ford I, Shepherd J, Gussekloo J, Buckley BM, Westendorp RG, de Craen AJ. Statins and Blood Pressure. Arch Intern Med. 2008 Nov 24;168(21):2383.

Stott DJ, Falconer A, Kerr GD, Murray HM, **Trompet S**, Westendorp RG, Buckley BM, de Craen AJ, Sattar N, Ford I. Does low-moderate alcohol intake protect against cognitive decline in older people? J Am Geriatr Soc. 2008 Dec;56(12):2217-24.

Trompet S, van Vliet P, de Craen AJ, Jolles J, Buckley BM, Murphy MB, Ford I, Macfarlane PW, Sattar N, Packard CJ, Stott DJ, Shepherd J, Bollen EL, Blauw GJ, Jukema JW, Westendorp RG. Pravastatin and cognitive function in the elderly. Results of the PROSPER study. J Neurol. 2010 Jan;257(1):85-90.

Trompet S, Jukema JW, Katan MB, Blauw GJ, Sattar N, Buckley B, Caslake M, Ford I, Shepherd J, Westendorp RG, de Craen AJ. Apolipoprotein E genotype, plasma cholesterol, and cancer: a Mendelian randomization study. Am J Epidemiol. 2009 Dec 1;170(11):1415-21.

Trompet S, de Craen AJ, Mooijaart S, Stott DJ, Ford I, Sattar N, Jukema W, Westendorp RG. High Innate Production Capacity of Proinflammatory Cytokines Increases Risk for Death from Cancer: Results of the PROSPER Study. Clin Cancer Res. 2009 Dec 15;15(24):7744-7748.

Stott DJ, Robertson M, Rumley A, Welsh P, Sattar N, Packard CJ, Shepherd J, **Trompet S**, Westendorp RG, de Craen AJ, Jukema JW, Buckley B, Ford I, Lowe GD. Activation of Hemostasis and Decline in Cognitive Function in Older People. Arterioscler Thromb Vasc Biol. 2010 Mar;30(3):605-11.

Trompet S, de Craen AJ, Jukema JW, Pons D. Slagboom PE, Kremer D, Bollen EL, Westendorp RG. Variation in the CBP gene involved in epigenetic control associates with cognitive function. Neurobiol Aging. 2010 Jan 21. [Epub ahead of print]

Willems JM, **Trompet S**, Blauw GJ, Westendorp RG, de Craen AJ. White blood cell count and C-reactive protein are independent predictors of mortality in the oldest old. J Gerontol A Biol Sci Med Sci. 2010 Jan 27. [Epub ahead of print]

van Nies JA, Marques RB, **Trompet S**, de Jong Z, Kurreeman FA, Toes RE, Jukema JW, Huizinga TW, van der Helm-van Mil AH. TRAF1/C5 polymorphism is not associated with increased mortality in rheumatoid arthritis; two large longitudinal studies. Arthritis Res Ther. 2010 Mar 5;12(2):R38. [Epub ahead of print]

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Curriculum Vitae

Stella Trompet werd op 4 juli 1981 geboren te ‘s-Gravenhage. Na het behalen van haar VWO diploma in 1999 aan het Atlascollege te Rijswijk studeerde zij vanaf 1 september 1999 Biomedische Wetenschappen aan de Faculteit Geneeskunde van de Universiteit Leiden. Tijdens haar afstudeeropdracht is onderzoek gedaan naar het effect van calcium niveaus op de cognitieve achteruitgang bij ouderen onder begeleiding van Dr. Ton de Craen op de afdeling Ouderengeneeskunde van het Leids Universitair Medisch Centrum (LUMC). Zij behaalde haar masterdiploma in september 2005.

Na een overgangsperiode van 5 maanden waar zij werkervaring heeft opgedaan als epidemiologe, is zij op 1 januari 2006 begonnen aan haar promotieonderzoek “Genes, inflammation, and age-related diseases” onder begeleiding van Dr. Ton de Craen en Professor Rudi Westendorp van de afdeling Ouderengeneeskunde en Professor Wouter Jukema van de afdeling Hartziekten van het Leids Universitair Medisch Centrum. De resultaten van dit onderzoek worden beschreven in dit proefschrift.

Sinds 1 januari 2009 werkt zij als project manager op het PHASE project (PHArmacogenomic study of Statins in the Elderly at risk for cardiovascular disease).