Genes, inflammation, and age-related diseases
Trompet, S.

Citation
Trompet, S. (2010, June 2). *Genes, inflammation, and age-related diseases*. Retrieved from https://hdl.handle.net/1887/15579

Version:	Corrected Publisher's Version
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Note: To cite this publication please use the final published version (if applicable).

Chapter 8

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

Douwe Pons[*], Stella Trompet[*], Anton JM. de Craen, Peter E Thijssen, Paul HA Quax, Rutger J Wierda, Peter J van den Elsen, Pascalle S Monraats, Mark M Ewing, Willemijn Passtoors, Joris M Deelen, Aeilko H Zwinderman, Pieter AFM Doevendans, René A Tio, Robbert J de Winter, Moniek PM de Maat, Bastiaan T Heijmans, P Eline Slagboom, Olga A Iakoubova, Naveed Sattar, Jim Shepherd, Rudi GJ Westendorp, J Wouter Jukema, also on behalf of the PROSPER, WOSCOPS and GENDER study groups.

* Both authors contributed equally to this work

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 [33P]-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, $\Delta\Delta$Ct was determined by substracting the average ΔCt at timepoint 0 hours from the ΔCt at each other timepoint. This $\Delta\Delta$Ct 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 (0.56-1.15)	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.
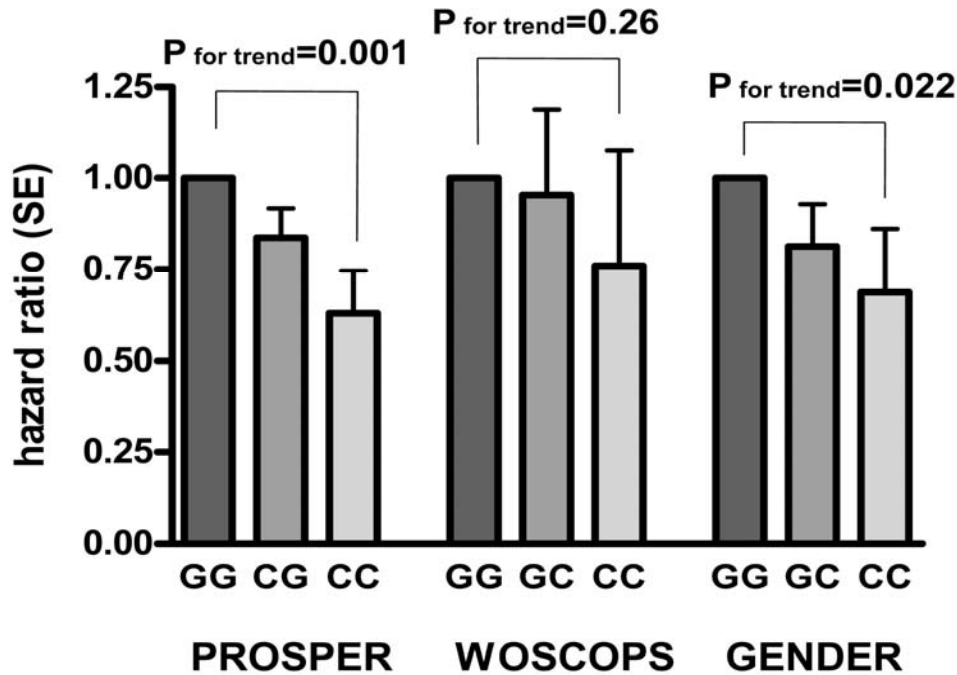
Figure 1: Hazard ratios for all-cause mortality in PROSPER, for coronary heart disease death in WOSCOPS, and for clinical restenosis in GENDER by PCAF -2481 G/C genotype at all ages.

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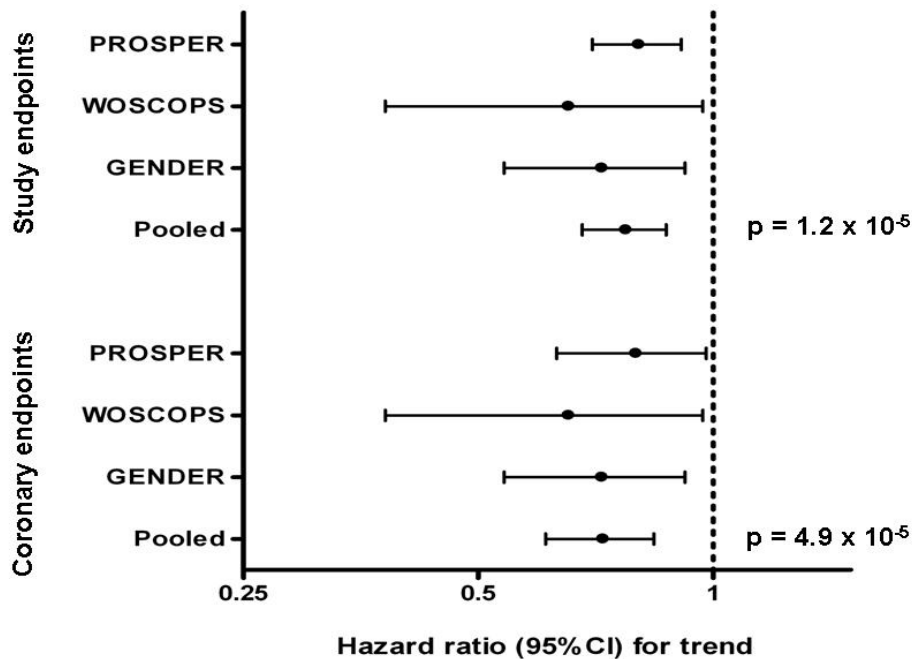
Figure 2: Combined effect estimate of the hazard ratios of the PROSPER, WOSCOPS, and GENDER studies for the PCAF -2481 G/C polymorphism.

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).
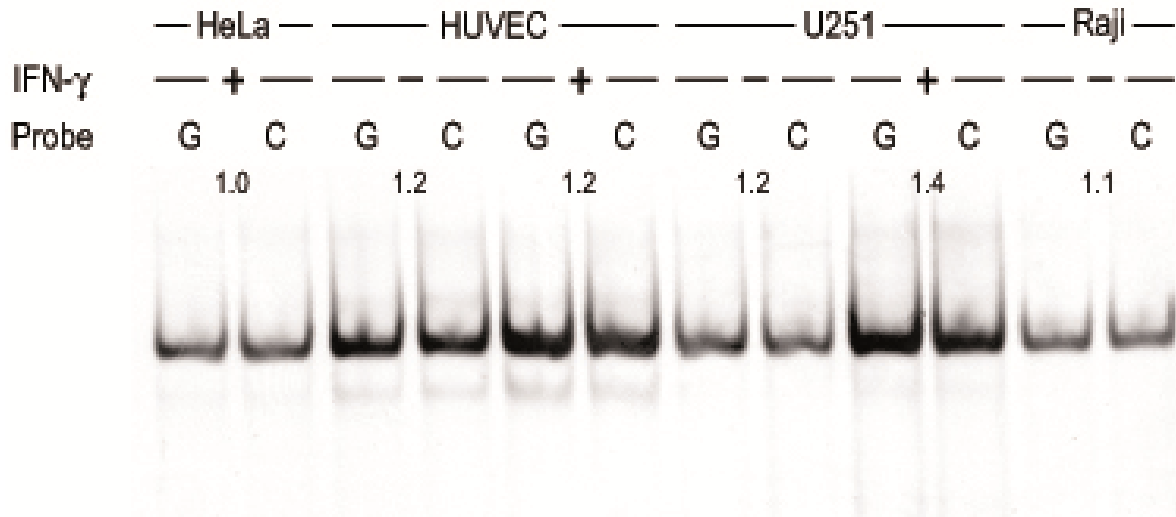

Figure 3: Protein binding to the C- and G-variant of the -2481 region.

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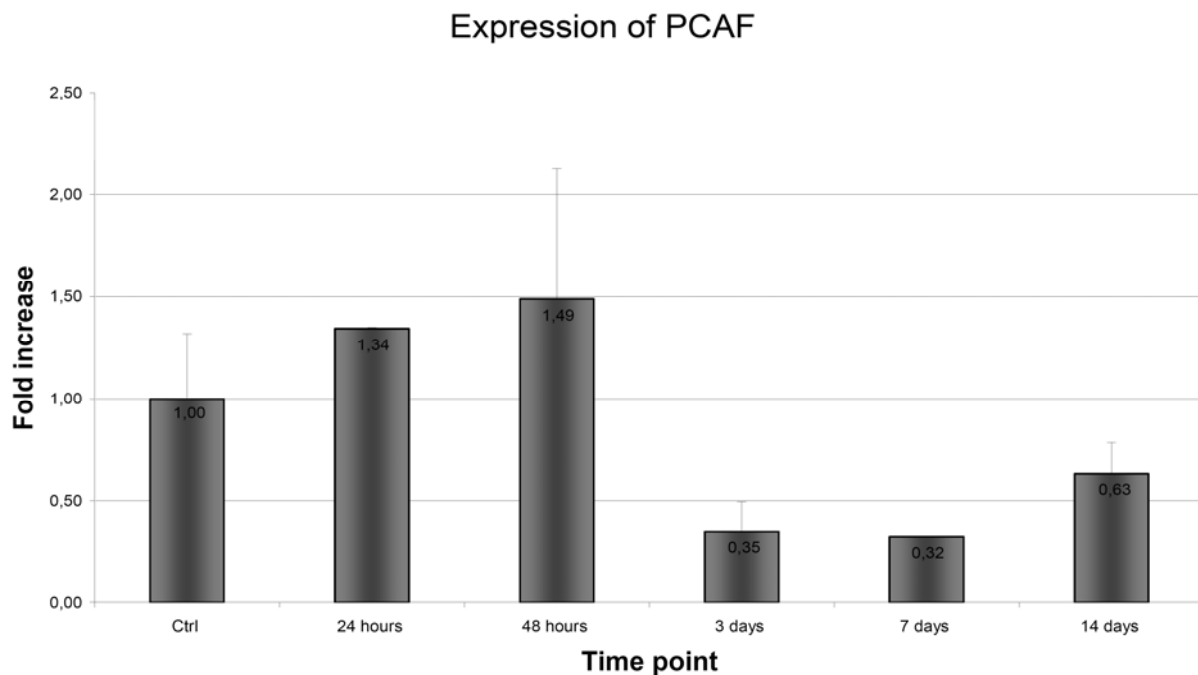
Figure 4: Expression of PCAF mRNA in a cuff-induced reactive stenosis mouse model.

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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