# Genes, inflammation, and age-related diseases

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## 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.

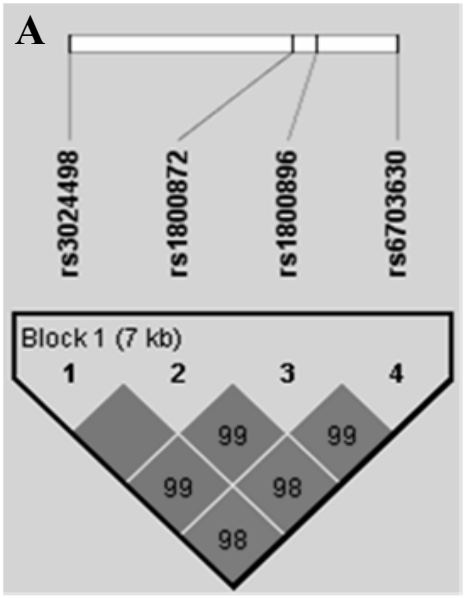
**Results**

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 $p>0.05$).

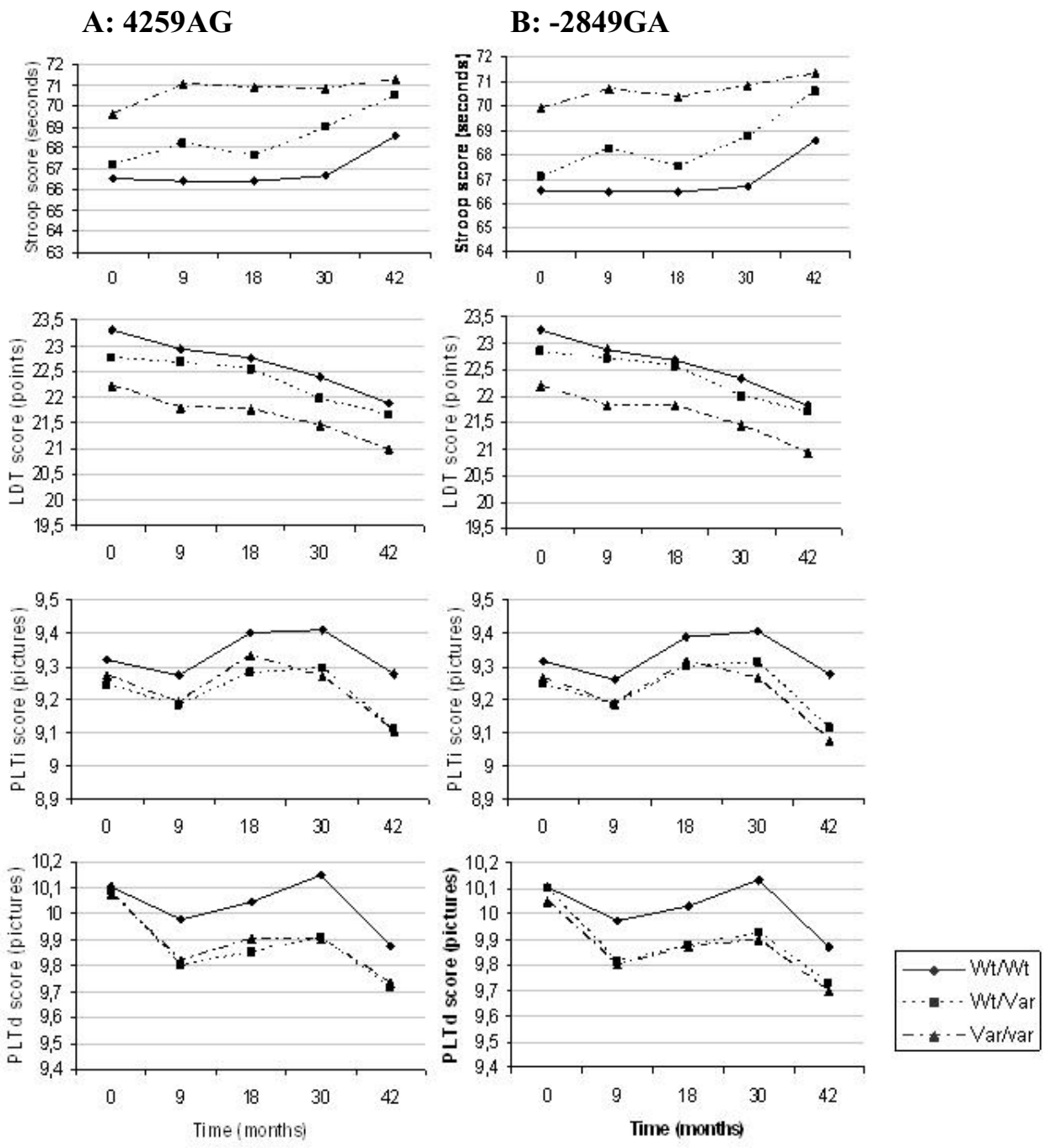



**Figure 2**: Representation of association between two IL-10 polymorphisms and cognition.
Figure2A represents the association between the 4259AG polymorphism with cognition and 2B the association between the -2849GA polymorphism with cognition. The straight line represents the homozygous wild-type carriers,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

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