Genetic Analysis of the Interleukin-18 System Highlights the Role of the Interleukin-18 Gene in Cardiovascular Disease

Laurence Tiret, PhD; Tiphaine Godefroy, BS; Edith Lubos, MD; Viviane Nicaud, MA; David-Alexandre Tregouet, PhD; Sandrine Barbaux, PhD; Renate Schnabel, MD; Christoph Bickel, MD; Christine Espinola-Klein, MD; Odette Poirier, PhD; Claire Perret, MSc; Thomas Münnzel, MD; Hans-Jurgen Rupprecht, MD; Karl Lackner, MD; François Cambien, MD; Stefan Blankenberg, MD; for the AtheroGene Investigators*

Background—Interleukin (IL)-18 plays a key role in atherosclerosis and its complications. The present study investigated the genetic variability of 4 genes of the IL-18 system—IL18, IL18R1, IL18RAP, and IL18BP—in relation to circulating IL-18 levels and cardiovascular mortality.

Methods and Results—Twenty-two polymorphisms were genotyped in 1288 patients with coronary artery disease prospectively followed up during a median period of 5.9 years. The end point was death from cardiovascular causes (n=142). Baseline IL-18 levels were predictive of cardiovascular deaths occurring during ≤4 years of follow-up (HR=2.96, 95% CI 1.54 to 5.70, P=0.001 for the top compared with the bottom quartile) but not of later deaths. Haplotypes of the IL18 gene were associated with IL-18 levels (P=0.002) and cardiovascular mortality (P=0.006) after adjustment for cardiovascular risk factors. The same haplotype was associated with both a 9% lowering effect on IL-18 levels and a protective effect on risk (HR=0.57, 95% CI 0.36 to 0.92). IL18 haplotypes explained only 2% of IL-18 variability. Adjustment for baseline IL-18 levels abolished the association of haplotypes with cardiovascular risk. The haplotype associated with phenotypes was the only one carrying the minor allele of the IL18/A+183G polymorphism located in the 3′ untranslated region and potentially affecting mRNA stability. The other genes of the system were not related to IL-18 levels or cardiovascular outcome.

Conclusion—Variations of the IL18 gene consistently influence circulating levels of IL-18 and clinical outcome in patients with coronary artery disease, which supports the hypothesis of a causal role of IL-18 in atherosclerosis and its complications. (Circulation. 2005;112:643-650.)

Key Words: coronary disease | genetics | interleukins | inflammation | prognosis

In recent years, increasing evidence has emerged from experimental and epidemiological data that interleukin-18 (IL-18), a proinflammatory cytokine involved in both innate and acquired immune responses,1–3 plays a key role in the inflammatory response that contributes to atherosclerosis. Increased IL-18 expression has been localized in human atherosclerotic plaque and associated with plaque instability.4,5 Animal models support the role of IL-18 in atherosclerotic lesion development and plaque vulnerability6,7 as well as the beneficial effect of inhibiting IL-18 on plaque progression and composition.8 Recently, we have shown that baseline circulating IL-18 levels were strongly predictive of future cardiovascular mortality in the AtheroGene prospective cohort of patients with coronary artery disease (CAD).9 This predictive role of IL-18 levels was further confirmed in the large population-based cohort of initially healthy men of the Prospective Epidemiological Study of Myocardial Infarction (PRIME).10 However, it remains unclear whether elevation of IL-18 has a causative role or is simply the consequence of an ongoing inflammatory process that is associated with the development of atherosclerotic lesions.

One important step to further evaluate the causality of IL-18 in atherosclerosis is to study its potential implication at the genetic level. Besides the gene encoding IL-18 itself, several genes are involved in the IL-18 pathway and might contribute, alone or in combination, to IL-18 variability and
in turn affect disease risk. IL-18 acts by binding to a heterodimeric receptor (IL-18R) comprising a binding α chain, termed IL-18Rα or IL-18R1, and a signal-transducing β chain, termed IL-18Rβ or IL-18RAP (for receptor accessory protein).11–13 Both chains form a high-affinity complex required for inducing IL-18 signaling. IL-18R is expressed on a variety of cells including macrophages, T lymphocytes, and natural killer cells, which are ascribed a key role in atherosclerotic plaque rupture.14 Another important component of the IL-18 system is the IL-18–binding protein, a natural endogenous inhibitor of IL-18 that is present in high concentrations in the extracellular milieu and can bind IL-18 with high affinity, preventing its interaction with IL-18R and thereby neutralizing IL-18 activity.2,15

The present study was aimed at investigating the sequence variability of the 4 genes of the IL-18 system—IL18, IL18R1, IL18RAP, and IL18BP—and relating this global genetic variability to circulating IL-18 levels and future cardiovascular death in the AtheroGene prospective cohort of CAD patients.

Methods

Study Population
Detailed description of the study has been provided elsewhere.5,16 Between November 1996 and June 2000, 1299 CAD patients were recruited on the occasion of a diagnostic coronary angiography at the Department of Medicine II of the Johannes Gutenberg–University Mainz and the Bundeswehrzentralkrankenhaus Koblenz. A priori inclusion criterion was the presence of a diameter stenosis ≥30% in ≥1 major coronary artery. Exclusion criteria were evidence of significant concomitant diseases, in particular hemodynamically significant valvular heart disease, known cardiomyopathy, and malignant diseases, as well as febrile conditions. Since our previous report on IL-18,9 the follow-up was extended to a median period of 5.9 (maximum 7.6) years for 1288 of 1299 subjects. Follow-up information was obtained about death from cardiovascular causes (n = 142), death from noncardiovascular causes (n = 43), and nonfatal myocardial infarction (n = 64). Information about the cause of death or clinical events was obtained by the hospital or general practitioner. Death from cardiovascular causes might—besides fatal myocardial infarction—also include heart failure as a consequence of myocardial infarction and ventricular arrhythmia.

Study participants had German nationality, were inhabitants of the Rhein-Mainz area, and were of European ethnic origin. The study was approved by the ethics committee of the University of Mainz. Each participant gave written informed consent.

Laboratory Methods
Serum IL-18 was measured with a commercially available ELISA method (MLB, Co, Ltd). The within-run coefficients of variation were determined to be 3.2% at a mean of 43.7 pg/mL and 8.9% at a mean of 103.8 pg/mL; between-run coefficients of variation were 3.2% at 43.7 pg/mL and 8.1% at 58 pg/mL (n = 7 each). Samples for these methodological determinations were taken from the AtheroGene control population of apparently healthy individuals. C-reactive protein (CRP) was determined by a highly sensitive, latex particle–enhanced immunoassay (detection range of 0 to 20 mg/L, Roche Diagnostics), IL-6 by ELISA (EASIA, Biosource Europe), and fibrinogen by a derived method. Lipid serum levels were measured immediately.

Molecular Screening, Selection, and Genotyping of Polymorphisms
We performed a molecular screening of the regulatory, coding, and flanking intronic regions of the IL18 gene by comparing 190 chromosomes from 95 unrelated patients from the Etude Cas-Témoin de l’Infarctus du Myocarde (ECTIM) study.17 The method of detection was polymerase chain reaction/single-strand conformational polymorphism followed by direct sequencing. Afterward, the 4 genes under study were published in the Innate Immunity Programs for Genomic Applications (IPGA) database (http://innateimmunity.net/), and polymorphisms of the IL18R1, IL18RAP, and IL18BP genes were then taken from this database. The database reports for genes of the innate immunity pathway the common polymorphisms identified through sequencing and establishes their relative allele frequencies and linkage disequilibrium (LD) in 2 populations of European American and African American origins. For the present study, we selected all polymorphisms detected in 23 European Americans that were located in the coding sequence, the flanking intronic regions (~100 bp on each side), the 5′ untranslated region (5′UTR) and 3′ UTR, and within 1 kb of the 5′ and 3′ regions. For blocks of polymorphisms in complete association identified from the LD matrix, we selected only one polymorphism of the block for further genotyping.

All polymorphisms, either identified by our molecular screening for the IL18 gene or selected from the IPGA database for the IL18R1, IL18RAP, and IL18BP genes, were genotyped in the SIPLAC study by allele-specific oligonucleotide hybridization. SIPLAC is a subsample of 600 European subjects drawn from the ECTIM study and used to estimate allele and haplotype frequencies in the framework of our program of exploration of candidate genes for cardiovascular diseases17 (see our Web site GeneCanvas http://www.genecanvas.org). The SIPLAC study provides us with a tool for selecting the subset of polymorphisms that are further genotyped in larger studies, after exclusion of rare (frequency ≤0.02) and redundant polymorphisms.

Polymorphisms selected at this step were then genotyped in the AtheroGene study by using the TaqMan technology (Applied Biosystems [ABI]). Briefly, PCR primers and TaqMan MGB probes were designed with Primer Express version 2.0. Reactions were performed in 96-well microplates with GeneAmp 9700 thermal cyclers. Fluorescence was measured with an ABI Prism 7000 sequence detection system and analyzed with the ABI Prism 7000 SDS software version 1.0. Primer and probe sequences as well as amplification conditions for genotyping can be found at the GeneCanvas Web site (http://www.genecanvas.org).

Statistical Analysis
All polymorphisms were diallelic (21 single-nucleotide polymorphisms [SNPs] and one insertion/deletion). Allele frequencies and pairwise LD coefficients were estimated with the THESIAS software.18 Association of single polymorphisms with continuous IL-18 levels was tested by ANOVA adjusted for age and sex. IL-18 concentrations were log-transformed to remove positive skewness. Association of polymorphisms with continuous IL-18 levels was tested by Cox proportional-hazards regression analysis. In all these single-locus analyses, a general genetic model was considered. Association of cardiovascular mortality with baseline IL-18 levels, in quartiles, was tested by Cox regression analysis. The assumption of hazards proportionality according to IL-18 quartiles was tested by introducing terms of interaction with log(time) in the Cox model (χ² with 3 df).

Haplotype Analyses
Haplotype analyses combining all polymorphisms of a gene were performed with the THESIAS software. The method allows one to simultaneously estimate haplotype frequencies and haplotype–phenotype association parameters, possibly adjusted for covariates, by using a Stochastic-EM algorithm for likelihood maximization.19 Covariates included in haplotype analyses were those, among traditional cardiovascular risk factors, that were significantly related to IL-18 levels or cardiovascular outcome by prior multivariate analysis. Haplotype frequencies were estimated assuming Hardy-Weinberg equilibrium at the haplotype level. The relationship between the phenotype and haplotypes was modeled by using a regression linear model for serum IL-18 levels and a Cox regression model for cardiovascular outcome. Models assumed additive effects of haplotypes on phenotype. Covariate-adjusted haplotype–phenotype association parameters (95% CI) were estimated for each
haplotype by comparison to a reference haplotype chosen as the most frequent one. Parameters were expressed as mean effects for IL-18 levels and hazard ratios (HRs) for cardiovascular outcome. A global test of association between haplotypes and the phenotype was performed by a likelihood ratio test ($\chi^2$ with $m$-1 df in the case of $m$ haplotypes). Effects associated with rare haplotypes (frequency <0.02) were not estimated and fixed to 0.

Multilocus Exploration

We applied the detection of informative combined effects (DICE) method\(^\text{20}\) to investigate high-order interactions between polymorphisms of the IL-18 system on serum IL-18 levels. The DICE algorithm explores in an automated way all combinations of polymorphisms acting either in an additive or in an interactive way (3-order interaction at maximum). Models of increasing complexity are successively fitted to the data in a stepwise manner, and the inter-step difference $\Delta$ of the Akaike's information criterion between models indicates whether the fit is substantially improved.

Polymorphisms of the IL-18 System

**IL18 Gene**

The organization of the *IL18* gene is shown in Figure 1. We identified 11 polymorphisms by molecular screening that were further confirmed in the IIPGA database. Except for the most distal one, the SNPs located in the 5' and 5'UTR had already been described with a different numbering starting from exon 1 (-656, -607, -137, +113, and +127, respectively).\(^\text{21}\) Three blocks of SNPs in almost complete association were observed (Figure 1). The C+167G and G+230A polymorphisms were relatively frequent (frequency of 0.02 in the SIPLAC study) and were not further studied. Five polymorphisms were finally genotyped in the AtheroGene study.

**IL18R1 Gene**

The organization of the *IL18R1* gene is shown in Figure 2. Among the 10 polymorphisms identified in the IIPGA database, only the T+956G polymorphism was not genotyped because it was in complete association with the F259F. Allele frequencies, LD coefficients, and haplotype frequencies are given in Data Supplement Table II. The 9 polymorphisms generated 7 common haplotypes accounting for 96.6% of all chromosomes.

**IL18RAP Gene**

The organization of the *IL18RAP* gene is shown in Figure 3. All 8 polymorphisms identified in the IIPGA database were genotyped. Allele frequencies, LD coefficients, and haplotype frequencies are given in Data Supplement Table III. The 8 polymorphisms generated 8 common haplotypes accounting for 98.8% of all chromosomes.

**Results**

**Polymorphisms of the IL-18 System**

### IL18 Gene

The organization of the *IL18* gene is shown in Figure 1. We identified 11 polymorphisms by molecular screening that were further confirmed in the IIPGA database. Except for the most distal one, the SNPs located in the 5' and 5'UTR had already been described with a different numbering starting from exon 1 (-656, -607, -137, +113, and +127, respectively).\(^\text{21}\) Three blocks of SNPs in almost complete association were observed (Figure 1). The C+167G and G+230A polymorphisms were relatively frequent (frequency of 0.02 in the SIPLAC study) and were not further studied. Five polymorphisms were finally genotyped in the AtheroGene study (in bold in Figure 1). They were in strong LD one with each other and defined 6 major haplotypes accounting for 99.0% of all chromosomes (Data Supplement Table I).

### IL18R1 Gene

The organization of the *IL18R1* gene is shown in Figure 2. Among the 10 polymorphisms identified in the IIPGA database, only the T+956G polymorphism was not genotyped because it was in complete association with the F259F. Allele frequencies, LD coefficients, and haplotype frequencies are given in Data Supplement Table II. The 9 polymorphisms generated 7 common haplotypes accounting for 96.6% of all chromosomes.

### IL18RAP Gene

The organization of the *IL18RAP* gene is shown in Figure 3. All 8 polymorphisms identified in the IIPGA database were genotyped. Allele frequencies, LD coefficients, and haplotype frequencies are given in Data Supplement Table III. The 8 polymorphisms generated 8 common haplotypes accounting for 98.8% of all chromosomes.
IL18BP Gene

Different splicing of the human IL18BP gene generates 4 distinct isoforms varying in size but sharing a common translation start site. In the IIPGA database, only 1 relatively frequent polymorphism was described in 3'/H11032 UTR of the isoform IL18BPc (C+1136T) with a frequency of 0.08 in European American individuals. This polymorphism was genotyped in the SIPLAC study, and because of its low frequency in this study (0.02), it was not further genotyped in the AtheroGene study.

Baseline Characteristics of Cases and Controls

Baseline characteristics of CAD patients are presented in Table 1. Serum IL-18 levels were increased in patients having experienced a cardiovascular death during follow-up compared with those who had not (69.0 versus 59.7 pg/mL, P<10^-4), as were all other markers of inflammation.

Association of IL18, IL18R1, and IL18RAP Gene Polymorphisms With Baseline Serum Levels of IL-18

IL18 Gene

By single-locus analysis, the A+183G and the T+533C polymorphisms were significantly associated with circulating IL-18 levels (P=0.002 and 0.003, respectively). The +183G and the +533T alleles were both associated with a decrease of IL-18 in a fairly additive fashion (age- and sex-adjusted IL-18 levels [log]: 4.14±0.02, 4.07±0.02, and 3.97±0.05 in AA, AG, and GG patients; 4.22±0.04, 4.10±0.02, and 4.07±0.02 in CC, CT, and TT patients, respectively). To account for the LD between polymorphisms, a haplotype-based regression analysis was performed. The global association between haplotypes and age- and sex-adjusted IL-18 levels was highly significant (P=0.002, Figure 4, gray

Baseline Characteristics of Patients With CAD According to Occurrence of Cardiovascular Death During Follow-Up

<table>
<thead>
<tr>
<th></th>
<th>Without Cardiovascular Death</th>
<th>With Cardiovascular Death</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, y</strong></td>
<td>61.0±0.3</td>
<td>67.2±0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Males, %</strong></td>
<td>74.9</td>
<td>71.1</td>
<td>0.33</td>
</tr>
<tr>
<td><strong>Body mass index, kg/m²</strong></td>
<td>27.2±0.1</td>
<td>27.1±0.3</td>
<td>0.74</td>
</tr>
<tr>
<td><strong>Current smoker, %</strong></td>
<td>13.6</td>
<td>19.7</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Diabetes mellitus, %</strong></td>
<td>14.9</td>
<td>31.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Hypertension, %</strong></td>
<td>71.9</td>
<td>78.2</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Statin, %</strong></td>
<td>35.5</td>
<td>26.8</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>β-Blocker, %</strong></td>
<td>61.1</td>
<td>45.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>ACE inhibitor, %</strong></td>
<td>46.3</td>
<td>62.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Total cholesterol, mg/dL</strong></td>
<td>219.1±1.3</td>
<td>216.5±3.8</td>
<td>0.52</td>
</tr>
<tr>
<td><strong>LDL cholesterol, mg/dL</strong></td>
<td>140.6±1.2</td>
<td>138.5±3.3</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>HDL cholesterol, mg/dL</strong></td>
<td>49.0±0.4</td>
<td>45.7±1.2</td>
<td>0.011</td>
</tr>
<tr>
<td><strong>LDL/HDL ratio</strong></td>
<td>3.06±0.03</td>
<td>3.26±0.09</td>
<td>0.046</td>
</tr>
<tr>
<td><strong>Triglycerides, mg/dL</strong></td>
<td>143.3 (139.1–147.6)</td>
<td>157.0 (144.3–170.9)</td>
<td>0.047</td>
</tr>
<tr>
<td><strong>hs-CRP, mg/L</strong></td>
<td>5.2 (4.8–5.7)</td>
<td>7.9 (6.2–10.0)</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Fibrinogen, mg/dL</strong></td>
<td>339.7 (333.5–345.9)</td>
<td>373.6 (354.5–393.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>IL-6, pg/mL</strong></td>
<td>10.1 (9.3–10.9)</td>
<td>15.5 (12.4–19.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>IL-18, pg/mL</strong></td>
<td>59.7 (58.3–61.2)</td>
<td>69.0 (64.4–74.0)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Categorical variables are presented as percentages, continuous variables are presented as age- and sex-adjusted mean±SE, except for *log-transformed variables, which are presented as geometric means (95% CI).
IL18R1 Gene
IL18R1 polymorphisms were not associated with IL-18 levels, either by single-locus analysis (all \( P > 0.18 \)) or by haplotype analysis (\( P = 0.55 \)) (Data Supplement Figure I).

IL18RAP Gene
Single-locus analyses of the IL18RAP polymorphisms revealed a borderline association of the A-992G polymorphism with IL-18 levels, the \(-992G \) allele being associated with a 7% decrease of levels in a fairly codominant fashion (\( P = 0.06 \)). There was a global association of haplotypes with IL-18 levels (\( P = 0.04 \)), but none of the individual haplotype effects reached statistical significance (Data Supplement Figure II).

Multilocus Exploration
We explored potential combined effects of polymorphisms of the different genes on IL-18 levels by using the DICE algorithm. Except the main effect of the IL18/A+183G polymorphism leading to a \( \Delta \), of 8.0, no other polymorphism or combination of polymorphisms exceeded the threshold of detection fixed at \( \Delta > 6 \).

Association of Baseline Circulating IL-18 Levels With Cardiovascular Mortality During Follow-Up
Since our first publication reporting a strong association between baseline IL-18 levels and future cardiovascular mortality,\(^a\) the follow-up period was extended and the number of cardiovascular deaths increased from 95 to 142. Baseline IL-18 levels were still predictive of future cardiovascular mortality, with a clear elevation of risk in patients whose level was higher than the median (Figure 5). The raw HRs (95% CI) estimated by Cox regression analysis were 0.98 (0.55 to 1.72, \( P = 0.93 \)), 1.78 (1.07 to 2.94, \( P = 0.025 \)), and 2.31 (1.42 to 3.73, \( P < 10^{-3} \)) for the second, third, and fourth quartiles, respectively, as compared with the first quartile of baseline IL-18. However, the hypothesis of proportionality of hazards assumed in the Cox model was rejected (\( P = 0.028 \)). Actually, baseline IL-18 levels appeared no longer predictive of outcome after 4 years of follow-up, as shown by the plateau of the Q3 and Q4 survival curves after 210 weeks of follow-up (Figure 5). When excluding the cardiovascular deaths having occurred beyond 4 years (n = 24), the hypothesis of proportionality of hazards was no longer rejected (\( P = 0.30 \)). When restricting events to those occurring during \( \leq 4 \) years of follow-up and adjusting for risk factors related to cardiovascular mortality (age, history of diabetes, smoking status, HDL cholesterol, \( \beta \)-blocker intake, and ejection fraction), the HRs were 1.00 (0.46 to 2.21, \( P = 0.99 \)), 1.71 (0.85 to
3.44, \( P = 0.13 \), and 2.96 (1.54 to 5.70, \( P = 0.001 \)) for quartiles 2 to 4 compared with the first quartile.

**Association of IL18, IL18R1, and IL18RAP Gene Polymorphisms With Cardiovascular Mortality During Follow-Up**

**IL18 Gene**

By single-locus analysis, none of the polymorphisms of the IL18 gene was associated with cardiovascular mortality during follow-up. However, a haplotype-based survival analysis revealed a global association of haplotypes with the risk of future cardiovascular death (\( P = 0.045 \), Figure 6, gray squares). Exclusion of cardiovascular deaths after 4 years strengthened the significance of the global test (\( P = 0.013 \), Figure 6, black circles). After adjustment on cardiovascular risk factors (age, smoking status, diabetes, HDL cholesterol, and \( \beta \)-blocker intake), the global association between haplotypes and cardiovascular outcome persisted (\( P = 0.006 \)), and haplotypes GCAGT and GCCAT were associated with opposite effects on risk (HR = 0.57, 95% CI 0.36 to 0.92, \( P = 0.021 \), and HR = 3.01, 95% CI 1.27 to 7.13, \( P = 0.012 \), respectively) (Figure 6, gray diamonds). Additional adjustment for baseline IL-18 levels markedly weakened the effect of haplotype GCAGT (HR = 0.85, 95% CI 0.43 to 1.67, \( P = 0.63 \)) but increased that of haplotype GCCAT (HR = 5.25, 95% CI 1.11 to 24.9, \( P = 0.037 \)). However, this haplotype was quite rare, and the estimation of its effect was affected by a large imprecision. The global association between haplotypes and outcome was no longer significant after adjustment on IL-18 levels (\( P = 0.30 \), Figure 6, black squares).

**IL18R1 Gene**

IL18R1 polymorphisms were associated with cardiovascular mortality neither by single-locus analysis (all \( P > 0.20 \)) nor by haplotype analysis (\( P = 0.79 \)), even when restricting the analysis to deaths occurring in \( \leq 4 \) years (\( P = 0.98 \)) (Data Supplement Figure III).

**IL18RAP Gene**

IL18RAP polymorphisms were associated with cardiovascular mortality neither by single-locus analysis (all \( P > 0.40 \)) nor by haplotype analysis (\( P = 0.42 \)), even when restricting the analysis to deaths occurring in \( \leq 4 \) years (\( P = 0.98 \)) (Data Supplement Figure IV).

**Discussion**

The present study reports a comprehensive analysis of the genetic variability of the IL-18 system in relation to baseline circulating levels of IL-18 and cardiovascular mortality in a large prospective cohort of CAD patients with a median follow-up of nearly 6 years. The genetic system investigated included the 4 main genes involved in the processing and signaling of IL-18—namely the gene encoding IL-18 itself, its 2 receptors, and its main endogenous inhibitor. This approach based on the genetics of systems rather than on single genes represents an important step for progressing in the understanding of biological mechanisms underlying complex diseases. All functionally important regions of the genes were investigated, and the overall genetic variability of the system was related to the different phenotypes by means of haplotype analysis. High-order interactions between polymorphisms within a gene or between different genes were also explored to detect potential combined effects of polymorphisms that would have escaped by single-gene analysis.\(^{20}\) This latter analysis was, however, restricted to serum IL-18 levels to have sufficient power to detect interactions, and also because circulating levels represent an intermediate phenotype for which genetic effects are expected to be stronger than for more distal and complex phenotypes such as mortality.

Unlike our original report,\(^9\) the hypothesis of proportionality of hazards according to baseline IL-18 levels was no longer supported after inclusion of the new events resulting from the extension of follow-up. The rejection of the assumption was due to the fact that baseline IL-18 levels were no longer predictive of cardiovascular deaths occurring after 4 years of follow-up. To rule out the possibility of an original chance finding, we compared baseline IL-18 levels in patients having died within \( \leq 4 \) years during the initial follow-up period and those having died within the same delay during the subsequent follow-up, and these levels were very similar between the 2 periods (73.4 versus 73.2 pg/mL, \( P = 0.87 \)). We also checked whether this observation was made for other inflammatory markers and found similar findings for IL-6 and CRP levels (data not shown). This is at variance with the relative stability generally reported for the predictive value of CRP over time,\(^{23,24}\) although in the Reykjavik Study, a weakening of this predictive value was observed after 10

![Figure 6. Hazard ratios (HRs) for cardiovascular death associated with IL18 gene haplotypes by reference to the most common haplotype. Gray squares indicate raw HRs estimated in all subjects (\( P = 0.045 \) for the global association); black circles indicate raw HRs when excluding cardiovascular deaths after 4 years (\( P = 0.013 \)); gray diamonds indicate HRs adjusted for cardiovascular risk factors (age, smoking status, diabetes, HDL cholesterol, \( \beta \)-blocker intake) (\( P = 0.006 \)); black squares indicate HRs additionally adjusted for baseline IL-18 levels (\( P = 0.30 \)).](http://circ.ahajournals.org/doi/10.1161/CIRCULATIONAHA.105.582357)
years of follow-up.25 Because IL-18 was measured only at baseline, it was not possible to relate the mortality to changes of IL-18 levels over time. Nevertheless, it is conceivable that, in this cohort of patients referred for clinical manifestations of CAD, a transient elevation of inflammatory markers might be more predictive of earlier complications than of distant events.

Of the 4 genes, 1—the IL18BP gene—had no common polymorphism in its regulatory or coding regions. Whether this lack of genetic diversity is the consequence of a selective pressure is unknown. By contrast, the 3 other genes were highly polymorphic, leading to a total number of 22 polymorphisms genotyped in the present study. As observed for most human genes,17 these polymorphisms generated only a limited number of common haplotypes because of the strong LD within genes.

A significant association of IL18 haplotypes was observed both with circulating IL-18 levels and clinical outcome. The same haplotype (GCAGT) was associated with a lowering effect on circulating IL-18 levels and a reduced risk of cardiovascular death during follow-up, both findings concurring to suggest that this haplotype carries a polymorphism having a protective effect. The lack of association of this haplotype with cardiovascular risk after adjustment for IL-18 levels indicates that this effect is mainly mediated by the activity of IL-18, either circulating in the blood or present in the plaque. The hypothesis of a genetic modulation of IL-18 activity at the site of lesions would deserve further investigation because the effect might be enhanced by comparison to what can be measured in the circulation. Because haplotype GCAGT is the only one carrying the +183G allele, it points toward a potential functional role of the A+183G polymorphism. This SNP is located in the 3’UTR of the gene and therefore might be involved in mRNA stability and regulation of the translation or regulation of the expression by interaction with the promoter. Alternatively, this haplotype might be only a marker for a functional mutation located in unexplored regions of the gene, even though the molecular screening focused on the regions a priori the most likely to be functional. Although polymorphisms in the promoter of the IL18 gene have been reported to influence gene expression,21 we could not identify by univariate or haplotype analysis any single promoter SNP that was significantly associated with IL-18 levels or cardiovascular outcome. However, this does not preclude subtle effects in the regulation of IL-18 gene expression that were not detected by our analyses.

At variance with the IL18 gene, the IL18R1 and IL18RAP genes did not appear to have any significant impact on cardiovascular risk or IL-18 levels. Because in complex systems, absence of marginal effect is not sufficient to rule out the role of a gene, we investigated in an extensive way all possible interactions between polymorphisms of the 3 genes using the DICE software,20 but no significant interaction of polymorphisms on IL-18 levels was detected by this approach. The 2 receptor chains form a high-affinity heterodimeric complex required for inducing the signaling of IL-18, and any genetic variation leading to a quantitative or a qualitative modification of the complex might have a functional impact. There was no nonsynonymous coding polymorphism in either receptor subunit that could have modified the binding affinity of the 2 chains. However, several SNPs were present in regulatory regions of the 2 genes, suggesting that functional polymorphisms, if any, might rather act by modulating the receptor expression and in turn the functionality of the complex, if, for example, one of the 2 chains was not available in sufficient amount.

Several limitations of the present study have to be addressed. Overall, the association of IL18 haplotypes with circulating IL-18 levels and cardiovascular risk was rather weak. Haplotypes explained <2% of IL-18 interindividually variability, and the association with clinical outcome was mainly observed when events were restricted to those ≤4 years. Owing to the multiple analyses performed, we cannot exclude the possibility that our findings were due to chance. However, the consistency of the results observed both on the risk of disease and an intermediate phenotype rather argues against a chance finding by providing a mechanistic interpretation. In the absence of functional proof, the causal variant(s) could not be formally identified, even though the IL18/A+183G polymorphism seems to be a good candidate for further experiments of functionality. For all these reasons, the present results have to be considered as hypothesis generating and will have to be replicated in further studies. Although investigation of the whole IL-18 system was motivated by the search for potential interactions between the different genes, the multilocus exploration did not reveal the existence of such interactions. However, the present study had limited power to detect high-order interactions, and this emphasizes the need for very large studies to explore complex systems. Finally, the present cohort was composed of European subjects, and the results might not be generalizable to other ethnic groups.

In conclusion, our results indicate that variations of the IL18 gene influence IL-18 levels and clinical outcome in patients with CAD, suggesting that IL-18 is causally involved in the development of atherosclerosis and its complications. These results should prompt functional experiments aimed at elucidating the causal variant(s).

Acknowledgments

This work was supported by a grant of the “Stiftung Rheinland-Pfalz für Innovation,” Ministry for Science and Education (AZ 15202-386261/545), Mainz; by the MAIFOR grant 2001 of the Johannes Gutenberg-University Mainz, Germany; by a grant from the Fondation de France (No. 2002004994); and by a grant from the French Ministry of Research (ACI IMPBIO No. 032619).

References


Genetic Analysis of the Interleukin-18 System Highlights the Role of the Interleukin-18 Gene in Cardiovascular Disease

Laurence Tiret, Tiphaine Godefroy, Edith Lubos, Viviane Nicaud, David-Alexandre Tregouet, Sandrine Barbaux, Renate Schnabel, Christoph Bickel, Christine Espinola-Klein, Odette Poirier, Claire Perret, Thomas Münzel, Hans-Jurgen Rupprecht, Karl Lackner, François Cambien and Stefan Blankenberg
for the AtheroGene Investigators

Circulation. 2005;112:643-650; originally published online July 25, 2005;
doi: 10.1161/CIRCULATIONAHA.104.519702

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/112/5/643

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2005/07/12/CIRCULATIONAHA.104.519702.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/