Association of IL-6 and a Functional Polymorphism in the IL-6 Gene with Cardiovascular Events in Patients with CKD

Belinda Spoto,* Francesco Mattace-Raso,† Eric Sijbrands,‡ Daniela Leonardis,* Alessandra Testa, * Anna Pisano,* Patrizia Pizzini,* Sebastiano Cutrupi,* Rosa M. Parlongo,* Graziella D’Arrigo,* Giovanni Tripepi,* Francesca Mallamaci,* and Carmine Zoccali*†

Abstract

Background and objectives High serum IL-6 is a major risk factor for cardiovascular disease (CVD) in the general population. This cytokine is substantially increased in patients with CKD, but it is still unknown whether the link between IL-6 and CVD in CKD is causal in nature.

Design, setting, participants, & measurements In a cohort of 755 patients with stages 2–5 CKD, consecutively recruited from 22 nephrology units in southern Italy, this study assessed the relationship of serum IL-6 with history of CVD, as well as with incident cardiovascular (CV) events (mean follow up ±SD, 31 ±10 months) and used the functional polymorphism (−174 G/C) in the promoter of the IL-6 gene to investigate whether the link between IL-6 and CV events is causal.

Results In adjusted analyses, serum IL-6 above the median value was associated with history of CVD (P=0.001) and predicted the incidence rate of CV events (hazard ratio, 1.66; 95% confidence interval [95% CI], 1.11 to 2.49; P=0.01). Patients homozygous for the risk allele (C) of the −174 G/C polymorphism had higher levels of IL-6 than did those with other genotypes (P=0.04). Homozygous CC patients more frequently had a history of CVD (odds ratio, 2.15; 95% CI, 1.15 to 4.00; P=0.02) as well as a 87% higher rate of incident CV events (hazard ratio, 1.87; 95% CI, 1.02 to 3.44; P=0.04) compared with other genotypes.

Conclusions In patients with stages 2–5 CKD, high serum IL-6 is associated with history of CVD and predicts incident CV events. The parallel relationship with history of CVD and incident CV events of the −174 G/C polymorphism in the IL-6 gene suggests that IL-6 may be causally involved in the high CV risk in this population.


Introduction

Classic experimental studies by Russel Ross et al. in the 1990s solidly established inflammation as a critical component of the atherosclerosis process (1). During the last two decades, large cohort studies in the general population have shown strong links between biomarkers of inflammation and cardiovascular (CV) outcomes in the general population (2–5) and in patients with CV disease (CVD) (6,7). Observational studies are methodologically vulnerable for testing causality because these studies are open to various sources of bias and confounding. Mendelian randomization—i.e., the random assortment of alleles at conception—offers an intriguing opportunity to limit problems inherent to observational studies because categorization of patients according to pertinent alleles is a sort of genetic randomization. Genetic variants may thus be used as indicators of environmental exposures in the observational context (8). Large-scale Mendelian randomization studies applying genetic polymorphisms of inflammatory cytokines (9) strongly support the hypothesis that, as seen in experimental animals (10,11), the link between inflammation and atherosclerosis complication is causal in nature.

CV risk is a multifactorial problem in patients with CKD (12). Systemic inflammation is common in these patients, particularly those with stage 5 CKD undergoing dialysis (13,14). In line with studies in the general population (5,15), IL-6, a major proinflammatory cytokine, is an established strong predictor of adverse clinical outcomes in patients with stage 5D CKD (16–20). However, the relationship between IL-6 and CVD at earlier CKD stages was investigated in just one relatively small study by Barreto et al. (21), which was based on a limited number of CV events (just 22 events). In addition, to date we lack specific proof that this relationship in the CKD population is causal.

Circulating levels of IL-6 are genetically regulated. Because transmission of genes is a random phenomenon, gene polymorphisms modulating IL-6 synthesis may represent an unbiased means for testing whether the link between IL-6 and CV outcomes in patients
with CKD is causal (Mendelian randomization). The −174 G/C single-nucleotide polymorphism is a functional variant located in the promoter region of the IL-6 gene that regulates the rate of IL-6 gene transcription (22–28) and therefore represents a reliable research tool for testing the nature (causal versus noncausal) of the link between IL-6 and CV outcomes in CKD. With this background in mind, we set out to confirm findings by Barreto et al. (21) in a large observational study with a carefully characterized cohort of 755 patients with stages 2–5 CKD and to test whether this relationship may underlie a causal link by applying the Mendelian randomization approach (i.e., by stratifying the study population according to the functional −174 G/C polymorphism in the IL-6 gene).

Materials and Methods

Study Protocol

The study protocol conformed with the ethical guidelines of our institution and was approved by ethical committees of all participating units. Each participant provided written informed consent.

Patients with CKD

Our study population included a genetically homogenous series of 755 white patients from the same geographic area (southern Italy) (29), consecutively recruited from 22 nephrology units between October 2005 and September 2008. Eligible patients were age 18–75 years and in stable clinical condition. Exclusion criteria included acute or rapidly evolving renal disease, kidney transplant, acute intercurrent infections or acute inflammatory processes, pregnancy, cancer, or diseases in the terminal phase. This cohort was described in detail elsewhere (30).

Control Population

To compare the allelic frequencies of the −174 G/C polymorphism observed in patients with CKD, we studied a sample of 463 consecutive blood donors of the general population from the same geographical area of patients with CKD.

Follow-up and Study Outcome

After the initial assessment, patients were monitored for a mean ±SD of 31 ±10 months (range, 0.3–48 months). The study endpoint was fatal and nonfatal CV events as described elsewhere (30). These events included myocardial infarction, documented by electrocardiography and biomarkers of myocardial injury; heart failure, defined as dyspnea in addition to two of the following conditions: raised jugular pressure, bi-basilar crackles, pulmonary venous hypertension, or interstitial edema on chest radiography requiring hospitalization; electrocardiography-documented arrhythmia; stroke; peripheral vascular disease; and major arterial or venous thrombotic episodes. These events were accurately recorded during the follow-up period.

The history of CVD was defined as the presence of at least one of the following comorbidities at enrollment: myocardial infarction, heart failure, peripheral vascular disease, stroke, transient ischemic attack, or coronary surgery/angioplasty.

Laboratory Measurements

In the whole study population, blood sampling was performed in the early morning after an overnight fast and plasma was stored at −80°C until analysis. Serum glucose, lipids, hemoglobin, albumin, creatinine, and C-reactive protein (CRP) were measured by standard methods in the routine clinical laboratory. Serum IL-6 was measured by ELISA (R&D Systems, Inc., Minneapolis, MN). The eGFR was calculated by using the four-variables Modification of Diet in Renal Disease study equation (31) and not by the CKD-Epidemiology Collaboration formula because the creatinine data were not traceable by isotope dilution mass spectrophotometry during the study period. All patients with CKD underwent a 24-hour urinary collection for the measurement of proteinuria.

Genotyping of −174G/C Polymorphism

Allelic discrimination of −174 G/C polymorphism was performed using a custom TaqMan SNP Genotyping Assay provided by Applied Biosystems (Foster City, CA). In this assay, primers were designed to amplify a region that included the mutation site specifically recognized by a few probes able to discriminate wild-type and mutated alleles. The sequences of primers and probes were: 5’-CGACC-TAAAGCTGCACCTTTCC-3’ (forward primer) and 5’-GGGCTGATTGAAACCTTATTAAGATTG-3’ (reverse primer); 5’-CCITTAGCAT[G]GCAAGAC-3’ (C allele-specific probe) and 5’-CCITTAGCAT[G]GCAAGAC-3’ (G allele-specific probe). Allelic discrimination was performed on a 7900HT Fast Real-Time PCR platform and its accompanying Sequence Detection System software, version 2.4 (Applied Biosystems). Briefly, genomic DNA was extracted from peripheral blood mononuclear cells using standard salting out procedure (32). The reaction system contained 20 ng of genomic DNA, 12.5 μl of 2X TaqMan Universal PCR Master Mix No AmpErase UNG, 1.25 μl of 40X Assay Mix (including unlabeled PCR primers, FAM and VIC dye-labeled TaqMan MGB probes), and H2O for a total volume of 25 μl. A random 10% of samples were independently repeated to confirm genotyping results. The genotype results for these samples were completely consistent. All analyses were done blinded to clinical information.

Statistical Analyses

Data were expressed as mean ±SD, median and interquartile range, or percentage frequency. Comparisons between two groups were made by t-test, Mann–Whitney test, or chi-square test, as appropriate. The comparison among more than two groups was performed by ANOVA for log-transformed variables, when appropriate. The deviation from Hardy–Weinberg equilibrium was assessed by the chi-square test comparing observed and expected genotype frequencies. The 95% confidence interval (95% CI) of the risk allele frequency was calculated as suggested by the standard method (33).

The functional form of serum IL-6 (as continuous, binary, quartile, or quintile data) was formally investigated by the analysis of Martingale residuals, and the binary form (below/above the median) provided the best data fitting. The relationships between serum levels of IL-6 and CVD was evaluated by two approaches. First, we analyzed the baseline association between serum IL-6 and history of
CVD. Second, we investigated the predictive power of serum IL-6 in the prospective cohort study. In both logistic and survival analyses, we considered variables that met criteria to be confounders (i.e., variables related \( P \leq 0.10 \) to both the exposure under investigation [serum IL-6 levels below/above the median value] and history of CVD or incident fatal and nonfatal CV events, which are not an effect of the exposure and are not in the causal pathway between the exposure and outcome) (34). Tested covariates included traditional risk factors (age, sex, smoking, diabetes and glucose, cholesterol, and BP), factors specific to CKD (hemoglobin, albumin, eGFR, and urinary protein), antihypertensive treatment, body mass index, and CRP. In both logistic and Cox regression models, eGFR was always forced because of the strong and significant correlation between eGFR and serum IL-6 (Table 1). To further investigate the causal role of IL-6 in the pathway leading to CV events in patients with CKD, we applied a Mendelian randomization approach (i.e., we stratified the study population according to the \(-174\) G/C polymorphism). These analyses were appropriately adjusted for variables that differed between CC and GC or GG patients and that appeared to be potential confounders (i.e., age, sex, cholesterol) for the interpretation of the link between the risk genotype (CC) and the study outcomes.

In the prospective cohort study, the potential distortion on the study results due to the competing risks of death was assessed by comparing the incidence rate of death in exposed patients (high IL-6 levels or CC risk genotype) and unexposed patients (low IL-6 levels or GC/GG genotype). If a difference was found, the competing risk of death was accounted for by carrying out a survival analysis considering a combined outcome: death/CV events (35). Data were expressed as odds ratios (ORs; logistic regression analysis), hazard ratios (HRs; Cox regression model), 95% CIs, and \( P \) values. To internally validate the independent relationships of serum IL-6 and \(-174\) G/C polymorphisms with history of CVD and incident CV events, a bootstrap resampling technique of 1000 samples (randomly extracted from the original sample) was performed (36). All potential effect modifications exerted by covariates on the relationship between the key exposures (serum IL-6 and \(-174\) G/C polymorphism) and study outcomes were formally tested by introducing a multiplicative term into the models; no significant interaction was found.

All calculations were made by using a standard statistical package (IBM SPSS Statistics for Windows, version 21.0. 0.1, IBM, Armonk, NY).

**Results**

We studied 755 patients with stages 2–5 CKD (stage 2, 3%; stage 3a, 22%; stage 3b, 38%; stage 4, 34%; stage 5, 3%). Four hundred fifty-three patients were male (60%), 263 had type 2 diabetes (35%), and 98 were current smokers (13%). Two-hundred twenty-one patients (29%) had a history of CVD (Table 1). One hundred nine patients had only one previous CV event, and 112 of them had two or more previous CV events. The first CV event in this population was myocardial infarction in 19 patients, heart failure in 26 patients, peripheral vascular disease in 29 patients, stroke in 12 patients, transient ischemic attack in 12 patients, and coronary artery surgery/angioplasty in 11 patients. Six hundred ninety-one patients (92%) were receiving antihypertensive treatment: one medication in 19%, two medications in 29%, three medications in 28%, and four or more medications in 16%. The mean eGFR was 36±13 ml/min per 1.73 m\(^2\), and the median 24-hour urinary protein excretion was 0.6 mg/24 hours (interquartile range, 0.2–1.5 mg/24 hours). The median IL-6 level was 2.5 pg/ml (interquartile range, 1.6–4.0 pg/ml).

**Analyses Based on Serum IL-6 Levels**

Patients with IL-6 above the median value were significantly older, more frequently had diabetes, and had higher serum glucose and 24-hour urinary protein values than those with IL-6 below this threshold (Table 1). Values for eGFR, hemoglobin, and albumin were lower in patients with higher IL-6 than in those with IL-6 below the median value. Systolic BP was higher and diastolic BP was lower in patients with IL-6 levels above the median (Table 1). As expected, serum CRP levels were directly associated with IL-6 levels (\( r=0.57; P<0.001 \)). On bivariate, multivariate, and bootstrapping validation analyses, patients with an IL-6 level above the median were more likely to have had a history of CVD (\( P=0.01 \)) than those with an IL-6 level below the median (Table 2).

During the follow-up, 42 patients died. The incidence rate of mortality was three times higher (\( P=0.01 \)) in patients with IL-6 above the median (3 deaths per 100 person-years; 95% CI, 2.0 to 4.1 deaths per 100 patient-years) than in those with IL-6 below this threshold (1 death per 100 person-years; 95% CI, 0.6 to 2.1 deaths per 100 patient-years). Overall, 117 patients had fatal and nonfatal CV events. As shown in Table 3, on bivariate, multivariate, and bootstrapping validation analyses, the incidence rate of fatal and nonfatal CV outcomes was significantly higher (\( P=0.02 \)) in patients with IL-6 levels above the median (8.6 CV events per 100 person-years; 95% CI, 2.5 to 5.0 CV events per 100 patient-years) than in those with IL-6 levels below this threshold (3.6 CV events per 100 person-years; 95% CI, 6.9 to 10.8 CV events per 100 patient-years) (Figure 1, left panel). CKD stages did not modify the relationship between IL-6 and CV risk (P for interaction=0.33). Further analyses investigating IL-6 as a continuous variable confirmed this biomarker as a strong and independent risk factor for study outcomes (history of CVD: bivariate analysis OR (1 pg/ml), 1.16 [95% CI, 1.10 to 1.26], \( P<0.001 \), and multivariate analysis OR, 1.10 [95% CI, 1.02 to 1.20], \( P=0.02 \); incident CV events, bivariate analysis HR (1 pg/ml), 1.14 [95% CI, 1.07 to 1.22], \( P<0.001 \), and multivariate analysis HR, 1.12 [95% CI, 1.03 to 1.22], \( P<0.001 \)).

To account for the potential effect of competing risks due to death on the relationship between IL-6 and CV outcomes, an additional adjusted analysis that considered a combined endpoint of death/CV events was performed. This multivariate analysis showed that the HR of the combined endpoint was about 1.5 times higher (HR, 1.54; 95% CI, 1.05 to 2.26; \( P=0.03 \)) in patients with IL-6 levels above the median as compared with the remaining ones.

**Analyses Based on the \(-174\) G/C Polymorphism**

In patients with CKD, the genotype distribution of \(-174\) G/C polymorphism (GG, \( n=333 \) [44%]; GC, \( n=370 \) [49%];
Table 1. Main demographic and clinical characteristics of the study population according to serum IL-6 levels and −174 G/C polymorphism

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Whole Group (n=755)</th>
<th>Serum IL-6 Level</th>
<th>Genotype</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;2.5 pg/ml (n=380)</td>
<td>≥2.5 pg/ml (n=375)</td>
<td>P Value</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>62±11</td>
<td>59±12</td>
<td>64±9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Men (%)</td>
<td>453 (60)</td>
<td>224 (59)</td>
<td>229 (61)</td>
<td>0.55</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>263 (35)</td>
<td>105 (28)</td>
<td>158 (42)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>98 (13)</td>
<td>55 (14)</td>
<td>43 (11)</td>
<td>0.22</td>
</tr>
<tr>
<td>CV comorbidities (%)</td>
<td>221 (29)</td>
<td>82 (22)</td>
<td>139 (37)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>28.2±4.7</td>
<td>27.3±4.3</td>
<td>29.0±4.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>134±18</td>
<td>132±17</td>
<td>135±19</td>
<td>0.03</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>78±11</td>
<td>79±10</td>
<td>77±11</td>
<td>0.01</td>
</tr>
<tr>
<td>Antihypertensive treatment (%)</td>
<td>691 (92)</td>
<td>341 (90)</td>
<td>350 (93)</td>
<td>0.89</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>116±49</td>
<td>109±41</td>
<td>122±56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>187±45</td>
<td>189±43</td>
<td>184±46</td>
<td>0.12</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>12.8±1.8</td>
<td>13.1±1.8</td>
<td>12.6±1.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>4.2±0.5</td>
<td>4.2±0.5</td>
<td>4.1±0.5</td>
<td>0.01</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>2.4 (1.0–5.5)</td>
<td>1.4 (0.7–2.8)</td>
<td>4.3 (1.9–9.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Phosphate (mg/dl)</td>
<td>3.7±0.8</td>
<td>3.6±0.7</td>
<td>3.8±0.8</td>
<td>0.02</td>
</tr>
<tr>
<td>eGFR (ml/min per 1.73 m²)</td>
<td>36±13</td>
<td>38±13</td>
<td>34±13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urinary protein (mg/24 hr)</td>
<td>0.6 (0.2–1.5)</td>
<td>0.5 (0.2–1.2)</td>
<td>0.6 (0.2–1.7)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD, median and interquartile range, or percentage frequency, as appropriate. CV, cardiovascular; CRP, C-reactive protein.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Bivariate</th>
<th></th>
<th>Multivariate</th>
<th></th>
<th>Bootstrapping Validation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P Value</td>
<td>OR (95% CI)</td>
<td>P Value</td>
<td>OR (95% CI)</td>
<td>P Value</td>
</tr>
<tr>
<td>Serum IL-6 value (0 [&lt;2.5 pg/ml]; 1 [≥2.5 pg/ml])&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.14 (1.55 to 2.95)</td>
<td>&lt;0.001</td>
<td>1.58 (1.11 to 2.24)</td>
<td>0.01</td>
<td>1.58 (1.12 to 2.24)</td>
<td>0.01</td>
</tr>
<tr>
<td>Age (in yr)</td>
<td>1.05 (1.03 to 1.07)</td>
<td>&lt;0.001</td>
<td>1.05 (1.03 to 1.07)</td>
<td>&lt;0.001</td>
<td>1.05 (1.03 to 1.07)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes (0=no; 1=yes)</td>
<td>2.83 (1.99 to 4.03)</td>
<td>&lt;0.001</td>
<td>2.83 (1.97 to 4.08)</td>
<td>&lt;0.001</td>
<td>2.83 (1.97 to 4.08)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic BP (in mmHg)</td>
<td>1.00 (0.99 to 1.01)</td>
<td>0.93</td>
<td>1.00 (0.99 to 1.01)</td>
<td>0.94</td>
<td>1.00 (0.99 to 1.01)</td>
<td>0.94</td>
</tr>
<tr>
<td>Hemoglobin (in g/dl)</td>
<td>1.04 (0.94 to 1.16)</td>
<td>0.47</td>
<td>1.04 (0.93 to 1.16)</td>
<td>0.49</td>
<td>1.04 (0.93 to 1.16)</td>
<td>0.49</td>
</tr>
<tr>
<td>Albumin (in g/dl)</td>
<td>0.74 (0.50 to 1.11)</td>
<td>0.15</td>
<td>0.74 (0.50 to 1.11)</td>
<td>0.14</td>
<td>0.74 (0.50 to 1.11)</td>
<td>0.14</td>
</tr>
<tr>
<td>Phosphate (in mg/dl)</td>
<td>1.02 (0.80 to 1.30)</td>
<td>0.86</td>
<td>1.02 (0.78 to 1.34)</td>
<td>0.87</td>
<td>1.02 (0.78 to 1.34)</td>
<td>0.87</td>
</tr>
<tr>
<td>Urinary protein (in mg/24 hr)</td>
<td>1.01 (0.89 to 1.13)</td>
<td>0.93</td>
<td>1.01 (0.88 to 1.14)</td>
<td>0.93</td>
<td>1.01 (0.88 to 1.14)</td>
<td>0.93</td>
</tr>
<tr>
<td>eGFR (ml/min per 1.73 m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>1.00 (0.98 to 1.01)</td>
<td>0.57</td>
<td>1.00 (0.98 to 1.01)</td>
<td>0.57</td>
<td>1.00 (0.98 to 1.01)</td>
<td>0.57</td>
</tr>
</tbody>
</table>

OR, odds ratio; 95% CI, 95% confidence interval.

<sup>a</sup>Below/above the median value (in parentheses). The description of the model building strategy is reported in the Materials and Methods section.

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<tr>
<th>Variable</th>
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<tr>
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<td>HR (95% CI)</td>
<td>P Value</td>
<td>HR (95% CI)</td>
<td>P Value</td>
<td>HR (95% CI)</td>
<td>P Value</td>
</tr>
<tr>
<td>Serum IL-6 value (0 [&lt;2.5 pg/ml]; 1 [≥2.5 pg/ml])&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.37 (1.61 to 3.51)</td>
<td>&lt;0.001</td>
<td>1.66 (1.11 to 2.49)</td>
<td>0.01</td>
<td>1.66 (1.10 to 2.52)</td>
<td>0.02</td>
</tr>
<tr>
<td>Age (in yr)</td>
<td>1.07 (1.04 to 1.10)</td>
<td>&lt;0.001</td>
<td>1.07 (1.03 to 1.10)</td>
<td>&lt;0.001</td>
<td>1.07 (1.03 to 1.10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes (0=no; 1=yes)</td>
<td>1.59 (1.09 to 2.32)</td>
<td>0.02</td>
<td>1.59 (1.06 to 2.37)</td>
<td>0.02</td>
<td>1.59 (1.06 to 2.37)</td>
<td>0.02</td>
</tr>
<tr>
<td>Systolic BP (in mmHg)</td>
<td>1.00 (1.00 to 1.02)</td>
<td>0.31</td>
<td>1.00 (1.00 to 1.02)</td>
<td>0.31</td>
<td>1.00 (1.00 to 1.02)</td>
<td>0.31</td>
</tr>
<tr>
<td>Hemoglobin (in g/dl)</td>
<td>0.91 (0.80 to 1.02)</td>
<td>0.10</td>
<td>0.91 (0.81 to 1.02)</td>
<td>0.09</td>
<td>0.91 (0.81 to 1.02)</td>
<td>0.09</td>
</tr>
<tr>
<td>Albumin (in g/dl)</td>
<td>0.82 (0.53 to 1.27)</td>
<td>0.38</td>
<td>0.82 (0.52 to 1.29)</td>
<td>0.39</td>
<td>0.82 (0.52 to 1.29)</td>
<td>0.39</td>
</tr>
<tr>
<td>Phosphate (in mg/dl)</td>
<td>1.13 (0.88 to 1.44)</td>
<td>0.34</td>
<td>1.13 (0.88 to 1.45)</td>
<td>0.35</td>
<td>1.13 (0.88 to 1.45)</td>
<td>0.35</td>
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<tr>
<td>Urinary protein (in mg/24 hr)</td>
<td>1.05 (0.94 to 1.17)</td>
<td>0.38</td>
<td>1.05 (0.93 to 1.18)</td>
<td>0.44</td>
<td>1.05 (0.93 to 1.18)</td>
<td>0.44</td>
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<tr>
<td>eGFR (ml/min per 1.73 m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>1.01 (0.99 to 1.02)</td>
<td>0.44</td>
<td>1.01 (0.99 to 1.02)</td>
<td>0.49</td>
<td>1.01 (0.99 to 1.02)</td>
<td>0.49</td>
</tr>
</tbody>
</table>

HR, hazard ratio.

<sup>a</sup>Below/above the median value (in parentheses). The description of the model building strategy is reported in the Materials and Methods section.
IL-6 (median, 2.9 pg/ml [interquartile range, 1.7–26%]).

than in healthy study participants (22%; 95% CI, 18% to 3.8 pg/ml]) and GG (2.5 pg/ml [interquartile range, 1.7–6.9 pg/ml]) (Figure 1, right panel) and this was also true in a bootstrapping validation model (HR, 1.87; 95% CI, 1.02 to 3.44; P=0.04) than in those with the GC or GG genotypes (Figure 1, right panel) and this was also true in a bootstrapping validation model (HR, 1.87; 95% CI, 1.01 to 3.51; P=0.05).

Discussion

In this study, high serum IL-6 levels were associated with history of CVD and predicted the risk for incident CV events in patients with stages 2–5 CKD. Furthermore, the functional polymorphism –174 G/C in the promoter of the IL-6 gene is associated with history of CVD and predicts the risk for future CV events in this population. These results are compatible with the hypothesis that this inflammatory cytokine is causally implicated in the high CV risk in CKD.

Atherosclerosis is an inflammatory disease (1). Chronic inflammation is pervasive at all CKD stages, particularly stage 5D (13,14). IL-6 is considered an orchestrator of the inflammatory response (37) and a key player in atherosclerosis in humans (38). The IL-6 effect on the cardiovascular system might be mediated via downstream acute-phase proteins (39) or by IL-6 per se. IL-6 stimulates endothelial activation, vascular smooth muscle cell proliferation (40), and leukocyte recruitment (41), thereby contributing to the process of atherosclerotic plaque growth (42) and instability (43). IL-6 mRNA is overexpressed in atheromatous arteries, and IL-6 expression co-localizes with macrophages in areas of plaque rupture (43). In apoE-deficient mice, systemic administration of IL-6 accelerates atherosclerosis
Elevated levels of IL-6 are predictive of future CV events in healthy men (15) and women (44) and are markers of poor prognosis in patients with chronic angina (45) and acute coronary syndrome (46). IL-6 levels are markedly elevated in CKD, a phenomenon only in part explained by reduced renal clearance of this cytokine. Furthermore, the observation of a parallel increase in IL-6 and CRP in our study is in keeping with the notion that IL-6 drives the synthesis of CRP. High IL-6 has been solidly associated with mortality in patients with stage 5 CKD who are being maintained on long-term dialysis (16–20). To the best of our knowledge, only one study has tested the relationship between IL-6 and CV mortality in CKD (21). This seminal study was carried out in a small cohort of 125 patients, was based on a limited number of CV events (n=22), and also included dialysis patients (34% of the whole cohort) (21). Our study, based on a large CKD cohort composed exclusively of predialysis patients and including a large number of CV events, confirmed pilot data by Barreto et al. (21) and showed that high IL-6 is coherently associated both with history of CVD as well as with incident CV events.

Being observational in nature, findings in studies discussed above, including our analysis based on circulating IL-6, remain hypothesis-generating and as such leave unresolved the critical question of whether this cytokine is causally implicated in CV complications in patients with predialysis CKD. This question can be resolved only by a full-fledged clinical trial.

The Mendelian randomization approach is useful step in the pathway to discovery in clinical research. Since genetic polymorphisms are distributed randomly at gamete formation and since genotypes precede phenotypes and do not change over time, comparing individuals harboring a given risk allele for the expression of a corresponding risk factor with those without the risk allele in question may allow unbiased assessment of the link between the attendant risk factor and relevant clinical outcomes. In this perspective, we used the functional polymorphism −174 G/C in the promoter of the IL-6 gene as a marker to further investigate the link between IL-6 and CV events in patients with CKD. The −174 G/C polymorphism is a common variant that regulates the serum concentration of IL-6 (22–28). In keeping with previous studies in patients with CVD (28), coronary artery bypass grafting surgery (23,25), carotid atherosclerosis (24), abdominal aortic aneurysm (22), and dialysis (26), we found that patients with CKD and CC genotype had higher circulating levels of IL-6 and CRP than those harboring GC or GG genotypes, specifically legitimating the use of this genetic marker as an unbiased means for assessing the causal nature of the link between the gene product (IL-6) of this polymorphism and CV complications in CKD. Interestingly, this analysis showed that this polymorphism is independently associated both with the history of CVD as well as with incident CV events. Such associations, which went along with the previously described relationships of serum IL-6 with the same outcomes, further strengthen the hypothesis that IL-6 is a direct player in atherosclerotic complications in CKD. Moreover, the observation that the distribution of genotypes frequency of the −174 G/C polymorphism in patients with CKD was not in Hardy–Weinberg equilibrium and that the frequency of the C allele was significantly higher in patients CKD compared with general population of the same geographic area, offers additional circumstantial evidence that IL-6 is a causal risk factor for CV events in this population (47).

Mendelian randomization studies support causal interpretations but do not constitute definitive proof for causality, which demands specific experimental evidence (i.e., a formal randomized clinical trial). In this respect, meta-analytic data from two genetic consortia exploring the effect of a polymorphism in the IL-6 receptor on the risk of coronary artery disease showed the allele that attenuated IL-6 signaling was significantly associated with reduced risk of coronary heart disease (48). Notably, the relevance of this genetic association for the control of inflammation is shown by a meta-analysis of clinical trials testing a monoclonal antibody against the IL-6 receptor (tocilizumab) in rheumatoid arthritis and documenting that lowering serum levels of IL-6 is an effective strategy to induce the remission of this chronic inflammatory disease (49).
Some limitations should be acknowledged. First, even though our cohort study registered a sizable number of cardiovascular events, the number of deaths (n=42) was limited, preventing adequately adjusted analyses focusing on this major outcome. However, the analysis of the primary outcome in this study, incident CV events, which was robustly based on 117 events, showed parallel links between serum IL-6 and the genetic marker of this cytokine with the same events.

Second, although Mendelian randomization is a powerful approach for inferring causality in observational studies, its application has potential limitations. Genetic variants can be indicators of environmental exposures on condition that there is no genetic admixture, pleiotropy or linkage disequilibrium. However, it is reasonable to believe that in our study all these assumptions are fulfilled. Our population is genetically homogeneous (29) and the −174G/C polymorphism is a functional variant directly responsible for serum levels of IL-6 (22–28). Furthermore, pleiotropy seems highly unlikely because of the location of −174 G/C polymorphism in the promoter region of the gene.

Third, although we demonstrated that our findings had high internal validity by bootstrap modeling, replication of the results in a second cohort is a required proof for the external generalizability of findings in observational studies. In this respect, our observational study in a large southern European cohort confirms findings in a small central European cohort (21). Furthermore, our study is the first applying a genetic marker of IL-6 to express the nature (causal versus not causal) of the link between IL-6 and CV events in patients with CKD.

In conclusion, high serum IL-6 is associated with both a history of CVD and future CV events in patients with CKD, and these associations are fully confirmed by the application of a functional polymorphism in the IL-6 gene. Overall, this study is compatible with the hypothesis that the IL-6 plays a causal role in the high CV risk of patients with CKD.

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Disclosures
None.

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