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# Interleukin 8 gene polymorphisms and susceptibility to restenosis after percutaneous coronary intervention

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**Abstract** Interleukin-8 is a strong mediator of inflammation and has been implicated in the biochemical pathways involved in a wide range of inflammatory diseases including atherosclerosis. We investigated the potential influence of two common functional polymorphisms of the interleukin (IL)-8 gene: −251A/T and 781C/T on susceptibility to in stent restenosis (ISR) following percutaneous coronary intervention (PCI). The hypothesis was tested by screening for the prevalence of the above polymorphisms in 201 coronary artery disease (CAD) patients subjected to PCI and presenting with symptoms or signs of recurrent ischemia. Patients were angiographically re-evaluated and formed the ISR group ($n = 73$) and the non-ISR group ($n = 128$) based on the presence or absence of ISR. One hundred and forty-seven subjects without angiographic evidence of CAD formed a reference control group (non-CAD group). A borderline statistically significant higher frequency of the $TT_{251}TT_{781}$ combined genotype was observed in patients with ISR on re-evaluation compared with patients with normal follow-up angiography. The predominance of $TT_{251}TT_{781}$ was independent of conventional risk factors for cardiovascular disease. Consequently, $T_{251}T_{781}$ haplotype was significantly more common in the ISR group. The above observations indicate that the genetic diversity of the IL-8 gene influences patient susceptibility to ISR and suggests the implication of IL-8-mediated pathways in the process of ISR. However, the rarity of $T_{251}T_{781}$ haplotype makes any clinical application of the above observations unfeasible.

**Keywords** Interleukin 8 · Polymorphisms · Coronary artery disease · In stent restenosis

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## Introduction

The correlation between atherosclerosis and inflammation is well established [1]. Several pro-inflammatory factors have been implicated in the molecular pathways that lead to atherosclerosis [2]. Interleukin 8 (IL-8) or CXCL8 is the prototype member of the CXC chemokine family. It is a strong chemoattractant for neutrophils and T-lymphocytes and is produced by a wide range of cell types in response to various inflammatory stimuli. Interleukin-8 is a strong mediator of inflammation and has been implicated in the biochemical pathways involved in a wide range of inflammatory diseases including atherosclerosis [3, 4].

Disrupted gene expression or altered protein formation of the IL-8 gene, mainly induced by mutations, may contribute positively or negatively to the establishment or progression of coronary artery disease (CAD). Several polymorphisms have been detected in the IL-8 gene, and a common polymorphism in the −251 position (251A/T, rs4073) of the promoter region has been associated with the gene's transcriptional activity. The 251A allele in a

homozygous state has been associated with an increased expression of IL-8 [5, 6]. Another common polymorphism in position 781 of the IL-8 gene (781C/T, rs2227306) has been associated with altered transcription levels of IL-8. The common haplotype $A_{251}T_{781}$ has been found to have the maximum up-regulating effect on IL-8 expression [5]. The two polymorphisms have been evaluated in a wide range of clinical settings and several genotype–phenotype interactions have been established [7–10]. We have investigated a possible influence of IL-8 gene polymorphisms on susceptibility to CAD and we have previously reported an association between a common genotype of the IL-8 gene and a reduced risk for acute coronary syndrome (ACS) in CAD subjects independently of established cardiovascular risk factors [11].

In the present study, we further explored the effects of the two genetic polymorphisms of the IL-8 gene on patient susceptibility to in-stent restenosis (ISR) following pecutaneous coronary intervention (PCI).

We hypothesize that an altered IL-8 expression induced by certain genotypes would interfere with susceptibility to ISR. In order to explore the hypothesis a population consisting of CAD patients with ISR after PCI, CAD patients subjected to PCI with normal follow-up angiography and subjects without angiographic evidence of CAD, was genotyped for the two polymorphisms.

## Patients and methods

### Study population

Patients presenting clinical signs of recurrent ischemia one month to two years after PCI and stent placement were prospectively recruited over a two-year period. Clinical signs of recurrent ischemia were considered: relapse of effort angina, occurrence of ACS, new signs of myocardial ischemia on the stress electrocardiogram, stress echocardiography or SPECT myocardial perfusion imaging. All patients were angiographically re-evaluated and classified in the in-stent restenosis (ISR) and non-in-stent restenosis (non-ISR) group based on the angiographic status of the intervened lesion. One hundred and forty-seven age matched subjects (male:female ratio 2.5:1) without angiographic evidence of CAD form a reference control group (non-CAD group). All of the subjects enrolled were Caucasian. Clinical and epidemiological characteristics are summarized in Table 1.

### Clinical definitions

Smoking was defined as a current or prior history of tobacco use. Diabetes was defined as a fasting blood glucose level >126 mg/dl, or treatment with dietary modification, oral hypoglycemic agents or insulin at the time of

**Table 1** Demographic and clinical characteristics of patients with clinical signs of recurrent ischemia following percutaneous coronary intervention

| | ISR ($n = 73$) | Non-ISR ($n = 128$) | Non-CAD ($n = 147$) |
|---|---|---|---|
| Age mean (±SEM) | 64 (1.1) | 64 (0.8) | 62 (1.1) |
| Male | 67 (91.8) | 110 (85.9) | 105 (71.4) |
| Hypertension | 68 (93.2) | 105 (82) | 87 (59.2) |
| Diabetes | 38 (52.1) | 61 (47.7) | 25 (17) |
| Dyslipidemia | 73 (100) | 126 (98.4) | 99 (67.3) |
| Smoking | 50 (68.5) | 71 (55.5) | 59 (40.1) |
| Positive family history | 31 (42.5) | 54 (42.2) | 60 (40.8) |
| Median time (days) of follow-up CA post PCI | 265 (130–553) | 285 (184–602) | |
| Number of diseased vessels | | | |
| 1 | 15 (20.5) | 36 (28.1) | |
| 2 | 15 (20.5) | 38 (29.7) | |
| 3 | 43 (59) | 54 (42.2) | |
| Clinical presentation | | | |
| Unstable disease | 15 (20.5) | 29 (22.7) | |
| Stable disease | 58 (79.5) | 99 (77.3) | |
| Type of stent | | | |
| BMS | 39 (53.4) | 54 (42.2) | |
| DES | 33 (45.2) | 66 (51.6) | |
| BMS and DES | 1 (1.4) | 8 (6.3) | |

Values refer to the number of subjects (%), means (±SEM) or medians (range)

*ISR* in-stent restenosis, *CAD* coronary artery disease, *CA* coronary angiography, *DES* drug-eluting stent, *BMS* bare-metal stent

the study. Hypertension was defined as systolic blood pressure >140 mmHg and/or diastolic pressure >90 mmHg in at least three distinct measurements, or in cases in which such a diagnosis had been made in the past and the patient was being treated with medication or lifestyle modification. For CAD patients, dyslipidemia was defined as treatment with lipid-lowering medication, dietary modification or lipid levels greater than those recommended by the Third Joint Task Force of European and Other Societies on Cardiovascular Disease Prevention in Clinical Practice [14]. Parental history of myocardial infarction was considered as positive family history.

Coronary artery disease was classified as 1, 2 or 3 vessel disease based on the number of major epicardial vessels with >70% lumen stenosis. Based on the clinical presentation of CAD, patients were further classified as suffering from stable disease (silent ischemia or patients with stable angina) or unstable disease (unstable angina or acute myocardial infarction). In-stent restenosis was defined as ≥50% diameter reduction of the stented segment on follow-up angiography.

Informed consent was obtained from all the individuals that participated in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the ethics committee of University Hospital of Crete and Onassis Cardiac Surgery Centre.

## DNA extraction and genotyping

Genomic DNA was extracted from 10 ml ethylenediaminetetraacetic acid (EDTA)-treated venous blood using the standard phenol/chloroform protocol. Blood samples were taken before catheterization. DNA purity was assessed by a UV/VIS spectrophotometer evaluating the A260/A280 ratio. Genotyping for the 251A/T polymorphism was performed using allele-specific polymerase chain reaction (PCR) as previously described [11]. Results were verified by direct sequencing of the PCR product (Fig. 1). PCR/restriction fragment length polymorphism (RFLP) analysis was performed for the detection of the variation 781C/T. Initially, PCR was performed to frame the polymorphic region using primers and conditions as previously described [11]. PCR products were further subjected to digestion with 1 unit of *Eco*RI endonuclease.

## Statistical analysis

Genotype distributions for each polymorphism were first compared to values predicted by the Hardy–Weinberg equilibrium (HWE) through $\chi^2$ analysis. Haplotypes were calculated and linkage disequilibrium was measured using the classic statistic, disequilibrium coefficient. Estimation of haplotype frequencies and linkage disequilibrium analysis was performed by *CubeX* analysis software [12].

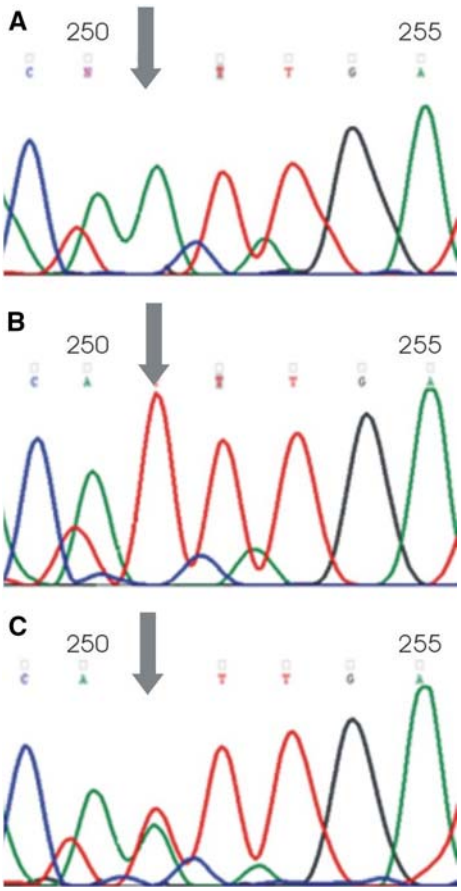


**Fig. 1** Representative chromatograms of 251 AA homozygote (**a**), 251 TT homozygote (**b**) and an heterozygote (**c**)

The correlation of each of the studied polymorphisms to clinical parameters was first evaluated by $\chi^2$ analysis with 2 degrees of freedom. The extent of each genotype's association with the disease was initially estimated by Pearson's $\chi^2$ or Fisher exact test. An adjustment for conventional risk factors of patients such as age, gender, smoking status, diabetes mellitus, hypertension, dyslipidemia and family history, was performed by including these covariates in a logistic regression model. To provide separate odds ratios (ORs) for each genotype, the most common genotype was considered as the reference group. Using our sample size, the study had a validity of 80% to detect a 1.55-fold increase of allele frequencies, assuming a 40% prevalence of the rare allele in the control group and a type I error probability of 0.05. Significant associations were further corrected for multiple testing by applying Bonferroni correction. The significance level was adjusted by dividing this level by the number of tested variables ($n$).

Numerical values are expressed as the mean ± SEM, and differences between means were compared by the 2-tailed

unpaired Student's *t*-test. In all cases, $P < 0.05$ was considered to be statistically significant. Analyses were performed using SPSSv10 (SPSS Inc., Chicago, IL, USA).

## Results

Two hundred and one CAD patients (median age 64, male:female ratio 7.4:1) presenting symptoms or clinical signs of ischemia after PCI were recruited. After angiographic re-evaluation, patients were classified into the in-stent restenosis (ISR) group ($n = 73$) or non-in-stent restenosis (non-ISR) group ($n = 128$).

### Hardy–Weinberg equilibrium and genetic interaction within the IL-8 gene

The prevalence of −251A/T and 781C/T polymorphisms of the IL-8 gene was analyzed in 73 patients with previous PCI presenting with angiographically established ISR, 128 patients subjected to PCI with normal follow-up angiography and 147 subjects without angiographic evidence of CAD. The genotypic and allelic frequencies for the studied variations are shown in Tables 2 and 3. Genotype frequencies of both polymorphisms were in agreement with those predicted by the Hardy–Weinberg Equilibrium (HWE) in controls. However, genotype frequencies of the 251A/T polymorphism both in the ISR and non-ISR groups deviated from those predicted by the HWE (Table 4). In accordance to our previous observation [11] disequilibrium analysis indicated a strong association between the two loci (Table 4). Four out of nine combined genotypes accounted for >90% of the sample, while haplotype analysis revealed three predominant haplotypes and the less common $T_{251}T_{781}$ haplotype, accounting for less than 2% of the total population gene pool (Table 3).

**Table 2** Genotype frequencies in patients and controls

| 251 A/T | 781 C/T | ISR *n* (%) | Non-ISR *n* (%) | Non-CAD *n* (%) |
|---|---|---|---|---|
| AA | TT | 7 (9.5) | 23 (17.9) | 15 (10.2) |
| AA | CT | 3 (4.1) | 2 (1.6) | 9 (6.1) |
| AA | CC | 0 | 2 (1.6) | 6 (4.1) |
| AT | TT | 1 (1.3) | 1 (0.8) | 2 (1.4) |
| AT | CT | 32 (43.8) | 59 (46.1) | 59 (40.1) |
| AT | CC | 15 (20.5) | 19 (14.8) | 16 (10.9) |
| TT | TT | 3 (4.1)* | 0* | 0* |
| TT | CT | 0 | 0 | 2 (1.4) |
| TT | CC | 12 (16.4) | 22 (17.2) | 38 (25.8) |
| AA | | 10 (13.7) | 27 (21.1) | 30 (20.4) |
| AT | | 48 (65.8) | 79 (61.7) | 77 (52.4) |
| TT | | 15 (20.5) | 22 (17.2) | 40 (27.2) |
| | CC | 27 (37) | 43 (33.6) | 60 (40.8) |
| | CT | 35 (47.9) | 61 (47.7) | 70 (47.6) |
| | TT | 11 (15.1) | 24 (18.8) | 17 (11.6) |
| Total | | 73 | 128 | 147 |

*ISR* in-stent restenosis, *CAD* coronary artery disease. Values refer to number of subjects (%)

ISR vs. non-ISR: OR = 1.2, 95% CI: 1–1.9, $P = 0.049$ (using as baseline risk the most common genotype, ATCT)

ISR vs. non-CAD: OR = 1.2, 95% CI: 1–1.9, $P = 0.049$ (using as baseline risk the most common genotype, ATCT)

*P*-values under the cut-off point of statistical significance in all other cases

**Table 3** Haplotype frequencies in patients and controls

| Haplotype | | ISR | Non-ISR | Non-CAD |
|---|---|---|---|---|
| h1 | $A_{251}C_{781}$ | 0.133 | 0.1 | 0.128 |
| h2 | $A_{251}T_{781}$ | 0.476 | 0.476 | 0.518 |
| h3 | $T_{251}C_{781}$ | 0.332 | 0.421 | 0.338 |
| h4 | $T_{251}T_{781}$ | 0.058* | 0.004* | 0.0159* |
| Total (n) | | 146 | 256 | 294 |

*ISR* in-stent restenosis, *CAD* coronary artery disease. *n* refers to the number of alleles

* ISR vs. non-ISR: OR = 15.7 95% CI: 1.9–126, $P = 0.001$ (using as baseline risk h4 haplotype)

ISR vs. non-CAD: OR = 3.9, 95% CI: 1.3–12, $P = 0.018$ (using as baseline risk h4 haplotype)

**Table 4** Allele frequencies and $\chi^2$ values of Hardy–Weinberg equilibrium in the studied sub-population

| | ISR | | Non-ISR | | Non-CAD | | $D'$ | $r^2$ |
|---|---|---|---|---|---|---|---|---|
| | *f* | $\chi^2$ HWE | *f* | $\chi^2$ HWE | *f* | $\chi^2$ HWE | | |
| 251A | 0.465 | 7.5 | 0.52 | 7.1 | 0.466 | 0.40 | 0.92 | 0.53 |
| 781C | 0.61 | 0.004 | 0.574 | 0.08 | 0.646 | 0.25 | | |
| Total (*n*) | 146 | | 256 | | 294 | | | |

*ISR* in-stent restenosis, *CAD* coronary artery disease, *HWE* Hardy–Weinberg Equilibrium. *n* refers to number of alleles

### Genotype–phenotype association studies

Genotype–phenotype interactions were evaluated for each genotype as well as for the 4 haplotypes and the 9 combined genotypes. No association was established when we assessed the impact of each of the studied polymorphisms independently on susceptibility to ISR. A combined genotype analysis revealed a statistical significant predominance of the $TT_{251}TT_{781}$ genotype (taking as baseline risk the $AT_{251}CT_{781}$ genotype) in the ISR group compared to the non-ISR group and the non-CAD group (Odds ratio 1.2 95% Confidence interval 1–1.9, $P = 0.049$). In fact the

$TT_{251}TT_{781}$ genotype was only detected in the ISR group. When the $TT_{251}TT_{781}$ genotype and conventional cardiovascular risk factors were included in a multiple logistic regression model, the effect of the $TT_{251}TT_{781}$ genotype on susceptibility to ISR proved to be independent of conventional cardiovascular risk factors (corrected $P$ value = 0.018). However, the predominance of the $TT_{251}TT_{781}$ genotype in the ISR group did not retain statistical significance when Bonferroni correction was applied for multiple testing (adjusted significance for 9 tested variables: $P = 0.3$).

Consequently, the $T_{251}T_{781}$ haplotype (h4) was significantly more common in the ISR group than in non-ISR and non-CAD group. In fact, using as baseline risk the most common haplotype $A_{251}T_{781}$ (h2), the predominance of the h4 in the ISR group produced the strongest statistical significance (odds ratio = 15.7; 95% confidence interval: 1.9–126; $P = 0.001$). The latter observation retained statistical significance after applying Bonferroni correction (adjusted significance for 4 tested variables: $P = 0.004$).

No phenotype–genotype or phenotype–haplotype association was detected between the studied genetic variations and clinical presentation of CAD or the angiographic severity of CAD.

No associations were established between conventional cardiovascular risk factors and risk for ISR, and between the studied genotypes and conventional risk factors of cardiovascular disease; nor did any other genotype grouping give statistically significant results.

## Discussion

This is the first study assessing the impact of −251A/T and 781C/T polymorphisms of the IL-8 gene on patient susceptibility to ISR. Our findings demonstrated a positive association between a rather uncommon genotype of the IL-8 gene and ISR. The $TT_{251}TT_{781}$ genotype was significantly more common in the ISR group, which exhibited a significantly higher frequency of the $T_{251}T_{781}$ haplotype. The latter association was established despite the small sample size and the low frequency of the $T_{251}T_{781}$ genotype. Undoubtedly the observed phenotype/genotype interaction is not expected to be clinically applicable in prognostic or therapeutic decision-making due to the relatively low frequency of the $TT_{251}TT_{781}$ genotype. It is, however, indicative of the implication of IL-8-mediated pathways in the pathogenesis of ISR.

The advent of stent technology has revolutionized the field of interventional cardiology by reducing the incidence of restenosis after balloon angioplasty. However, ISR remains a principal drawback with substantial clinical implications [12]. Several factors contribute to ISR including lesion- or procedure-; patient- and genetic-related factors [13–15].

Neointimal proliferation is the main mechanism involved in the development of stent restenosis and a number of previous investigations have consistently suggested that inflammation plays a significant role in the process [13].

Several investigators have assessed the impact of genetic alterations mainly single nucleotide polymorphisms (SNPs) on patient susceptibility to ISR [16–19]. Most recently Shah et al., [20] assessed the impact of 39 candidate atherosclerosis genes on susceptibility to ISR in 49 BMS-treated patients with subsequent ISR and 39 matched controls. They concluded that ALOX5AP, a gene within the inflammatory leukotriene pathway, which was previously associated with coronary atherosclerosis, is also associated with in-stent restenosis. Similarly, Oguri et al., [21] in a population comprised of 28 subjects who developed ISR two or more times and 499 subjects without restenosis, genotyped for 142 polymorphisms in 121 candidate genes. They concluded that the −55C/T polymorphism of the uncoupling protein 3 gene (UCP3) may contribute to the assessment of the genetic risk for recurrent ISR. In the field of inflammatory mediators, Miranda-Malpica et al., [22] explored the association between IL-1 family gene polymorphisms and the risk for restenosis after coronary stent placement in 165 patients who underwent coronary artery stenting. Basal and follow-up coronary angiography were analyzed in search of angiographic restenosis. The above-mentioned authors concluded that IL-1B-511 polymorphism may be involved in the development of restenosis following coronary stent placement. Several other studies have resulted in a positive or negative association between certain genotypes and susceptibility to restenosis post-PCI.

Interleukin-8 has been implicated in the pathways involved in stent restenosis by several investigators in different experimental settings. Most recently, Caixeta et al. reported that patients who experienced restenosis had higher levels of IL-8 at 6 h after stent placement than those without restenosis [23]. Similarly, Qi et al. reported that increased levels of IL-8 after PCI are a powerful prognostic factor for cardiac events and restenosis. The higher the peak level of post-procedure IL-8, the lower the event-free survival observed [24]. These observations are in accordance with our results and support a pivotal role of IL-8 in restenosis.

Nevertheless, there are several limitations in the study that may diminish the significance of the reported results. It is important to note that the study was conducted in a relatively small population. The small sample size significantly reduced the validity of the study and minimized the prospect of establishing negative associations. This is a

common drawback in studies assessing genetic predisposition to ISR mainly because stent placement (particularly DES) has significantly reduced the incidence of restenosis. Furthermore, the *gold standard* in the diagnosis of ISR is follow-up angiography which is an invasive procedure that is not easily applied for experimental purposes. To overcome these setbacks we included patients presenting with clinical evidence of restenosis one month to two years after successful PCI. In this clinically defined group, the rate of restenosis was significantly higher (35.6%) than the overall expected rate after PCI.

Another discrepancy in our findings is that if IL-8 was implicated in the ISR process we would expect more pronounced results regarding the genotypes with the strongest functional consequences, −251AA and 781TT. However, differences were noted only regarding the $TT_{251}TT_{781}$ combined genotype and the $T_{251}T_{781}$ haplotype. The up-regulating effect of the 781T allele is in agreement with our finding, while the up-regulating effect of the −251A allele has been observed only in the homozygote state. There is also much disagreement in the literature regarding the clinical consequences of −251A/T and 781C/T on inflammatory diseases. Furthermore, the impact of the two variations on the transcriptional activity of the IL-8 gene has largely been studied in respiratory epithelial cells in a pulmonary disease setting and not in the field of endothelial dysfunction or atherosclerosis [5].

Another limitation of the study is that it consists of a rather heterogeneous population. The fact that both BMS- and DES-treated patients were included is a complicating factor. The pathophysiological background of restenosis might differ in BMS and DES treated vessels [25]. Another confounding factor is that the presenting symptoms and signs of the recruited subjects varied, from asymptomatic patients to those presenting with acute coronary syndromes. Moreover, the fact that the study included only patients presenting symptoms or signs of restenosis is a limitation. Patients with asymptomatic restenosis or restenosis not producing ischemia in non-invasive tests might have been overlooked.

Finally, an observation that needs explanation is the significant deviation of 251A/T allele frequencies from those predicted by the HWE in both the ISR and non-ISR groups. Violation of HWE can be the result of genotyping errors, ethnic diversity or over-selected study populations. In our study protocol, genotyping was repeated twice and the validity of allele-specific PCR was re-evaluated by direct sequencing of the randomly selected samples. However, no deviation was observed between the allele-specific PCR and sequencing results. Ethnic diversity was also discarded as a possible cause of HWE disassociation since all the study participants were Caucasians. However, our study population, did not consist of randomly selected subjects from the general population, but were subjects with significant CAD subjected to PCI and presented with clinical evidence of restenosis one month to two years later. It is thus the over-selected population that may have resulted in the deviation from HWE. In a previous study of ours, conducted in a less selected population sample (CAD patients and CAD-free controls), the allele frequencies of the two polymorphisms did not deviate from the ones predicted by HWE [11].

## Conclusions

In conclusion, we demonstrated a phenotype/genotype interaction between a specific IL-8 gene genotype and susceptibility to ISR. This finding is in agreement with the functional consequences of 781T polymorphism and support the pivotal role of IL-8 chemokine in the development of ISR.

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