Tumor necrosis factor α plays an important role in restenosis development

Pascal S. Monraats, Nuno M.M. Pires, Abbey Schepers, Willem R.P. Age
ma MD, Lianne S.M. Boesten, Margreet R. de Vries, Aeilko H. Zwinderman,
Moniek P.M. de Maat, Pieter A.F.M. Doevendans, Robbert J. de Winter, René
A. Tio, Johannes Waltenberger, Leen M. ‘t Hart, Rune R. Frants, Paul H.A.
Quax, Bart J.M. van Vlijmen, Louis M. Havekes, Arnoud van der Laarse, Ernst
E. van der Wall, J. Wouter Jukema

Abstract

Background
Genetic factors appear to be important in the restenotic process after percutaneous coronary intervention (PCI), as well as in the process of inflammation, a pivotal factor in restenosis. TNFα, a key regulator of inflammatory responses, may exert critical influence on the development of restenosis after PCI.

Methods
The GENetic DEterminants of Restenosis (GENDER) project included 3,104 patients who underwent a successful PCI. Systematic genotyping for six polymorphisms in the TNFα gene was performed. The role of TNFα in restenosis was also assessed in ApoE*3-Leiden mice, TNFα knockout mice and by local delivery of a TNFα biosynthesis inhibitor, thalidomide.

Results
The -238G-1031T haplotype of the TNFα gene increased clinical and angiographic risk of restenosis (P=0.02 and P=0.002, respectively). In a mouse model of reactive stenosis, arterial TNFα mRNA was significantly, time-dependently, upregulated. Mice lacking TNFα or locally treated with thalidomide showed a reduction in reactive stenosis (P=0.01 and P=0.005, respectively).

Conclusions
Clinical and preclinical data indicate that TNFα plays an important role in restenosis. Therefore, TNFα genotype may be used as a risk marker for restenosis and may contribute to individual patient screening prior to PCI in clinical practice. Inhibition of TNFα may be an anti-restenotic target strategy.
**Introduction**

Percutaneous coronary intervention (PCI) has become the main treatment for patients with atherosclerotic lesions. However, it is limited by restenosis.\(^1\) Restenosis is not a random event, but it affects selectively a subset of patients prone to develop lumen renarrowing after PCI. Inherited factors may explain part of the risk of restenosis in certain patients, which cannot be attributed to conventional clinical variables.\(^2\) Inflammatory responsiveness plays a pivotal role in restenosis. Therefore, it is plausible that differences in genetic make-up of inflammatory-genes between individuals may explain part of the risk of restenosis.\(^3-5\) An important mediator in the inflammatory response is tumor necrosis factor-alpha (TNF\(\alpha\)). TNF\(\alpha\) is a key pro-inflammatory cytokine produced by a number of cells, including macrophages, neutrophils, endothelial cells, and VSMCs. TNF\(\alpha\) acts locally at sites of tissue injury induced by vessel wall damage and has many biological functions.\(^6\) Therefore, we believe TNF\(\alpha\) is a key factor in regulating restenosis development. The promoter of the gene encoding TNF\(\alpha\) contains polymorphic sites that are associated with different responsiveness to regulatory signals.\(^7,8\) The aim of our study is to examine in a large patient-population if functionally relevant polymorphisms in the promoter region of the TNF\(\alpha\) gene are related to unfavorable outcomes after PCI. To further investigate the involvement of TNF\(\alpha\)-gene in restenosis development, we examined an established mouse model of reactive stenosis.

**Methods**

**GENDER-project**

**Study design**

The GENetic DEterminants of Restenosis (GENDER) project was designed to study the association between various gene polymorphisms and clinical restenosis, defined by Target Vessel Revascularization (TVR).\(^9\) Patients eligible for inclusion were treated successfully for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia by PCI in four interventional cardiology centers in the Netherlands. Experienced operators using a radial or femoral approach performed standard angioplasty and stent placement. Before the procedure, patients received aspirin 300mg and heparin 7500IU. The use of intra coronary stents and additional medication was at the operator’s discretion. No
drug-eluting stents were used. TVR by PCI or coronary artery bypass-grafting (CABG) was the primary end point. An independent events committee adjudicated clinical events. The protocol conforms to the Declaration of Helsinki and was approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from each participant before PCI-procedure.

Genetic methodology

DNA was extracted using standard procedures from blood collected in EDTA-tubes. Genotyping for four TNFα-promoter polymorphisms; -238G/A, -244G/A, -308G/A, and -376G/A was performed by a previously described multilocus genotyping assay for genetic markers of inflammation and cardiovascular disease (Roche Molecular Systems, Alameda, USA). The -857C/T and -1031T/C polymorphisms were genotyped using Taqman-based assays. Primers and probes were synthesized by Applied Biosystems (Nieuwerkerk a/d IJssel, the Netherlands).

To confirm genotype assignments, PCR-analysis was randomly performed in replicate on 10% of the samples. Two independent observers carried out scoring. Disagreements (<1%) were resolved by joint reading, when necessary, a repeated genotyping reaction was performed.

Angiographic assessment

Quantitative computer-assisted angiographic analysis was performed off-line on angiograms obtained just before, immediately after stenting, and at follow-up in a subpopulation of patients from the GENDER-study, who were scheduled for re-angiography at six-months, according to previously described standard procedures. Identical projections were used for all angiograms. Binary restenosis was defined as a stenosis diameter >50% within the stent or in the 5-mm segments proximal or distal to the stent at follow-up angiography. Quantitative analysis of angiograms was performed by operators not involved in the stenting procedure and unaware of the genetic data (Heartcore, Leiden, the Netherlands).

Animal experiments

Since the association found sheds no definite light on a causal relationship between TNFα and restenosis we, in order to obtain a better understanding of the role of TNFα-gene in the development of restenosis, investigated the effect of this gene in an established mouse model of reactive stenosis.
Tumor necrosis factor-α plays an important role in restenosis development

Femoral artery cuff mouse model
The institutional committee on animal welfare of TNO approved all animal experiments. For all experiments (unless stated otherwise), hyperlipidemic male ApoE*3-Leiden mice were fed a high-cholesterol diet (ArieBlok, Woerden, The Netherlands). Blood samples to determine plasma cholesterol were collected at time of surgery. After 3 weeks on diet, mice were anaesthetized with intraperitoneal injection of 5mg/kg Dormicum (Roche, Basel, Switzerland), 0.5mg/kg Dormitor (Orion, Helsinki, Finland) and 0.05mg/kg Fentanyl (Janssen, Geel, Belgium). A non-constricting polyethylene cuff (Portex, Kent, UK) was placed around the femoral artery.

Real time (RT)-PCR TNFα-mRNA analysis
Animals were sacrificed at different time-points after cuff-placement (6, 24, 48h, and 7d), 4 mice for each time-point. Both cuffed right and non-cuffed sham operated left femoral arteries were isolated, harvested and snap frozen. Femoral arteries, either cuffed or non-cuffed sham operated, were pooled (two arteries/sample, two samples/time point), total RNA was isolated using Trizol (Invitrogen, Carlsbad, USA) and cDNA was made using Ready-To-Go RT-PCR beads (Amersham Biosciences, Uppsala, Sweden). Intron-spanning primers and probe were designed for mouse TNFα cDNA using Primer-ExpressTM1.5 (Applied Biosystems). Housekeeping genes (HPRT, Cyclophilin and GAPDH) were used as controls. RT-PCR was performed on an ABI-PrismTM7700 system (Perkin-Elmer Biosystems, Boston, USA) using RT-PCR Mastermix (Eurogentec, Seraing, Belgium). For each time-point RT-PCR was performed in duplicate and the signals were averaged and corrected using the average signal of the housekeeping gene (∆Ct). ∆∆Ct was defined as difference between ∆Ct value of healthy and cuffed femoral artery. Data are presented as fold induction, calculated as 2-∆∆Ct.

ApoE*3-LeidenTNFα-/- mice
Experimental mice (ApoE*3-LeidenTNFα-/- and control littermate ApoE*3-LeidenTNFα+/- mice) were obtained by crossbreeding TNFα-/- with ApoE*3-Leiden mice. Six male mice underwent cuff-placement.

Perivascular delivery of TNFα-inhibitor
Thalidomide was purchased from Sigma (St Louis, USA). Poly(ε-caprolactone) (PCL) based drug-delivery cuffs were manufactured as previously described.
Thalidomide was extracted from 1% Thalidomide/PCL-cuffs (w/w) (n=4) using DMSO (Merck, Darmstadt, Germany) and quantified spectrophotometrically before and 14d after in vivo placement at 295nm, the specified wavelength for thalidomide. The total release was quantified.\(^{(18)}\)

Murine femoral artery was dissected from its surroundings and an empty or a 1% thalidomide/PCL-cuff (w/w) was placed loosely around it (n=6).\(^{(16)}\) The TNF\(\alpha\) presence in the vessel wall was visualized by immunohistochemistry using antibodies against TNF\(\alpha\) (1:100, Abcam, Cambridge, UK).

Statistical analysis
Statistical analysis was performed using SPSS-11.5. Continuous variables were expressed as mean±SD and compared by unpaired, two-sided Student t-test. Discrete variables are expressed as counts or percentages and were compared with the Chi-square test. Deviations of genotype distribution from that expected for a population in Hardy-Weinberg equilibrium were tested using Chi-squared tests with one degree of freedom. Allele frequencies were determined by gene counting, the 95% confidence intervals of allele frequencies were calculated from sample allele frequencies, based on the approximation of binomial and normal distributions in large sample sizes.

In the first stage, association between TNF\(\alpha\)-polymorphisms and TVR was assessed using the Cox proportional regression model under a co-dominant genetic model without adjustments for covariates so that we could assess their possible involvement in the causal pathway. All polymorphisms were also assessed using dominant and recessive models, and the model with the lowest Akaike-information criterion was used in multivariable regression analysis.\(^{(19)}\) The polymorphisms were combined into haplotypes, and effect of haplotypes on restenosis risk was estimated according to methods developed by Tanck et al.\(^{(20)}\) Multivariable regression analysis of the TVR-risk was performed with the TNF-polymorphisms, using a stepwise backward selection algorithm. In the final step clinical variables associated with TVR were entered into the regression model. Animal data are presented as mean±SEM, and analyzed using the Mann-Whitney U-test. P-values < 0.05 were regarded as statistically significant.
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Results

GENDER-project

A total of 3,146 patients had a complete follow-up (99.3%) with a median duration of 9.6 months (interquartile range 3.9). Out of 3,146 patients 42 had an event in the first 30 days. These patients were excluded from further analysis, according to the protocol. Baseline characteristics of the population are shown in table 1. DNA-genotyping was successful in 3,012 patients (97%) for the multilocus assay and in 2,727 patients (87.9%) for the Taqman-procedure. Results of the remaining patients are missing, due to lack of DNA or inconclusive results. The genotypes distributions (Table 2) were consistent with the Hardy-Weinberg equilibrium (p>0.05), except for the -308G/A polymorphism, which was therefore excluded from further analysis. Allele frequencies were in concordance with previously described frequencies.\(^{(8,21)}\)

During follow-up 304 patients (9.8%) had to undergo TVR. Patients with the -238A/A genotype (R.R.=0.59, 95%CI: 0.37-0.94) and patients with the -1031C/C genotype (R.R.=0.77, 95%CI: 0.59-1.00) needed TVR less frequently. The other TNFα-polymorphisms did not show a significant association with TVR (p>0.5) (Table 2).

Table 2. Distributions and univariate analysis of TNFα polymorphisms in association with TVR

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Allele freq (%)</th>
<th>12 month TVR-rate (%)</th>
<th>Best fitting genetic model</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-244G/A</td>
<td>0.99/0.01</td>
<td>9.8% GG 0% GA/AA</td>
<td>Dominant</td>
<td>0.37</td>
</tr>
<tr>
<td>-238G/A</td>
<td>0.95/0.05</td>
<td>10.2% GG 6.3% GA/AA</td>
<td>Dominant</td>
<td>0.03</td>
</tr>
<tr>
<td>-376G/A</td>
<td>0.98/0.02</td>
<td>9.7% GG 9.0% GA 50.0% AA*</td>
<td>Additive</td>
<td>0.77</td>
</tr>
<tr>
<td>-857C/A</td>
<td>0.91/0.09</td>
<td>9.6% CC/CA 19.2% AA</td>
<td>Recessive</td>
<td>0.099</td>
</tr>
<tr>
<td>-1031T/C</td>
<td>0.80/0.20</td>
<td>10.3% TT 8.3% TC/CC</td>
<td>Dominant</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Only two patients were homozygous for the A allele
### Table 1. Demographic, clinical and lesion characteristics of patients with TVR and without TVR during the follow-up

<table>
<thead>
<tr>
<th></th>
<th>TVR (n=304)</th>
<th>No TVR (n=2,800)</th>
<th>Total (n=3,104)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>61.7 ± 10.1</td>
<td>62.2 ± 10.8</td>
<td>62.1 ± 10.7</td>
<td>0.56</td>
</tr>
<tr>
<td>BMI (kg.m⁻²)</td>
<td>26.9 ± 3.7</td>
<td>27.0 ± 3.9</td>
<td>27.0 ± 3.9</td>
<td>0.73</td>
</tr>
<tr>
<td>Male sex</td>
<td>220 (72.4%)</td>
<td>1,996 (71.3%)</td>
<td>2,216 (71.4%)</td>
<td>0.73</td>
</tr>
<tr>
<td>Diabetes</td>
<td>63 (20.7%)</td>
<td>390 (13.9%)</td>
<td>453 (14.6%)</td>
<td>0.002</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>188 (61.8%)</td>
<td>1,702 (60.8%)</td>
<td>1,890 (60.9%)</td>
<td>0.75</td>
</tr>
<tr>
<td>Hypertension</td>
<td>138 (45.4%)</td>
<td>1,121 (40.0%)</td>
<td>1,259 (40.6%)</td>
<td>0.05</td>
</tr>
<tr>
<td>Current smoker</td>
<td>62 (20.4%)</td>
<td>700 (25.0%)</td>
<td>762 (24.5%)</td>
<td>0.06</td>
</tr>
<tr>
<td>Family history of MI</td>
<td>121 (39.8%)</td>
<td>977 (34.9%)</td>
<td>1,098 (35.4%)</td>
<td>0.13</td>
</tr>
<tr>
<td>Previous MI</td>
<td>109 (35.9%)</td>
<td>1,130 (40.4%)</td>
<td>1,239 (39.9%)</td>
<td>0.12</td>
</tr>
<tr>
<td>Previous PCI</td>
<td>64 (21.1%)</td>
<td>493 (17.6%)</td>
<td>557 (17.9%)</td>
<td>0.14</td>
</tr>
<tr>
<td>Previous CABG</td>
<td>36 (11.8%)</td>
<td>340 (12.1%)</td>
<td>376 (12.1%)</td>
<td>0.97</td>
</tr>
<tr>
<td>Stable angina</td>
<td>198 (65.1%)</td>
<td>1,881 (67.2%)</td>
<td>2,079 (67.0%)</td>
<td>0.46</td>
</tr>
<tr>
<td>Multivessel disease</td>
<td>148 (48.7%)</td>
<td>1,284 (45.9%)</td>
<td>1,432 (46.1%)</td>
<td>0.26</td>
</tr>
<tr>
<td>Peripheral vessel disease</td>
<td>12 (3.9%)</td>
<td>92 (3.3%)</td>
<td>104 (3.4%)</td>
<td>0.34</td>
</tr>
<tr>
<td>Lipid lowering medication</td>
<td>171 (56.3%)</td>
<td>1,516 (54.1%)</td>
<td>1,687 (54.3%)</td>
<td>0.53</td>
</tr>
<tr>
<td>Restenotic lesions</td>
<td>27 (8.9%)</td>
<td>181 (6.5%)</td>
<td>208 (6.7%)</td>
<td>0.12</td>
</tr>
<tr>
<td>Total occlusions</td>
<td>56 (18.4%)</td>
<td>372 (13.3%)</td>
<td>428 (13.8%)</td>
<td>0.05</td>
</tr>
<tr>
<td>Type C lesion</td>
<td>94 (30.9%)</td>
<td>708 (25.3%)</td>
<td>802 (25.8%)</td>
<td>0.18</td>
</tr>
<tr>
<td>Proximal LAD</td>
<td>70 (23.0%)</td>
<td>619 (22.1%)</td>
<td>689 (22.2%)</td>
<td>0.60</td>
</tr>
<tr>
<td>RCX</td>
<td>75 (24.7%)</td>
<td>764 (27.3%)</td>
<td>839 (27.0%)</td>
<td>0.35</td>
</tr>
<tr>
<td>Residual stenosis &gt;20%</td>
<td>51 (16.8%)</td>
<td>299 (10.7%)</td>
<td>350 (11.3%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Stent length (mm)</td>
<td>10.3 (0-82)</td>
<td>13.0 (0-93)</td>
<td>15 (0-146)</td>
<td>0.80</td>
</tr>
<tr>
<td>Diameter stenosis pre-intervention</td>
<td>89% (10%)</td>
<td>89% (10%)</td>
<td>89% (10%)</td>
<td>0.83</td>
</tr>
</tbody>
</table>

BMI: body mass index, MI: myocardial infarction, LAD: left anterior descending branch of the left coronary artery, RCX: circumflex branch of the left coronary artery, MI: myocardial infarction, CABG: coronary artery bypass grafting. Age is mean ± SD; other variables are percentage of patients. *P-value, determined by Cox regression analysis

Strong linkage-disequilibrium was found between both associated polymorphisms. Therefore, we evaluated the four different haplotypes: -238G/-1031T, -238G/-1031C, -238A/-1031T and -238A/-1031C, relative frequencies of these haplotypes were 79%, 16%, 1% and 4%, respectively. Twelve month TVR-rates associated with these haplotypes were 10.1%, 8.0%, 6.6% and 0%, respectively.
Haplotypic analysis showed that patients with the -238G/-1031T haplotype had a higher risk for restenosis (RR: 1.33, 95% CI: 1.05-1.69, \(p=0.02\)), compared to the patients with the other three haplotypes. Finally, in the regression model we included patient and intervention-related characteristics that were previously found to be related to TVR-risk, such as diabetes, stenting, residual stenosis >20%, current smoking, hypertension, and total occlusion. When both the -238G/A and the -1031T/C polymorphisms were entered into the multivariable analysis, only one showed a significant association, due to their strong linkage, however, when analyzed separately both were significantly associated (Table 3).

**Table 3. Multivariable analysis of TNFα polymorphisms in association with TVR, including clinical factors**

<table>
<thead>
<tr>
<th>Clinical factor</th>
<th>RR (95% CI)**</th>
<th>P-value</th>
<th>RR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>1.62 (1.20-2.19)</td>
<td>0.001</td>
<td>1.67 (1.23-2.25)</td>
<td>0.001</td>
</tr>
<tr>
<td>Total occlusion</td>
<td>1.46 (1.07-1.99)</td>
<td>0.02</td>
<td>1.47 (1.07-2.01)</td>
<td>0.02</td>
</tr>
<tr>
<td>Residual stenosis &gt;20%</td>
<td>1.38 (0.97-1.96)</td>
<td>0.08</td>
<td>1.41 (0.98-2.02)</td>
<td>0.06</td>
</tr>
<tr>
<td>Stenting</td>
<td>0.77 (0.58-1.03)</td>
<td>0.07</td>
<td>0.75 (0.56-1.01)</td>
<td>0.06</td>
</tr>
<tr>
<td>TNFα-238A/A*</td>
<td>0.60 (0.37-0.98)</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα-1031C/C*</td>
<td></td>
<td></td>
<td>0.75 (0.57-0.98)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* Results of the multivariable regression analyses performed with either TNFα -238G/A or TNFα -1031T/C in the analysis

** RR= relative risk, 95% CI= 95% confidence interval**

Additionally, six-month follow-up angiography was performed in a predefined subpopulation (478 patients) and a significant protective association between the -238A allele and angiographic restenosis was again observed (OR: 0.08, 95% CI: 0.01-0.60, \(p=0.002\)). Angiographic restenosis rates were 22.9% for the -238G/G genotype, 2.5% for the -238G/A genotype and 0% for the -238A/A genotype. No significant association for the -1031T/C polymorphism and angiographic restenosis was found (\(p=0.35\)). The -238G/-1031T and -238G/-1031C haplotypes were associated with increased angiographic restenosis risk when compared to the -238A/-1031T and -238A/-1031C haplotypes (\(p=0.002\)).
Animal experiments

TNFα-mRNA analysis with RT-PCR in a mouse model of reactive stenosis

We studied TNFα-mRNA expression in a mouse model of reactive stenosis. At the time of cuff-placement, plasma cholesterol level was 13.9±3.6mM. TNFα-transcription was upregulated time-dependently after the induction of the stenotic process (Figure 1). TNFα-mRNA showed a peak expression 24h after vascular injury (∼5,000-fold increase) compared with control arteries, after which the signal declined. Sham-operated vessels (femoral artery prepared free, but without cuff-placement) showed similar results as untreated non-operated vessels (data not shown).

Figure 1. Fold induction of TNFα mRNA in cuff-induced reactive stenosis mouse model in time.
Neointima formation in ApoE*3-LeidenTNFα/- mice
To analyze the impact of TNFα on restenosis development we generated ApoE*3-LeidenTNFα/- and control littermate ApoE*3-LeidenTNFα+/+ mice. At surgery, plasma cholesterol level was 21.3±4.2mM. No differences were seen between both groups. 14d after cuff-placement, morphometric quantification revealed significantly less neointima formation in ApoE*3-LeidenTNFα/- than in control littermate ApoE*3-LeidenTNFα+/+ mice (1927±622 vs. 8164±2803 μm², p=0.01, Figure 2). Intima/media ratio was also reduced in TNFα-knockout than in TNFα-expressing mice (0.20±0.05 vs. 0.97±0.28, p=0.014).

**Figure 2.** For color figures see back of book.

Representative cross sections of cuffed murine femoral arteries  HPS staining, magnification 400x. Arrow indicates the inner elastic lamina. **A:** ApoE*3 LeidenTNFα+/+ mice. **B:** ApoE*3 LeidenTNFα/- mice. **C:** Total intimal area of cuffed murine femoral arteries 14 days after cuff placement mean±SEM, n=6. *, P<0.05.
Effect of thalidomide perivascular delivery on neointima formation

To assess whether local delivery of a TNFα-biosynthesis inhibitor, thalidomide, could inhibit neointima formation, PCL-cuffs were loaded with 1%(w/w) thalidomide and placed around the femoral artery of ApoE*3-Leiden mice for 14d. At surgery, plasma cholesterol level was 11.4±0.6 mM. Total extraction of encapsulated thalidomide of PCL-cuffs before and 2w after placement in animals revealed a 67% release of thalidomide in the 14-day period (37.5μg released, i.e. 33.4% still present in the cuff). Neointima formation in the thalidomide-treated group was profoundly decreased compared to the empty PCL-cuffed arteries (1,885±285 vs. 4,629±625 μm², p=0.005, Figure3).

Figure 3. For color figures see back of book.

Representative cross sections of cuffed murine femoral arteries  HPS staining, magnification 400x. Arrow indicates the inner elastic lamina. A: empty PCL cuff. B: 1% (w/w) thalidomide eluting PCL cuff. C: Total intimal area of cuffed murine femoral arteries 14 days after cuff placement mean±SEM, n=6. **, P<0.01.
Perivascular delivery of thalidomide also resulted in a lower intima/media ratio compared to the empty counterparts (0.22±0.08 vs. 0.43±0.16, p=0.005). Immunohistochemical analysis of TNFα was performed in cuffed femoral arteries of mice receiving either an empty PCL-cuff or a 1% (w/w) thalidomide-eluting PCL-cuff to demonstrate decreased TNFα-protein levels in the vessel wall. During the stenotic process, TNFα is abundantly expressed in intimal and medial tissue of cuffed femoral arteries receiving an empty PCL-cuff at 14d. Sections of cuffed femoral arteries perivascularly treated with thalidomide showed considerably less TNFα (Figure 4).

**Figure 4.** For color figures see back of book.

TNFα immunostaining of cross sections of cuffed murine femoral arteries 14 days after placement of either **A** an empty PCL or **B** a 1% (w/w) thalidomide eluting PCL cuff. Magnification 400x. Arrowheads indicate the inner elastic lamina. Arrows indicate TNFα immunostaining

**Discussion**

The present study demonstrates that TNFα is involved in the process of restenosis (defined as TVR) after PCI in humans and in murine model of reactive stenosis. TNFα is a pleiotropic proinflammatory cytokine, involved in many aspects of inflammation. Advanced human atherosclerotic lesions express low levels of TNFα in the basal state, but high levels in response to injurious stimuli. In the present study we primarily assessed the role of six different TNFα-polymorphisms in the development of restenosis. In our large prospective multicenter follow-up study we demonstrated that TNFα -238A/A and the -1031C/C geno
types have a protective association with TVR after PCI. The -238A allele also showed a significant association with angiographic restenosis. The physiological significance of the -238 G/A polymorphism has not been analyzed in detail. However, one study found a significant decrease in promoter activity of patients with the -238A allele compared to patients with the -238G allele.\(^{(23)}\) Huizinga et al. found a lower TNF\(\alpha\)-production for the -238A allele compared to the -238G allele as measured in a whole blood culture.\(^{(24)}\) Fong et al. localized a repressor site to a 25bp stretch between positions -254 and -230 in the promoter. Therefore, these authors hypothesized that -238G/A exchange could lead to increased transcriptional repression.\(^{(25)}\) For the -1031T/C polymorphism the physiological significance has not yet been clearly elucidated. However, patterns of strong linkage disequilibrium between the -1031T/C and the -863C/A polymorphisms have been described.\(^{(8;21;26)}\) In a study on the effect of the -863C/A polymorphism and serum TNF\(\alpha\) concentration, the rare -863A allele had significantly lower serum TNF\(\alpha\)-levels than the -863C allele in healthy middle-aged men.\(^{(8)}\) To further explore the effect of this gene on restenosis development, we quantified TNF\(\alpha\)-transcripts in the stenotic vessel wall in a mouse model of reactive stenosis. During the stenotic process, TNF\(\alpha\)-gene transcription was time-dependently upregulated indicating that TNF\(\alpha\)-gene expression is activated upon vascular injury and suggesting that this cytokine is involved in the development of reactive stenosis, at least in the early stages. Furthermore, we demonstrated that mice that constitutively lack TNF\(\alpha\) present a reduction in neointima formation. Finally, thalidomide, a compound known to enhance TNF\(\alpha\) mRNA degradation\(^{(19;20)}\) caused, a reduction in intimal hyperplasia. Moreover, vascular TNF\(\alpha\)-protein levels are decreased upon local thalidomide administration suggesting that the decrease in neointima formation is due to inhibition of TNF\(\alpha\) biosynthesis in the injured vessel wall. Recently, Park et al. showed a significant reduction in neointima formation and proliferative activity of VSMCs by orally administered thalidomide after carotid artery denudation in Sprague-Dawley rats.\(^{(27)}\) These results are in concordance with ours. However, they used Sprague-Dawley rats, a normolipidemic model that may be less suited to test clinical relevance. Furthermore, they delivered the drug orally whereas we applied it locally, which contributes to less side effects and higher localized action. Thalidomide may be a suitable candidate for locally applied anti-restenosis therapy through the development of a thalidomide-eluting stent. Thalidomide teratogenic side effects, previously reported on this drug, may be of less importance since patients undergoing PCI are usually beyond the reproductive age and the drug is applied locally and in low concentration.
Limitations of the study
We made use of an atherosclerotic mouse model to study the effect of TNFα on restenosis, in this model we were not able to test the TNFα-polymorphisms found in humans. However, we believe that this model contributes to better understanding of the involvement of TNFα in restenosis. Furthermore, although mice studies can be used for the analysis, it should be realized that perivascular cuff-placement result initially in adventitial injury whereas in patients PCI results in intimal injury. It is not certain to what extent these apparently different ways of vascular injury differ in their reaction regarding vascular activation and the resulting intimal hyperplasia.
Another possible limitation of our study is the lack of plasma TNFα-data. However, we believe that plasma determinations are of little additive value, since pre-PCI plasma measurements of the protein do not reflect the genetically determined differences in reaction to a trauma such as PCI. Also it is conceivable that local differences in reactions are not represented systemically. In humans it is nearly impossible to measure gene products in the vessel wall locally in the acute phase after treatment.
Finally, the TNFα-gene is localized on chromosome six and belongs to the MHC Class III region. Its proximity to the MHC Class I and II raises the possibility that variations within the TNFα-locus are present because of linkage-disequilibrium with the MHC.

Conclusions
Taken together, we demonstrated a role of TNFα on restenosis development. Genetic variants in the TNFα-gene explain differences with regard to restenosis susceptibility after PCI. Therefore, when these results are confirmed in other studies, screening patients for this genotype could lead to a better risk stratification of patients at increased risk for restenosis and thereby individualize treatment, for instance by a drug-eluting stent strategy.

Sources of support that require acknowledgement:
P.S. Monraats and Dr. W. R. P. Agema are supported by grant 99.210 from the Netherlands Heart Foundation and a grant from the Interuniversity Cardiology Institute of the Netherlands ICIN.
N. M. M. Pires is supported by a Netherlands Heart Foundation grant, 2001T32.
A. Schepers and Dr. P. H. A. Quax Established Investigator are supported by the Molecular Cardiology Program of the Netherlands Heart Foundation M93.001.
Dr. J. W. Jukema is an Established Clinical Investigator of the Netherlands Heart Foundation 200D032.
We thank S. Cheng, L. Steiner and their colleagues at Roche Molecular Systems (Alameda, USA) for developing and providing their multilocus genotyping assays under a research collaboration.
Furthermore, we would like to thank Laura de Jong for her assistance with the Taqman analysis and Paul Schiffer from the University of Maastricht for assistance with the genotyping assay.
The contribution of the members of the clinical event committee, J.J. Schipperheyn MD PhD, J.W. Viersma MD PhD, D. Düren MD PhD, and J. Vainer MD is greatly acknowledged.
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