High throughput mRNA profiling highlights associations between myocardial infarction and aberrant expression of inflammatory molecules in blood cells

Stephanie Bezzina Wettinger, Carine J. M. Doggen, C. Arnold Spek, Frits R. Rosendaal, and Pieter H. Reitsma

Studies on the role of inflammation in cardiovascular disease focus on surrogate markers like plasma levels of C-reactive protein or interleukins that are affected by several factors. In this study we employ an approach in which the inflammatory mRNA profile of leucocytes is measured directly in a multigene system. We investigated the mRNA profile for 35 inflammatory markers in blood samples in a case-control study including 524 men with a history of myocardial infarction and 628 control subjects. Compared with controls, patients showed mRNA profiles with increased levels of most inflammatory mRNAs. The 2 most prominent mRNA risk indicators encoded the secreted protein macrophage migration inhibitory factor (crude odds ratio [OR], 3.4 for the highest quartile versus the lowest quartile (95% confidence interval [CI95], 2.3-4.9), and the intracellular regulator proteinase inhibitor 9 (OR, 2.5 for the highest versus the lowest quartile (CI95, 1.8-3.5), both showing an increase in odds ratio with increasing quartiles. Leucocytes in the blood of patients with myocardial infarction are more active in transcription of inflammatory genes, as evidenced by mRNA profiling. These data support the hypothesis that an inflammatory response involving leucocytes plays a role in the pathogenesis of myocardial infarction. (Blood. 2005;105:2000-2006)

© 2005 by The American Society of Hematology

Introduction

Inflammation plays a key role in the pathophysiology of atherosclerosis and in the development of acute coronary events.[1] Activated leucocytes, cytokines, and chemokines are prominent features of an atherosclerotic plaque. Moreover, plasma levels of markers of inflammation such as cell adhesion molecules, cytokines, proatherogenic enzymes, and C-reactive protein (CRP) were found to predict cardiovascular events in a variety of clinical settings.[2] However surrogate markers like CRP are far removed from the actual disease process since they reflect how the liver reacts to disease in the vasculature. Therefore, the nature of a chronic systemic inflammatory state and the role of circulating leucocytes in maintaining such a state remain unclear.

The inflammatory state of leucocytes may be the byproduct of the local inflammation in the vessel wall, or it may reflect an active or latent infection that is in part responsible for the atherosclerotic process. This is supported by observations of increased neopterin and procalcitonin levels in patients with cardiovascular disease.[3-6] Whatever the mechanism, some inflammatory mediators may directly influence the atherosclerotic process in several ways. Interleukin 6, for example, lowers high density lipoprotein and alters lipoprotein metabolism,[7] and CRP may facilitate low-density lipoprotein uptake by macrophages.[8]

Plasma protein levels do not fully reflect the inflammatory signature of leucocytes in whole blood. Tissue leucocytes and endothelial cells may also contribute to the plasma levels of inflammatory markers, and many inflammatory mediators are also produced by other cell-types. To overcome these obstacles in assessing the inflammatory status of circulating leucocytes, we have developed a sensitive quantitative assay that is capable of measuring a panel of mRNA levels in large series of whole blood samples in a single reaction. The panel was composed of target genes encoding cytokines, chemokines, their receptors (as representatives of the soluble mediators of the inflammatory response), genes encoding nuclear factor κB (NFκB) pathway components (as representatives of the main intracellular signal transduction route of inflammation), tissue factor (as the inducible component of the clotting system), and genes encoding several intracellular components involved in the link between NFκB and apoptosis, a link that is considered important for the survival of immune cells. We have applied this novel high throughput technology in a large population-based case-control study on myocardial infarction to assess whether inflammatory mRNA in circulating cells is increased in patients with myocardial infarction compared to control subjects.

Patients, materials, and methods

Patients and control subjects

Patients were men consecutively diagnosed with a first myocardial infarction before the age of 70 years between January 1990 and January 1996. Two of the following 3 characteristics had to be identifiable in the discharge

From the Laboratory for Experimental Internal Medicine, Academic Medical Center, Amsterdam, the Netherlands; and the Departments of Clinical Epidemiology and Haematology, Leiden University Medical Center, Leiden, the Netherlands.

Submitted August 26, 2004; accepted October 28, 2004. Prepublished online as *Blood* First Edition Paper, November 2, 2004; DOI 10.1182/blood-2004-08-3283.

Supported by the Netherlands Heart Foundation (grant no. 92.345) and the EU Fifth Framework Improving Human Potential Program (S.B.W.).

There is no conflict of interest to report, and all costs of the study were provided by nonprofit organizations. None of the authors has an interest, directly or indirectly, in the companies from which the reagents for these studies were acquired.

Reprints: P. H. Reitsma, Laboratory for Experimental Internal Medicine, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands; e-mail: p.h.reitsma@amc.uva.nl.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

© 2005 by The American Society of Hematology

record or hospital charts to confirm acute myocardial infarction: typical chest pain, electrocardiographical changes indicative of evolving myocardial infarction, or a transient rise in cardiac enzymes to more than twice the normal upper limit. Control subjects were men without a history of myocardial infarction who had a minor orthopaedic intervention between January 1990 and May 1996 and who had received prophylactic anticoagulation treatment after this intervention. Control subjects were identified via the Leiden Anticoagulation Clinic, which serves the same region as the hospitals where the patients were recruited and which was responsible for monitoring prophylactic anticoagulation for several weeks or months after the surgery. They had not used anticoagulants for at least 6 months prior to inclusion and control subjects were frequency matched to the patients on 10-year age groups. To ensure that inflammatory reactions surrounding the cardiac event or orthopaedic intervention had subsided, subjects were included in this study at least 6 months after the date of the event, or index date. Median time between index date and blood collection was 2.8 years (range, 0.6 years to 6.3 years) for cases and control subjects alike. Details of the population-based case-control Study of Myocardial Infarctions Leiden (SMILE), which included 1206 men, are described elsewhere.[9]

Medication use and history of diabetes prior to the index date were ascertained by interview with control subjects and retrieved from discharge letters for patients. At time of the blood draw they were assessed by using a structured interview. A person was classified as hypertensive or hypercholesterolemic when he was prescribed specific medications for these conditions. The study was approved by the review committee of the Leiden University Medical Center and the subjects gave written informed consent in accordance with institutional guidelines.

RNA analysis

Morning fasting–citrated blood samples were drawn from the antecubital vein. Immediately thereafter, aliquots of 100 μL were added to 900 μL lysis buffer.[10] The samples were stored at −70°C until further use. RNA was isolated using a silica-based method[10] and analyzed by multiplex ligation–dependent probe amplification (MLPA) using a kit developed in collaboration with MRC-Holland (Amsterdam, The Netherlands) for the simultaneous detection of 38 messenger RNA molecules.[11] This MLPA profiling method is insensitive to the total amount of mRNA that is included in the reaction; therefore, the profile is independent of the total white blood cell (WBC) count. All samples were tested with the same batch of reagents, and a negative and lipopolysaccharide-stimulated control sample were included on each plate. The final polymerase chain reaction (PCR) fragments amplified with carboxyfluorescein-labeled primers were separated by capillary electrophoresis on a 16-capillary ABI-Prism 3100 Genetic Analyzer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Peak area and height were processed using GeneScan analysis software (Applied Biosystems). The levels of mRNA for each gene were expressed as a normalized ratio of the peak area divided by the peak area of a control gene, resulting in the relative abundance of mRNAs of the genes of interest. Our probe set is listed in Table 1 and contains probes for mRNAs of 35 inflammation- and apoptosis-related proteins and 3 control genes, *B2M*, *CDKN1A*, and *PARN*. Expression levels of β2 microglobulin *(B2M)* were above the upper limit of detection in all samples, whereas *CDKN1A* and *PARN* mRNA levels were detectable in all 1152 samples and in 1135 samples, respectively. Therefore, areas were normalized to *PARN* and to *CDKN1A* resulting in similar expression profiles. We have opted to present the results relative to *CDKN1A* because of its insensitivity to in vitro lipopolysaccharide stimulation of an inflammatory response[11] (C.A.S., unpublished data, November, 2002).

Statistics

Because this mRNA profiling technique was not available when the SMILE study was initiated and the samples were collected, this study was not preceded by a sample size calculation.

The relative mRNA levels of cases and control subjects were compared using a Mann-Whitney test. Persons with values above the upper detection limit were assumed to have the highest levels, whereas persons with values below the detection limit were assumed to have the lowest mRNA level for that marker. Odds ratios (ORs) were calculated as an estimate of the relative risk for myocardial infarction. Quartiles were defined on the basis of the mRNA distribution among control subjects. The lowest quartile was used as a reference category for calculating odds ratios. 95% confidence levels (CI95) were calculated according to the method by Woolf[12] or were derived from the standard errors calculated by the logistic model. For variables with more than a quarter of the readings below the lower limit of detection, odds ratios were calculated for persons with detectable levels compared with those with nondetectable levels. About three-fourths of the samples had readings for *PTP4A2* above the upper detection limit; therefore, the relative risk of myocardial infarction was estimated by calculating the odds ratio for persons with levels above the upper detection limit compared with those within the detection limits. Odds ratios were adjusted for traditional cardiovascular risk factors such as age, smoking, hypertension, hypercholesterolemia, diabetes, body mass index (BMI), alcohol use, and quartile of CRP by using unconditional logistic regression. The odds ratios were further adjusted for time between the index date and blood sampling. Adjusted odds ratios were also calculated limited to samples drawn at least 2 years after the index date. Because 185 patients stopped smoking in the interval between the time of their myocardial infarction and blood withdrawal, and smoking may influence some of the inflammatory markers, odds ratios were also calculated restricted to men who were nonsmokers both before and after the index date. Odds ratios were also adjusted for use of medication at time of blood sampling. Separate restriction analyses to include only men who were not on lipid-lowering therapy, antihypertensives, and aspirin at time of myocardial infarction and at time of blood sampling were also performed.

Results

It was possible to isolate RNA and perform MLPA analysis on 524 cases and 628 control subjects that fit the criteria required for this study. Mean age was 56.4 years (range, 32.3-70.0 years) and 57.4 years (range, 27.2-74.8 years) for patients and controls, respectively. Out of the patients, 62% were smokers compared with 33% of control subjects (Table 2).

Men with a history of myocardial infarction had higher median leukocyte levels (normalized to *CDKN1A*) of most inflammatory mRNAs compared with control subjects (Table 1). Analysis based on mRNA levels after stratification in quartiles resulted in elevated odds ratios (Table 3) that were not influenced much by adjustment for traditional risk factors and plasma levels of CRP. The adjusted odds ratios increased 2- to 3-fold for the highest versus lowest quartiles of *MIF*, *PI9*, *CCL3*, *PTPN1*, and *BMI1*. *PDE4B*, *GSTP1*, *NFKB1*, *IL12A*, *PDGFB*, *MYC*, *IL1B*, *IL8*, *NFKB1A*, and *IL15(1)* had odds ratios between 1.5 and 1.9 after adjustment, while *LTA*, *IFNG*, *IL1RN*, *TNFRSF1A*, *IL18*, and *CCL4* had adjusted odds ratios of 1.3 or 1.4. *THBS1* and *PARN* were not associated with myocardial infarction (adjusted OR [OR_{adj}] 0.9 and 1.0, respectively). The full data in quartiles are shown in Table 4 for those markers that show increasing odds ratios with increasing levels of mRNA.

Odds ratios for markers with more than a quarter of the readings below detection levels are shown in Table 5. In this comparison of detectable versus undetectable levels, *IL15(2)*, *MCP-2*, and *IL2* were associated with an approximately 2-fold increase in odds ratio. *IL12B* had an adjusted odds ratio of 1.6 and IL6, *TNF*, *IL4(2)*, and *IL10* had a somewhat lower adjusted odds ratio of 1.4. Too few samples had detectable levels of IL13 and *TF* mRNA (9 and 15 samples, respectively). *MCP-1* and *NFKB2* had an odds ratio of 1.2 (CI95, 0.9-1.6 for both) and *IL4* was not associated with myocardial infarction. *IL1A* was peculiar because it gave an odds ratio below 1, indicating a protective effect (OR, 0.6; CI95, 0.5-0.8).

Table 1. Alphabetical listing of the mRNAs and median values of cases and control subjects and their range (normalized to *CDKN1A*)

Gene symbol	Descriptive name	Patients		Controls		P
		Median	Range	Median	Range	
B2M	Beta-2-microglobulin	NA*	NA*	NA*	NA*	NA
BMI1	BMI-1 oncogene homolog	0.84	0.09-2.18	0.76	0.00-2.41	< .001
CCL3	Chemokine (C-C motif) ligand 3	0.14	0.00-0.85	0.13	0.00-1.16	< .001
CCL4	Chemokine (C-C motif) ligand 4	1.30	0.23-*	1.26	0.23-*	.57
CDKN1A	Cyclin-dependent kinase inhibitor 1A	NA	NA	NA	NA	NA
GSTP1	Glutathione S-transferase	0.31	0.00-1.36	0.29	0.00-1.05	< .001
IFNG	Interferon, gamma	0.16	0.00-0.80	0.15	0.00-1.78	.21
IL10	Interleukin 10	0.00	0.00-0.73	0.00	0.00-0.45	.006
IL12A	Interleukin 12, subunit p35	3.04	0.16-*	2.76	0.16-*	< .001
IL12B	Interleukin 12, subunit p40	0.00	0.00-0.33	0.00	0.00-1.78	.06
IL13	Interleukin 13	0.00	0.00-0.26	0.00	0.0-0.08	.96
IL15 (1)	Interleukin 15, transcript variants 1 and 3	0.48	0.13-2.59	0.46	0.16-2.52	.014
IL15 (2)	Interleukin 15, transcript variant 2	0.00	0.00-0.26	0.00	0.00-0.08	.003
IL18	Interleukin 18	0.12	0.00-1.45	0.11	0.00-0.97	.009
IL1A	Interleukin 1, alpha	0.00	0.00-0.39	0.00	0.00-0.31	< .001
IL1B	Interleukin 1, beta	0.99	0.24-*	0.87	0.14-*	< .001
IL1RN	Interleukin 1 receptor antagonist	1.90	0.54-*	1.71	0.47-*	< .001
IL2	Interleukin 2	0.07	0.00-0.52	0.03	0.00-1.19	< .001
IL4 (1)	Interleukin 4, transcript variant 1	0.00	0.00-0.31	0.00	0.00-0.22	.79
IL4 (2)	Interleukin 4, transcript variants 1 and 2	0.03	0.00-0.46	0.00	0.00-0.51	< .001
IL6	Interleukin 6	0.00	0.00-0.24	0.00	0.00-0.28	.11
IL8	Interleukin 8	1.38	0.00-*	1.30	0.00-*	.06
LTA	Lymphotoxin alpha (Tumor necrosis factor, beta)	0.25	0.00-1.08	0.23	0.00-0.82	< .001
MCP-1	Monocyte chemotactic protein, 1	0.00	0.00-8.22	0.14	0.00-0.70	.31
MCP-2	Monocyte chemotactic protein, 2	0.00	0.00-10.36	0.00	0.00-0.66	.07
MIF	Macrophage migration inhibitory factor	0.91	0.00-3.95	0.75	0.00-3.98	< .001
MYC	v-myc oncogene homolog	1.41	0.43-*	1.25	0.28-*	< .001
NFKB1	nuclear factor kappa-B, subunit 1	1.12	0.43-*	1.02	0.19-*	< .001
NFKB2	nuclear factor kappa-B, subunit 2	0.00	0.00-0.29	0.00	0.00-0.20	.049
NFKBIA	nuclear factor kappa-B inhibitor, alpha	2.98	0.65-*	2.66	0.55-*	< .001
PARN	Polyadenylate-specific ribonuclease	2.27	0.71-*	2.34	0.67-*	.41
PDE4B	Phosphodiesterase 4B, cAMP-specific	2.23	0.54-*	2.12	0.58-*	< .001
PDGFB	Platelet-derived growth factor, beta polypeptide	0.15	0.00-0.65	0.13	0.00-0.75	.002
PI9	proteinase inhibitor 9, ovalbumin type	1.81	0.24-*	1.58	0.00-*	< .001
PTP4A2	Protein-tyrosine phosphatase, type 4A, 2	NA*	NA*	NA*	NA*	NA
PTPN1	Protein-tyrosine phosphatase, nonreceptor-type, 1	0.24	0.00-0.63	0.21	0.00-0.79	< .001
TF	Tissue factor	0.00	0.00-0.13	0.00	0.00-0.07	.54
THBS1	Thrombospondin 1	0.29	0.00-1.47	0.30	0.00-1.40	.75
TNF	Tumor necrosis factor, alpha	0.00	0.00-0.32	0.00	0.00-0.21	.014
TNFRSF1A	Tumor necrosis factor receptor superfamily, 1A	2.77	1.02-*	2.57	0.77-*	< .001

P values are according to the (2-tailed) Mann-Whitney test.
NA indicates not applicable.
*mRNA levels above the detection limit.

Most of the readings for *PTP4A2* were above the upper detection limit. The OR_{adj} for persons with levels above the upper detection limit compared with those with levels within the detection limits was 1.7 (CI95, 1.2-2.3).

Few of the crude odds ratios were changed by adjustment for cardiovascular risk factors, and only 7 mRNA markers (*LTA*, *MIF*, *IL1B*, *IL1RN*, *MYC*, *NFKB1*, and *CCL4*) showed differences between the crude and adjusted odds ratios greater than 0.2. Multivariate analysis indicated that smoking accounted for most of these differences (Tables 3 and 5). A stratified analysis showed that the inflammatory markers have elevated odds ratios even in nonsmokers (Tables 3 and 5), and, in most cases, the odds ratios were higher in men who did not smoke on and after the index date even after adjustment for traditional risk factors. This was particularly striking for the highest compared with the lowest quartile of mRNA levels of *MIF* with an age-adjusted odds ratio of 5.2 (95CI, 2.9-9.5) in nonsmokers.

Analysis excluding persons with levels of CRP above 10 mg/L did not materially change the odds ratios (data not shown). Adjustment for use of medication at time of blood sampling in the logistic regression model had little effect on the odds ratios. Odds ratios remained elevated even after restriction to subjects who never used aspirin or medications for hypercholesterolemia or hypertension. Adjustment for traditional risk factors had little effect on these odds ratios. Time since the index date did not change the adjusted odds ratios for the highest quartiles, except for those of *MIF*, *PI9*, and *PTPN1*, which increased further to 3.5 (95CI, 2.3-5.2), 2.9 (95CI, 2.0-4.2), and 2.5 (95CI, 1.7-3.7), respectively (Tables 3 and 5). The odds ratios of these markers were elevated even when cases who had a myocardial infarction less than 2 years before sample collection were excluded from the analysis (*MIF*, 2.0 [1.2-3.1]; *PI9*, 2.0 [1.3-3.0]; and *PTPN1*, 2.4 [1.5-3.8]). Besides the odds ratios of *MIF* and *PI9*, in this analysis, only the odds ratios of *MYC*, *IL8*, and *IL2* decrease somewhat (Tables 3 and 5).

Table 2. Characteristics of patients and control subjects

	Patients, n = 524	Controls, n = 628
Age, y, mean (range)	56.4 (32.3-70.0)	57.4 (27.2-74.8)
Current smokers, no. (%)	323 (61.6)	209 (33.3)
Alcohol users, no. (%)	420 (80.2)	543 (86.5)
Obesity no. (%)*	90 (17.2)	104 (16.6)
BMI, kg/m^2, mean (range)*	27.1 (17.3-45.8)	26.9 (17.1-40.6)
Diabetes, no. (%)	23 (4.4)	21 (3.3)
Hypertension, no. (%)†	97 (18.5)	101 (16.1)
Hypercholesterolemia, no. (%)†	12 (2.3)	10 (1.6)

*A person was defined as obese if his BMI exceeded 30 kg/m^2. Data on height and weight were not available for 2 people.

†A person was classified as hypertensive or hypercholesterolemic if he was taking prescription drugs for these conditions.

Discussion

Patients with a history of myocardial infarction have a different mRNA signature for inflammatory markers than healthy control subjects, with higher expression of circulating inflammatory RNA. More specifically, increased mRNA levels of *MIF*, *PI9*, *CCL3*, *PTPN1*, *BMI1*, and *IL15(2)* were found to be associated with myocardial infarction, with odds ratios in the range of 2 to 3 for patients compared with control subjects. Elevated mRNA levels of *PDGFB*, *IL12A*, *IL12B*, *MYC*, *NFKB1*, *GSTP1*, *IL18*, *IL15(2)*, *IL1B*, *IL1RN*, *IL8*, *NFKBIA*, *PDE4B*, *MCP-2*, and *IL2* all yielded odds ratios for myocardial infarction varying between 1.5 and 2.

The highest odds ratio was observed for patients with high mRNA levels of macrophage migration inhibitory factor *(MIF)*. Recently, this protein has been associated with an array of autoimmune and inflammatory diseases including severe sepsis,[13] arthritis,[14] bronchial asthma,[15] and acute respiratory distress syndrome.[16,17] *MIF* counteracts the immunosuppressive effects of glucocorticoids,[18] and prolongs the inflammatory response by inhibiting apoptosis of macrophages.[19] Its plasma levels increase up to 5-fold in the acute phase of myocardial infarction, but decrease to levels similar to those in healthy persons within 3 weeks. The rapid increase is thought to be due to the production or release of *MIF* by necrotic tissue in the heart, cellular infiltrate at local sites, and peripheral blood mononuclear cells.[20,21] These studies were performed on a small number of patients and controls, and aimed at the inflammatory response during and shortly after the myocardial event. The major source of *MIF* may differ in the acute and subacute stages of myocardial infarction.[21] Our study shows that expression of *MIF* in circulating cells is higher in men with myocardial infarction even in the stable state long after the event.

PI9 was also prominently associated with myocardial infarction. This protein protects cells from apoptosis due to granzyme B released by cytotoxic lymphocytes to kill abnormal cells.[22] It also inhibits the conversion of the inactive precursors of IL1B and IL18 into the active forms. Its involvement in atherosclerosis

Table 3. Odds ratios for highest versus lowest quartile

Marker	Patients N = 524‡	Crude OR	OR adjusted for age and smoking	Adjusted OR*	OR for nonsmokers only, age corrected N = 617	OR adjusted for traditional risk factors and for time since the event	Adjusted OR* for samples collected more than 2 years after the event N = 852	OR adjusted for traditional risk factors and for use of medication at time of sample collection
MIF	193	3.4 (2.3-4.9)	3.1 (2.1-4.5)	3.0 (2.0-4.4)	5.2 (2.9-9.5)	3.5 (2.3-5.2)	2.0 (1.2-3.1)	3.2 (2.1-4.9)
PI9	195	2.5 (1.8-3.5)	2.5 (1.8-3.6)	2.6 (1.8-3.8)	3.1 (1.8-5.3)	2.9 (2.0-4.2)	2.0 (1.3-3.0)	2.7 (1.8-4.1)
CCL3	183	2.2 (1.5-3.0)	2.2 (1.5-3.1)	2.3 (1.6-3.3)	2.8 (1.7-4.6)	2.3 (1.6-3.3)	2.5 (1.6-3.8)	2.4 (1.6-3.5)
PTPN1	170	2.3 (1.6-3.3)	2.2 (1.5-3.2)	2.2 (1.5-3.2)	3.6 (2.1-6.2)	2.5 (1.7-3.7)	2.4 (1.5-3.8)	2.2 (1.4-3.2)
BMI1	159	1.9 (1.3-2.7)	1.8 (1.3-2.6)	2.0 (1.4-2.9)	1.7 (1.0-2.8)	2.1 (1.4-3.0)	1.9 (1.3-3.0)	2.0 (1.3-3.0)
PDE4B	162	1.9 (1.3-2.6)	1.9 (1.3-2.7)	1.9 (1.3-2.8)	2.7 (1.6-4.6)	2.2 (1.5-3.2)	1.9 (1.2-2.9)	1.9 (1.3-2.8)
GSTP1†	162	1.9 (1.3-2.6)	1.7 (1.2-2.4)	1.8 (1.2-2.5)	1.7 (1.0-2.8)	1.6 (1.1-2.4)	1.9 (1.2-2.9)	1.9 (1.2-2.8)
NFKB1	182	2.0 (1.4-2.8)	1.7 (1.2-2.5)	1.7 (1.2-2.4)	2.0 (1.2-3.3)	1.8 (1.2-2.6)	1.5 (1.0-2.3)	1.4 (1.0-2.1)
IL12A	165	1.8 (1.3-2.5)	1.7 (1.2-2.5)	1.7 (1.2-2.4)	2.2 (1.3-3.6)	1.7 (1.2-2.4)	1.9 (1.3-3.0)	1.6 (1.1-2.4)
PDGFB	156	1.6 (1.1-2.2)	1.6 (1.1-2.3)	1.7 (1.2-2.4)	1.3 (0.8-2.2)	1.8 (1.2-2.6)	1.5 (0.9-2.3)	1.7 (1.2-2.6)
MYC	182	2.0 (1.4-2.8)	1.5 (1.1-2.2)	1.6 (1.1-2.3)	1.6 (1.0-2.6)	1.5 (1.1-2.2)	1.1 (0.7-1.7)	1.4 (0.9-2.0)
IL1B	193	2.0 (1.5-2.9)	1.8 (1.2-2.5)	1.6 (1.1-2.3)	2.7 (1.6-4.5)	1.6 (1.1-2.2)	1.7 (1.1-2.6)	1.7 (1.2-2.5)
IL8	152	1.6 (1.1-2.2)	1.5 (1.1-2.1)	1.6 (1.1-2.3)	2.0 (1.2-3.3)	1.7 (1.2-2.4)	1.2 (0.8-1.9)	1.6 (1.1-2.4)
NFKBIA	174	1.7 (1.2-2.3)	1.6 (1.1-2.2)	1.5 (1.0-2.1)	1.9 (1.2-3.1)	1.4 (1.0-2.0)	1.3 (0.9-2.0)	1.6 (1.1-2.3)
IL15(1)	140	1.4 (1.0-2.0)	1.5 (1.0-2.1)	1.5 (1.0-2.2)	1.5 (0.9-2.6)	1.5 (1.0-2.1)	1.4 (0.9-2.1)	1.3 (0.8-1.9)
LTA	163	1.7 (1.2-2.3)	1.3 (0.9-1.9)	1.4 (1.0-2.0)	1.4 (0.8-2.3)	1.3 (0.9-2.0)	1.4 (0.9-2.1)	1.4 (1.0-2.1)
IFNG	146	1.3 (0.9-1.8)	1.4 (1.0-2.0)	1.4 (1.0-2.1)	1.5 (0.9-2.4)	1.4 (1.0-2.0)	1.4 (0.9-2.2)	1.4 (1.0-2.1)
IL1RN	160	1.7 (1.2-2.3)	1.4 (1.0-2.0)	1.3 (0.9-1.9)	1.4 (0.8-2.3)	1.3 (0.9-1.9)	1.3 (0.8-1.9)	1.1 (0.8-1.7)
TNFRSFIA	153	1.4 (1.0-2.0)	1.4 (1.0-1.9)	1.3 (0.9-1.8)	1.9 (1.2-3.1)	1.3 (0.9-1.8)	2.0 (1.3-3.0)	1.2 (0.8-1.8)
IL18	134	1.3 (0.9-1.8)	1.3 (0.9-1.8)	1.3 (0.9-1.9)	1.5 (0.9-2.4)	1.3 (0.9-1.9)	2.0 (1.3-3.2)	1.3 (0.9-2.0)
CCL4	135	1.0 (0.7-1.4)	1.2 (0.9-1.8)	1.3 (0.9-1.9)	0.9 (0.6-1.5)	1.3 (0.9-1.8)	1.5 (1.0-2.3)	1.5 (1.0-2.2)
THBSI	126	0.9 (0.6-1.2)	0.9 (0.7-1.3)	0.9 (0.6-1.3)	0.7 (0.5-1.2)	1.0 (0.7-1.4)	1.2 (0.8-1.8)	0.9 (0.6-1.4)
PARN	117	0.8 (0.6-1.2)	1.0 (0.7-1.4)	1.0 (0.7-1.5)	0.7 (0.4-1.1)	1.0 (0.7-1.4)	1.3 (0.8-1.9)	1.0 (0.7-1.5)

Number of cases in the highest quartiles are shown in column 2. Results of a stratified analysis on men who were nonsmokers both at the index date and the sampling date are shown in column 6 after adjustment for age. Subsequent columns show odds ratios adjusted for traditional risk factors and for time since the event (column 7), adjusted odds ratios using only samples collected more than 2 years after the event (column 8), and adjustment for use of medication at time of sample collection (last column). 95% confidence intervals are shown in parentheses.

*Adjusted for age, smoking, use of medication for hypertension and hypercholesterolemia, diabetes, BMI, alcohol habit, and quartile of CRP.

†Out of 518 cases and 597 controls.

‡The number of controls in each quartile is 157 except for *GSTP1*. These numbers apply when restrictions are not used.

Table 4. Odds ratios with increasing quartiles of mRNA levels for a selection of markers

Inflammatory marker	Quartile	Patients N = 524	Controls N = 628	Crude OR	Adjusted OR*
MIF	1	57	157	1	1
	2	131	157	2.3 (1.6-3.4)	2.1 (1.4-3.2)
	3	143	157	2.5 (1.7-3.7)	2.4 (1.6-3.6)
	4	193	157	3.4 (2.3-4.9)	3.0 (2.0-4.4)
PI9	1	78	157	1	1
	2	106	157	1.4 (1.0-2.0)	1.4 (1.0-2.1)
	3	145	157	1.9 (1.3-2.6)	1.8 (1.2-2.6)
	4	195	157	2.5 (1.8-3.5)	2.6 (1.8-3.8)
PTPN1	1	73	157	1	1
	2	122	157	1.7 (1.2-2.4)	1.7 (1.1-2.4)
	3	159	157	2.2 (1.5-3.2)	2.0 (1.4-2.9)
	4	170	157	2.3 (1.6-3.3)	2.2 (1.5-3.2)
PDGFB	1	98	157	1	1
	2	124	157	1.3 (0.9-1.8)	1.3 (0.9-1.9)
	3	146	157	1.5 (1.1-2.1)	1.5 (1.0-2.1)
	4	156	157	1.6 (1.1-2.2)	1.7 (1.2-2.4)
IL12A	1	92	157	1	1
	2	126	157	1.4 (1.0-1.9)	1.3 (0.9-1.9)
	3	141	157	1.5 (1.1-2.2)	1.4 (1.0-2.1)
	4	165	157	1.8 (1.3-2.5)	1.7 (1.2-2.4)
NFKB1	1	90	157	1	1
	2	108	157	1.2 (0.8-1.7)	1.2 (0.8-1.7)
	3	144	157	1.6 (1.1-2.3)	1.4 (1.0-2.1)
	4	182	157	2.0 (1.4-2.8)	1.7 (1.2-2.4)
MYC	1	91	157	1	1
	2	96	157	1.0 (0.7-1.5)	1.0 (0.7-1.4)
	3	155	157	1.7 (1.2-2.4)	1.5 (1.0-2.1)
	4	182	157	2.0 (1.4-2.8)	1.6 (1.1-2.3)
LTA	1	97	157	1	1
	2	107	157	1.1 (0.8-1.6)	1.0 (0.7-1.5)
	3	157	157	1.6 (1.2-2.3)	1.3 (0.9-1.9)
	4	163	157	1.7 (1.2-2.3)	1.4 (1.0-2.0)

95% confidence intervals are shown in parentheses. The 1st quartile is always the reference category.

*Adjusted for age, smoking, use of medication for hypertension or hypercholesterolemia, diabetes, BMI, alcohol habit, and quartile of CRP.

has been suggested by its altered expression in atherosclerotic lesions.[23] The present finding of increased expression in the circulation of men with a history of myocardial infarction adds evidence to this and suggests that it may exert an influence outside the plaque itself.

The absence of a noticeable effect of adjustment or of restrictions to people on particular groups of medication shows that these associations are not brought about by traditional risk factors or by medication use. The only cardiovascular risk factor that had an effect on some of the odds ratios (*LTA*, *MIF*, *IL1B*, *IL1RN*, *MYC*, *NFKB1*, and *CCL4*) was smoking. This finding is not surprising since it is generally accepted that smoking enhances vascular inflammation, and some systemic inflammatory markers, including CRP, are higher in smokers than former or never smokers.[24,25] The results of the stratified analysis on nonsmokers show that the inflammatory markers have an effect themselves, and that smoking masked some of this effect.

Adjustment for levels of CRP did not change the odds ratios. This underlines that in cardiovascular disease inflammation plays a role in 3 compartments. The first is in the inflamed vascular wall where many inflammatory cells are known to accumulate. The second is in the liver where acute phase reactants such as CRP are synthesized, most likely in response to cytokines that are produced elsewhere in the body. The results from our study indicate that there is a third inflammatory compartment in circulating leucocytes. Apparently, the relationship between events in leucocytes on the one hand and acute-phase protein production in the liver on the other, if there is any, is not a simple one.

In quantitative or semiquantitative mRNA profiling methods accuracy is generally poor. As described in our original methods paper reproducibility of the MLPA using independent duplicate samples was satisfactory: interassay correlation between 3 representative data sets of independent samples was 0.96 and intra-assay variation between 4 independent samples was 0.97.[11] With respect to the reproducibility of the presented profiling results we would like to add the following. Based on the repeat measurements of the lipopolysaccharide samples in the present study we find coefficients of variation for individual mRNA species of around 0.25. Second, we recently measured a second mRNA profile, centered on the expression of members of the Toll-like receptor family of proteins, in the SMILE samples (S.B.W., P.R.H., unpublished results, April 2004). *PI9*, *MIF*, and *PARN* were also included in the second study, and we were able to replicate the findings reported here.

Our study evaluated mRNA-based profiles in a case-control design. The inflammatory profile that we have observed may reflect a chronic inflammatory state in circulating blood cells that is predictive for myocardial infarction. Alternatively, it may be a result of the myocardial infarction itself either directly, since several of the mRNA markers that we assessed may also increase during the acute phase of a myocardial infarction (*TNFA*,[26] *IL6*[27]; *IL1RN* and *IL10*[28]; *MIF*[20,21]), or indirectly through decreased left ventricular function in myocardial infarction patients,[29] a variable that was not assessed in the SMILE study. Support for the hypothesis that we may indeed be dealing with causative risk factors comes from the observation that the increased odds ratios were observed in blood samples drawn at least 6 months (median, 2.8 years) after the index date, and from the observation that time since the event did not decrease the odds ratios. In any case, an inflammatory state of cells in the circulation may well have implications on the outcome and progress of inflammation-related diseases.

After the human genome effort, gene expression profiling is rapidly developing into a powerful tool. It has shown promising results in identifying patterns of aberrant expression in cancer patients, and in determining subtypes of leukemias.[30] These studies have been based on microarray techniques that, due to their cost, have limited analysis to typically tens of samples. To study more complex diseases a substantially larger number of samples need to be analyzed to get reproducible and meaningful results. The present RNA profiling study utilizes more than 1000 samples to study myocardial infarction, the largest number of samples for such a study to date. This greatly reduces noise and effects of factors not directly related to the disease and it also decreases the chances of false positives, which is a major problem in microarray studies.

In conclusion, this study presents the direct measurement of molecular signatures from more than 30 inflammatory genes in almost 1200 individuals. These large-scale mRNA measurements give direct insight into the inflammatory status of circulating leucocytes without the limitations of the more common measurements of CRP and cytokine/chemokine levels in blood or other smaller RNA studies. Leucocytes in the blood of patients with myocardial infarction are more active in transcription of inflammatory genes. Our results therefore

Table 5. Odds ratios for markers with readings above or below the detection limits

Marker	Patients N = 524	Controls N = 628	Crude OR	OR adjusted for age and smoking	Adjusted OR*	OR for nonsmokers only, age corrected N = 617	OR adjusted for traditional risk factors and for time since the event	Adjusted OR* samples collected more than 2 years after the event N = 852	OR adjusted for traditional risk factors and for use of medication at time of sample collection
Detectable versus nondetectable levels of relative mRNA									
IL15(2)	42	25	2.1 (1.3-3.5)	2.2 (1.3-3.7)	2.2 (1.3-3.7)	2.7 (1.4-5.5)	2.1 (1.2-3.6)	2.4 (1.4-4.4)	2.3 (1.2-4.1)
MCP-2	31	23	1.7 (1.0-2.9)	1.7 (1.0-3.1)	1.8 (1.0-3.2)	2.1 (1.0-4.5)	2.1 (1.1-3.7)	2.0 (1.1-3.8)	2.0 (1.0-3.8)
IL2	334	316	1.7 (1.4-2.2)	1.7 (1.3-2.1)	1.7 (1.3-2.2)	2.0 (1.4-2.8)	1.9 (1.5-2.5)	1.2 (0.9-1.6)	1.9 (1.5-2.6)
IL12B	57	48	1.5 (1.0-2.2)	1.5 (1.0-2.4)	1.6 (1.1-2.5)	1.5 (0.8-2.6)	1.7 (1.1-2.6)	1.5 (0.9-2.5)	1.6 (1.0-2.6)
IL6	49	43	1.4 (0.9-2.1)	1.4 (0.9-2.1)	1.4 (0.9-2.3)	1.4 (0.7-2.6)	1.4 (0.9-2.2)	1.8 (1.0-3.0)	1.9 (1.1-3.2)
TNF	76	62	1.5 (1.1-2.2)	1.5 (1.0-2.1)	1.4 (1.0-2.1)	1.7 (1.0-2.9)	1.5 (1.0-2.2)	1.6 (1.0-2.4)	1.4 (0.9-2.1)
IL4(2)	266	248	1.6 (1.3-2.0)	1.5 (1.2-1.9)	1.4 (1.1-1.8)	1.7 (1.2-2.4)	1.5 (1.1-1.9)	1.4 (1.0-1.9)	1.4 (1.1-1.8)
IL10	58	42	1.7 (1.1-2.6)	1.5 (1.0-2.3)	1.4 (0.9-2.1)	2.3 (1.2-4.4)	1.4 (0.9-2.1)	1.3 (0.8-2.2)	1.2 (0.7-1.9)
IL13	4	5	1.0 (0.3-3.6)	1.0 (0.2-3.9)	1.2 (0.3-5.0)	0.5 (0.1-4.9)	1.2 (0.3-5.0)	0.4 (0.0-4.0)	2.1 (0.4-10.1)
TF	8	7	1.4 (0.5-3.8)	1.2 (0.4-3.4)	1.2 (0.4-3.7)	0.4 (0.0-3.7)	1.2 (0.4-3.6)	1.1 (0.3-3.8)	1.3 (0.4-4.4)
MCP-1	120	128	1.2 (0.9-1.5)	1.1 (0.8-1.4)	1.2 (0.9-1.6)	1.0 (0.6-1.5)	1.1 (0.8-1.5)	1.3 (0.9-1.9)	1.2 (0.8-1.6)
NFKB2	152	152	1.3 (1.0-1.7)	1.2 (0.9-1.6)	1.2 (0.9-1.6)	1.4 (0.9-2.0)	1.2 (0.9-1.6)	1.4 (1.0-1.9)	1.3 (0.9-1.8)
IL4(1)	110	138	1.0 (0.7-1.3)	0.9 (0.7-1.2)	0.9 (0.7-1.2)	0.9 (0.6-1.4)	1.0 (0.7-1.3)	0.8 (0.6-1.2)	0.9 (0.6-1.2)
IL1A	189	291	0.7 (0.5-0.8)	0.6 (0.5-0.8)	0.6 (0.5-0.8)	0.5 (0.4-0.7)	0.7 (0.5-0.9)	0.5 (0.4-0.7)	0.6 (0.4-0.8)
Marker with levels above the upper detection limit versus levels within the detection limits									
PTP4A2†	428†	437†	1.8 (1.3-2.3)	1.7 (1.2-2.2)	1.7 (1.2-2.3)	1.8 (1.2-2.7)‡	1.8 (1.3-2.5)	2.1 (1.4-3.1)#	1.9 (1.4-2.6)

Results of a stratified analysis on men who were nonsmokers at the index date and sampling date are shown in column 7. Subsequent columns show odds ratios adjusted for traditional risk factors and for time since the event (column 8), adjusted odds ratios using only samples collected more than 2 years after the event (column 9), and adjustment for use of medication at time of sample collection (last column). 95% confidence intervals are shown in parentheses.

*Adjusted for age, smoking, use of medication for hypertension or hypercholesterolemia, diabetes, BMI, alcohol habit, and quartile of CRP.

†Out of 518 cases and 599 controls.

‡Out of 592 samples.

#Out of 841 samples.

represent a further step in demonstrating that inflammation in activated leukocytes is a hallmark in the etiology of cardiovascular disease.

Acknowledgements

S.B.W. and C.A.S. were responsible for setting up the RNA assays. S.B.W. performed all laboratory analyses. F.R.R. and C.J.M.D. were responsible for setting up the SMILE study and for collecting all patient data and blood samples. S.B.W. performed all statistical data analyses, and F.R.R. and C.J.M.D. oversaw these. P.H.R. and F.R.R. initiated the study and carry overall responsibility for the laboratory work and for the writing of the paper. P.H.R. and F.R.R. also take responsibility for the integrity of the work as a whole, from inception to published article.

We would like to thank Hella Aberson for her help in developing and establishing the MLPA technique, Ank Ververs-Schreijer for data management, and Thea Visser-Oppelaar for technical help in the SMILE study. A special thanks goes to all the men who consented to participate in this study.

References

1. Libby P. Inflammation in atherosclerosis. Nature. 2002;420:868-874
2. Blake GJ, Ridker PM. Inflammatory bio-markers and cardiovascular risk prediction. J Intern Med. 2002;252:283-294.
3. Weiss G, Willeit J, Kiechl S, et al. Increased concentrations of neopterin in carotid atherosclerosis. Atherosclerosis. 1994;106:263-271.
4. Schumacher M, Eber B, Tatzber F, Kaufmann P, Esterbauer H, Klein W. Neopterin levels in patients with coronary artery disease. Atherosclerosis. 1992;94:87-88.
5. Tatzber F, Rabl H, Koriska K, et al. Elevated serum neopterin levels in atherosclerosis. Atherosclerosis. 1991;89:203-208.
6. Assicot M, Gendrel D, Carsin H, Raymond J, Guilbaud J, Bohuon C. High serum procalcitonin concentrations in patients with sepsis and infection. Lancet. 1993;341:515-518.
7. Ettinger WH, Jr., Sun WH, Binkley N, Kouba E, Ershler W. Interleukin-6 causes hypocholesterolemia in middle-aged and old rhesus monkeys. J Gerontol A Biol Sci Med Sci. 1995;50:M137-M140.
8. Zwaka TP, Hombach V, Torzewski J. C-reactive protein-mediated low density lipoprotein uptake by macrophages: implications for atherosclerosis. Circulation. 2001;103:1194-1197.
9. Doggen CJ, Berckmans RJ, Sturk A, Manger Cats V, Rosendaal FR. C-reactive protein, cardiovascular risk factors and the association with myocardial infarction in men. J Intern Med. 2000; 248:406-414.
10. Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, van der Noorda J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol. 1990;28:495-503.
11. Spek CA, Verbon A, Aberson H, et al. Treatment with an anti-CD 14 monoclonal antibody delays and inhibits lipopolysaccharide-induced gene expression in humans in vivo. J Clin Immunol. 2003; 23:132-140.
12. Woolf B. On estimating the relation between blood group and disease. Ann Hum Genet. 1955; 19:251-253.
13. Lehmann LE, Novender U, Schroeder S, et al. Plasma levels of macrophage migration inhibitory factor are elevated in patients with severe sepsis. Intensive Care Med. 2001;27:1412-1415.
14. Meazza C, Travaglino P, Pignatti P, et al. Macrophage migration inhibitory factor in patients with juvenile idiopathic arthritis. Arthritis Rheum. 2002; 46:232-237.
15. Yamaguchi E, Nishihira J, Shimizu T, et al. Macrophage migration inhibitory factor (MIF) in bronchial asthma. Clin Exp Allergy. 2000;30:1244-1249.
16. Donnelly SC, Haslett C, Reid PT, et al. Regulatory role for macrophage migration inhibitory factor in acute respiratory distress syndrome. Nat Med. 1997;3:320-323.
17. Lai KN, Leung JC, Metz CN, Lai FM, Bucala R, Lan HY. Role for macrophage migration inhibitory factor in acute respiratory distress syndrome. J Pathol. 2003;199:496-508.
18. Calandra T, Bucala R. Macrophage migration inhibitory factor: a counter-regulator of glucocorticoid action and critical mediator of septic shock. J Inflamm. 1995;47:39-51.
19. Hudson JD, Shoaibi MA, Maestro R, Carnero A,

Hannon GJ, Beach DH. A proinflammatory cytokine inhibits p53 tumor suppressor activity. J Exp Med. 1999;190:1375-1382.

20. Yu CM, Lau CP, Lai KW, Huang XR, Chen WH, Lan HY. Elevation of plasma level of macrophage migration inhibitory factor in patients with acute myocardial infarction. Am J Cardiol. 2001;88:774-777.
21. Takahashi M, Nishihira J, Shimpo M, et al. Macrophage migration inhibitory factor as a redox-sensitive cytokine in cardiac myocytes. Cardiovasc Res. 2001;52:438-445.
22. Hirst CE, Buzza MS, Bird CH, et al. The intracellular granzyme B inhibitor, proteinase inhibitor 9, is up-regulated during accessory cell maturation and effector cell degranulation, and its overexpression enhances CTL potency. J Immunol. 2003;170:805-815.
23. Young JL, Sukhova GK, Foster D, Kisiel W, Libby P, Schonbeck U. The serpin proteinase inhibitor 9 is an endogenous inhibitor of interleukin 1beta-converting enzyme (caspase-1) activity in human vascular smooth muscle cells. J Exp Med. 2000; 191:1535-1544.
24. Mendall MA, Patel P, Asante M, et al. Relation of serum cytokine concentrations to cardiovascular risk factors and coronary heart disease. Heart. 1997;78:273-277.
25. Bermudez EA, Rifai N, Buring J, Manson JE, Ridker PM. Interrelationships among circulating interleukin-6, C-reactive protein, and traditional cardiovascular risk factors in women. Arterioscler Thromb Vasc Biol. 2002;22:1668-1673.
26. Akatsu T, Nakamura M, Satoh M, Hiramori K. Increased mRNA expression of tumor necrosis factor-alpha and its converting enzyme in circulating leukocytes of patients with acute myocardial infarction. Clin Sci (Lond). 2003;105:39-44.
27. Sturk A, Hack CE, Aarden LA, Brouwer M, Koster RR, Sanders GT. Interleukin-6 release and the acute-phase reaction in patients with acute myocardial infarction: a pilot study. J Lab Clin Med. 1992;119:574-579.
28. Shibata M, Endo S, Inada K, et al. Elevated plasma levels of interleukin-1 receptor antagonist and interleukin-10 in patients with acute myocardial infarction. J Interferon Cytokine Res. 1997; 17:145-150.
29. Paulus WJ. How are cytokines activated in heart failure? Eur J Heart Fail. 1999;1:309-312.
30. Haferlach T, Kohlmann A, Kern W, Hiddemann W, Schnittger S, Schoch C. Gene expression profiling as a tool for the diagnosis of acute leukemias. Semin Hematol. 2003;40:281-295.