Molecular Cardiology

Cardioprotective Function of the Long Pentraxin PTX3 in Acute Myocardial Infarction

Monica Salio, PharmD; Stefano Chimenti, PharmD; Noeleen De Angelis, BiolD; Fabiola Molla, BiolD; Virginia Maina, BiolD; Manuela Nebuloni, MD; Fabio Pasqualini, BS; Roberto Latini, MD; Cecilia Garlanda, VetScD; Alberto Mantovani, MD

Background—Despite widespread clinical use as a prognostic marker in ischemic heart disorders, the actual pathogenetic role of the short pentraxin, C-reactive protein, has not undergone stringent genetic testing because of evolutionary divergence between mouse and humans. The long pentraxin PTX3 is conserved in evolution, is expressed in the heart under inflammatory conditions, and is a candidate prognostic marker in acute myocardial infarction. It was therefore important to assess whether PTX3 plays a pathogenetic role in acute myocardial infarction.

Methods and Results—In a model of acute myocardial infarction caused by coronary artery ligation and reperfusion, tissue mRNA expression and circulating levels of PTX3 increased. The interleukin-1R–MyD88 pathway plays a pivotal role in the induction of PTX3 transcript after ischemia. ptx3-deficient mice showed exacerbated heart damage (33% larger infarcts in null mice; P = 0.0047). Increased myocardial damage in ptx3-deficient mice was associated with a greater no-reflow area, increased neutrophil infiltration, decreased number of capillaries, and increased number of apoptotic cardiomyocytes. In addition, ptx3-deficient mice with acute myocardial infarction showed higher circulating levels of interleukin-6 and increased C3 deposition in lesional tissue. The phenotype was reversed by exogenous PTX3.

Conclusions—PTX3 plays a nonredundant, regulatory, cardioprotective role in acute myocardial infarction in mice. Our results suggest that modulation of the complement cascade contributes to the cardioprotective function of PTX3.

Key Words: heart diseases • infarction • inflammation • ischemia • reperfusion

Pentraxins are a superfamily of proteins highly conserved during evolution and characterized by a multimeric, usually pentameric, structure.1–3 The classic short pentraxin, C-reactive protein (CRP), is a prototypic acute-phase protein produced in the liver in response to inflammatory signals, most prominently interleukin (IL)-6, which serves as a marker of inflammation and infection.4 Evidence has linked CRP to risk of cardiovascular events, a reflection of the involvement of inflammation in atherosclerosis and its complications.5,6

Clinical Perspective p 1064

The involvement of CRP as a pathogenetic factor in cardiovascular diseases is debated.7,8 CRP regulation and sequence have not been conserved in evolution between mouse and humans. For instance, mouse CRP does not behave as an acute-phase protein, and human CRP does not bind mouse C1q.9 The divergence of CRP between mouse and humans has hampered assessment of its in vivo function with genetic approaches and inferences as to its role in human pathology. Injection of CRP in rats increases infarct size and cardiac dysfunction, an effect blocked by a specific inhibitor.10–12 Thus, exogenous administration has provided evidence that CRP acts as an amplification loop of tissue damage in acute myocardial infarction (AMI), although genetic evidence is lacking.

The prototypic long pentraxin, PTX3, shares similarities with the classic short pentraxins, but it has an unrelated long N-terminal domain coupled to the C-terminal pentraxin domain and differs in gene organization, cellular source, and ligands recognized.3 PTX3 is rapidly produced and released by several cell types, in particular by mononuclear phagocytes, dendritic cells, fibroblasts, and endothelial cells,3 in response to primary inflammatory signals (eg, Toll-like receptor engagement, tumor necrosis factor-α, IL-1β). PTX3 is stored in a ready-made form in neutrophils, localized in specific granules, and secreted in response to recognition of microbial moieties and inflammatory signals.13 PTX3 binds
with high affinity to C1q, to the extracellular matrix components tumor necrosis factor-α-induced protein 6 and inter-α-trypsin inhibitor, to selected microorganisms, and to apoptotic cells. PTX3 may exert a dual role and contrasting effects on complement activation: It supports clearance of microbes recognized, facilitating recognition by phagocytes, whereas, on the other hand, it may protect against unwanted complement activation in the fluid phase. Recent studies in gene-modified mice have shown that PTX3 plays complex nonredundant roles in vivo, ranging from the assembly of a hyaluronic acid-rich extracellular matrix and female fertility to innate immunity against diverse microorganisms.

There is evidence linking PTX3 to ischemic heart disorders. PTX3 is induced in vascular smooth muscle cells by atherosclerotic modified low-density lipoprotein and is present in human atherosclerotic lesions. PTX3 levels increase rapidly in the AMI, reaching a peak ∼7 hours after the onset of symptoms. In a series of 748 patients with ST-elevation AMI, PTX3, measured along with such established markers as CRP, emerged as the only independent predictor of AMI, PTX3. Thus, PTX3 is a candidate new prognostic marker.

The ptx3 gene was identified in a recent study.3,16,20–22 The ptx3 gene is expressed in the heart and the lung, kidney, spleen (not shown). In permanent ischemia, expression was significantly higher in the ischemic left ventricle compared with the septum at both 8 and 24 hours after ischemia (P=0.02 and P=0.005, respectively), whereas in ischemia and reperfusion, both the ischemic ventricle and the septum expressed ptx3 mRNA (Figure 1A).

ptx3 mRNA expression also was analyzed in other tissues excised from mice undergoing heart ischemia to evaluate their involvement in ptx3 production during the systemic inflammatory response accompanying AMI, but we failed to find ptx3 induction in any of them (skeletal muscle, liver, lung, kidney, spleen) (not shown).

PTX3 plasma concentrations increased significantly starting 8 hours after permanent coronary occlusion, peaked at 24 hours, and remained >30 ng/mL up to 72 hours (Figure 1B). Concentrations in sham-operated mice were <6 to 10 ng/mL at the corresponding time points. Similarly, in the ischemia and reperfusion model, ptx3 serum levels increased significantly at 8 hours (28.17±7.8 ng/mL, mean±SE) and 24 hours (34±4.2 ng/mL) after coronary ligation compared with control mice (4.65±0.7 ng/mL; P=0.03 and P=0.005, respectively; Figure 1C).

PTX3 expression is induced in cells of the myelomonocytic lineage, in endothelial cells, and in fibroblasts by proinflammatory cytokines and Toll-like receptor ligands through nuclear factor-κB activation. To address the involvement of this pathway in ptx3 induction in heart ischemia, ptx3 mRNA levels were analyzed in IL-1RI– and MyD88-
deficient mice at 8 and 24 hours after ischemia. As shown in Figure 1D, ptx3 mRNA induction was virtually abolished in IL-1RI– and MyD88-deficient mice compared with wild-type mice at both 8 and 24 hours after ischemia.

To address which cell types produced ptx3 during cardiac ischemia, immunohistochemistry and confocal microscopy were performed (Figures 2 and 3). At 24 hours of reperfusion after ischemia, the typical changes in early myocardial necrosis were observed: loss of cellular nuclei and striation, interstitial edema, and scattered neutrophils within the widened spaces between the dead fibers. At this time point, ptx3 expression was detected around the necrotic myocardium in the ischemic and reperfused area, with an interstitial pattern of distribution (Figure 2A). In addition, ptx3 immunostaining was found in the cytoplasm of granulocytes and endothelium (Figure 2C and 2F).

At 3 days after reperfusion, the necrotic and reperfused areas were characterized by a dense inflammatory infiltrate composed largely of granulocytes and macrophages. The damaged zone was progressively replaced by a highly vascularized granulation tissue, and the necrotic myocytes were removed by phagocytosis. At this time point, a large amount of ptx3 was found in the ischemic necrotic interstitium (Figure 2B). ptx3 was detected in most of the macrophages and endothelial cells but in only a few granulocytes localized mainly in the center of the lesion. In the outer part of the damaged tissue, ptx3 staining was only extracellular (Figure 2D, 2E, 2G, 2H). Cardiomyocytes were constantly negative for ptx3. In control mice, we did not observe the expression of ptx3. Specificity of the reactivity was confirmed by the absence of staining in ptx3/H11001/H11001 mice.

PTX3 Plays a Nonredundant Role in Myocardial Infarction

A possible functional role of PTX3 in AMI was first explored by comparing infarct size 24 hours after permanent coronary ligation in ptx3+/− and ptx3−/− mice. Infarct size, measured as the ratio of TTC-negative area to the area of the whole transverse section of the heart, was 37±4% and 34±4% in ptx3−/− (n=23) and ptx3+/+ (n=26) mice, respectively (P=0.445).

Reperfusion of a previously ischemic area of myocardium triggers a marked inflammatory response that reflects a cellular response to injury and includes complement activa-
tion, oxygen free radical and proinflammatory cytokine production, and activation of neutrophils and vascular endothelium. Moreover, because restoration of coronary blood flow is standard therapy in AMI, a more clinically relevant model of cardiac ischemia/reperfusion was used. The involvement of PTX3 in ischemia/reperfusion-induced cardiac damage was investigated by measuring the infarct size as TTC-negative area 24 hours after the onset of postischemia reperfusion (Figure 4A). Although no difference was observed in the area at risk (AAR) in the 2 groups (56.7±2.8% in ptx3+/− versus 52.6±2.9% in ptx3+/+ mice; P=NS), the infarcted area (TTC-negative) normalized by the AAR was significantly greater in ptx3+/− compared with ptx3+/+ mice (36.6±3.1% and 24.4±2.0%, respectively; P=0.0047; Figure 4B). We next examined the infarcted area at a later time point, 3 and 13 days after reperfusion, measured on hematoxylin and eosin-stained sections (Figure 4A). As shown in Figure 4C and 4D, the AAR was similar in the 2 experimental
groups ($43.04 \pm 3.8\%$ in $\text{ptx3}^{+/+}$ and $47.6 \pm 63\%$ in $\text{ptx3}^{-/-}$ mice at 3 days, $37.8 \pm 3.4\%$ in $\text{ptx3}^{+/+}$ and $32.4 \pm 3.8\%$ in $\text{ptx3}^{-/-}$ mice at 13 days), whereas the infarcted area normalized by the AAR was $43.7 \pm 3.8\%$ in $\text{ptx3}^{+/+}$ mice and $57.8 \pm 5.8\%$ in $\text{ptx3}^{-/-}$ mice at 3 days and $38.3 \pm 3.4\%$ in $\text{ptx3}^{+/+}$ mice and $47.6 \pm 2.7\%$ in $\text{ptx3}^{-/-}$ mice at 13 days ($P=0.04$ and $P=0.03$, respectively). All together, these results suggest that PTX3 is not involved in modulating the infarct size induced by ischemia, whereas it plays a nonredundant role in defective reperfusion or reperfusion-induced tissue damage.

The so-called no reflow, a consequence of microvascular injury during ischemia and activated neutrophil adhesion to the vascular wall, is a major determinant of infarct expansion. To address the involvement of PTX3 in the no-reflow phenomenon, the nonreperfused area, number of capillaries, percentage of perfused capillaries in the AAR, and number of infiltrating neutrophils and macrophages in the AAR were measured (Figure 5). As illustrated in Figure 5A, which shows 1 of 2 experiments performed with similar results, the degree of no reflow, measured as nonreperfused area after intravenous injection of colored particles, was significantly greater in $\text{ptx3}^{-/-}$ mice at 24 hours of reperfusion ($21.3 \pm 1.7\%$ and $31.0 \pm 2.5\%$ of the AAR in $\text{ptx3}^{+/+}$ and $\text{ptx3}^{-/-}$ mice, respectively; 2-way ANOVA followed by Bonferroni’s post test, $P=0.043$). Capillaries in the reperfused AAR stained by lectin were $2572 \pm 286$ per $1 \text{ mm}^2$ myocardium and $1599 \pm 131$ per $1 \text{ mm}^2$ in $\text{ptx3}^{+/+}$ and $\text{ptx3}^{-/-}$ mice, respectively, at 24 hours of reperfusion ($P=0.006$; Figure 5C and 5D). Capillary density was higher in $\text{ptx3}^{-/-}$ mice than in $\text{ptx3}^{+/+}$ mice up to 13 days after myocardial ischemia ($1276 \pm 116$ and $984 \pm 44$ per $1 \text{ mm}^2$, respectively; $P=0.04$). Because endothelial cells rapidly undergo apoptosis during reperfusion, the reduced number of capillaries in the reperfused area of $\text{ptx3}^{-/-}$ mice suggests increased susceptibility to apoptosis in PTX3 deficiency. The fraction of perfused capillaries containing green particles was similar in the 2 groups after 24 hours of reperfusion ($62\%$ and $58\%$ in $\text{ptx3}^{+/+}$ and $\text{ptx3}^{-/-}$ mice, respectively; $P=0.056$), thus supporting a marginal defect in capillary reperfusion. The number of infiltrating neutrophils was higher in $\text{ptx3}^{-/-}$ than in $\text{ptx3}^{+/+}$ mice after 24 hours of reperfusion ($549 \pm 44$ and $366 \pm 41$ per $1 \text{ mm}^2$ in $\text{ptx3}^{-/-}$ and $\text{ptx3}^{+/+}$ mice, respectively, in the nonreperfused area, $P=0.009$; $436 \pm 27$ and $329 \pm 26$ per $1 \text{ mm}^2$ in $\text{ptx3}^{-/-}$ and $\text{ptx3}^{+/+}$ mice in the reperfused area, $P=0.013$; Figure 5E and 5F). Infiltrating macrophages in the infarcted area after 3 days of reperfusion

Figure 3. $\text{ptx3}$ is expressed by neutrophils, macrophages, and endothelial cells in AMI. Immunohistochemical (magnification $\times 100$) and double immunofluorescence (magnification $\times 40$) analysis of $\text{ptx3}$ expression by neutrophils (A, B, and C), macrophages (D, E, and F), and endothelial cells (G, H, and I) (arrows). $\text{ptx3}$ in brown (A, D, and G) or green (C, F, and I); GR-1 (B and C), CD68 (E and F), and CD31 (H and I) in brown (B, E, and H) or red (C, F, and I).
IL-6 was analyzed in this study as one of the markers of the inflammatory response activated by myocardial ischemia and reperfusion. IL-6 serum levels 24 hours after the onset of reperfusion were higher ($P=0.05$) in $ptx3^{-/-}$ compared with $ptx3^{+/+}$ mice ($0.5\pm0.18$ ng/mL [n=8] and $0.14\pm0.045$ ng/mL [n=10], respectively; Figure 6).

Rescue of the Phenotype

To address the specificity of the observed phenotypes in terms of no reflow and systemic inflammatory response, $ptx3^{+/+}$ and $ptx3^{-/-}$ mice were treated with human recombinant PTX3 (1 mg/kg IP 1 hour before and 4 hours after coronary ligation) and analyzed 24 hours after the onset of reperfusion. The dose was chosen on the basis of results obtained in kinetic experiments and on the pharmacological effect of 1 mg/kg PTX3 in infections. Treatment with PTX3 rescued the phenotype, abolishing the difference in nonreperfused area between $ptx3^{+/+}$ and $ptx3^{-/-}$ mice (22.5±3.5% and 22.2±2.5%, respectively; $P=0.946$) and significantly reducing the extent of damage from 31.0±2.5% in untreated $ptx3^{+/+}$ to 22.2±7.9% in treated $ptx3^{+/+}$ mice ($P=0.023$) (Figure 5B). In contrast, treatment with PTX3 of $ptx3^{+/+}$ mice did not modify the extent of no reflow compared with untreated $ptx3^{+/+}$ mice. Similarly, treatment with PTX3 significantly reduced IL-6 levels in $ptx3^{+/+}$ mice (0.17±0.08 and 0.5±0.18 ng/mL in treated and untreated mice, respectively; $P=0.05$), rescuing the phenotype (Figure 6).

Role of Complement

Complement-mediated inflammation participates in the pathogenesis of tissue injury in myocardial infarction. Furthermore, CRP bound to damaged cells may lead to complement-mediated exacerbation of tissue damage.

PTX3 can activate the classic pathway of complement activation when bound to a solid phase; however, PTX3 plays a dual role because its binding to C1q in the fluid phase prevents complement activation. It was therefore important to assess whether the increased damage observed in the absence of PTX3 correlated with complement activation. To this aim, C3 deposition in the ischemic tissue was assessed by immunofluorescence, quantified, and expressed as mean intensity per pixel in randomly selected fields of the reperfused and infarcted areas of $ptx3^{+/+}$ and $ptx3^{-/-}$ mice after 24 hours of reperfusion. Staining for C3 was localized mainly in the ischemic AAR (Figure 7). Although the fluorescence intensity in the reperfused AAR was similar in $ptx3^{+/+}$ and $ptx3^{-/-}$ mice, the fluorescence intensity in the ischemic AAR was significantly higher in $ptx3^{-/-}$ mice compared with $ptx3^{+/+}$ mice ($P=0.0006$; Figure 7B). Similar to what was observed when other parameters were assessed, treatment with recombinant PTX3 before ischemia/reperfusion significantly reduced C3 deposition in the ischemic AAR of $ptx3^{-/-}$ mice ($P=0.008$ between PTX3 treated and untreated $ptx3^{-/-}$ mice; Figure 7A and 7B).

To assess the role of complement activation in myocardial tissue damage observed in PTX3 deficiency, mice were depleted of complement with CVF before undergoing ischemia/reperfusion. After complement depletion, the area of no reflow relative to the AAR was similar in
ptx3−/− and ptx3+/+ mice (21.0±8.4% and 18.3±7.4%, respectively; P=NS; Figure 7C). The efficacy of the treatment with CVF was confirmed by immunofluorescence. No staining for C3 was observed in the left ventricle of complement-depleted mice (Figure 7A).

**Discussion**

Despite widespread interest in the measurement of pentraxins in ischemic heart disorders, there is no genetic evidence as to their actual role in vivo in the pathogenesis. In this study, we took advantage of the conservation from...
mouse to humans of the long pentraxin, PTX3, in terms of primary sequence, gene organization, and regulation. The present investigation was designed to assess the role of PTX3 in AMI, taking advantage of gene-targeted mice. It was found that ptx3-deficient mice have greater myocardial lesions in a model of coronary artery ligation/reperfusion damage. Increased tissue damage in ptx3-deficient mice was associated with a greater no-reflow area, increased neutrophil and macrophage infiltration, decreased number of capillaries, and increased number of apoptotic cardiomyocytes. Thus, PTX3 plays a nonredundant cardioprotective function in a model of AMI in mice.

Circulating ptx3 levels were elevated in mice with myocardial ischemia, a finding reminiscent of the early rise of this molecule in ischemic heart disorders in humans. Circulating ptx3 levels were elevated in mice with myocardial ischemia, a finding reminiscent of the early rise of this molecule in ischemic heart disorders in humans.25–27 Myocardial infarction was associated with induction of ptx3 mRNA in the ischemic area by endothelial cells, macrophages, and neutrophils (Figures 1 through 3). Expression of PTX3 in the heart had already been observed after systemic administration of lipopolysaccharide.31 To address the issue of inducer(s) of PTX3 mRNA in AMI, MyD88-deficient mice were used. MyD88 is an adaptor protein downstream of Toll-like receptor and IL-1R, both of which are capable of inducing PTX3.3 MyD88-deficient mice showed defective induction of ptx3. Moreover, IL-1R-deficient mice also were unable to upregulate ptx3 expression. Thus, the IL-1R–MyD88 pathway plays a primary dominant role in ptx3 mRNA induction in heart ischemia. We recently found that PTX3 is stored in specific granules and undergoes release in response to microbial recognition and inflammatory signals.3 Thus, we cannot exclude that rapid release of stored PTX3 by activated neutrophils contributes in the early phases to protein elevation after ischemia, preceding gene expression–dependent production.

ptx3-deficient mice showed no difference in infarct size in the absence of reperfusion. In ischemia and reperfusion, the inflammatory response leading to leukocyte recruitment, endothelial dysfunction, blood flow defects, and apoptosis is much higher than in permanent ischemia.32 The more striking difference in phenotype of ptx3-deficient mice is a defective reperfusion of the ischemic myocardium (no-reflow phenomenon). This can be better appreciated when reperfusion is surgically induced because spontaneous reperfusion is absent in this model and collateral circulation in the mouse is scarce. Exacerbated myocardial damage in ptx3-deficient mice was associated with higher IL-6 levels. PTX3 binds to FGF2 and inhibits FGF2-dependent activities.39 However, we observed decreased capillary density in ptx3-deficient mice up to 13 days after reperfusion. These data suggest that the decreased perfusion in PTX3 deficiency is the net result of endothelial...
cell death during ischemia/reperfusion and angiogenic response with generation of new vessels on trigger by ischemia via vascular endothelial growth factor, fibroblast growth factor-2, and other angiogenic factors. Similarly, PTX3 binds to apoptotic cells and decreases their ingestion by dendritic cells. The effect observed of increased apoptotic cardiomyocytes in PTX3-deficient hosts is likely the net result of increased cell death caused by increased inflammation and lack of perfusion versus a diminished clearance, which remains to be shown in vivo.

All together, these results suggest that PTX3 modulates the reperfusion-associated inflammation and tissue damage. Under different conditions, there is evidence for PTX3-mediated modulation of inflammation-associated tissue damage. For instance, transgenic overexpression of ptx3 resulted in increased resistance to lipopolysaccharide toxicity and cecal ligation and puncture.

PTX3 binds the complement component C1q and activates the classic pathway of complement activation when bound to immobilized ligands. The structural basis for PTX3 recognition of C1q has recently been defined. However, fluid-phase PTX3 can sequester C1q and prevent complement activation. Thus, PTX3 may exert a dual role and contrasting effects on complement activation: It supports clearance of material that is able to bind PTX3 such as microbes, whereas, on the other hand, it may protect against unwanted complement activation in the fluid phase. The short pentraxin, CRP, in addition to binding C1q, also interacts with the regulatory molecule factor H, but this possibility has not been explored yet for PTX3. Strong evidence suggests that complement components amplify ischemia-reperfusion injury by causing tissue damage and recruiting leukocytes and do so in AMI. In the present study, we found that ptx3−/− mice had increased C3 deposition in ischemic myocardium and that exogenous PTX3 reduced complement deposition. Inhibition of complement activation by fluid-phase PTX3 in vitro occurred at concentrations in the same range as those observed in blood 24 hours after reperfusion. It is therefore likely that modulation of the complement cascade contributes to the cardioprotective function of PTX3.

Despite widespread clinical use as a diagnostic in ischemic heart disorders, the actual role of CRP in the pathogenesis of heart damage is still debated. Recently, direct injection of human CRP into rats resulted in amplification of tissue injury that was blocked by an inhibitor. However, because of species-specific differences in ligand recognition and interaction with complement components, it remains unclear whether CRP is a marker of or a pathogenic component of ischemic heart disease. Indeed, the evolutionary divergence of CRP and its different regulation in mice and humans has prevented stringent genetic testing of its role in vivo. The results reported here provide the first stringent genetic demonstration of a regulatory role of a member of the pentaxin superfamily, PTX3, in AMI. The evidence for a regulatory role in the pathogenesis provides further impetus to the assessment of the clinical relevance of PTX3 measurement in ischemic heart disorders.

Sources of Funding
This study is supported by the FP6 of the European Union (Network of Excellence MUGEN LSBB-CT-2005-005203 and Network of Excellence EVGN LSMM-CT-2003-503254), Ministero dell’Istruzione, Universitá e Ricerca (project FIRB and “Piano Nazionale Ricerche–Biotecnologie Avanzate, tema 2”), Fondazione CARIPL (project Nobel), and the Italian Association for Cancer Research.

Disclosures
Dr Mantovani has filed a patent application for the use of PTX3 as a therapeutic as an affiliate of the University of Milan, Italy, and is member of the Advisory Board of Perseus Proteomics. The other authors report no conflicts.

References


---

**CLINICAL PERSPECTIVE**

The short pentraxin C-reactive protein and the long pentraxin PTX3 are markers of risk or severity in cardiovascular disorders. However, the actual role of pentraxins in pathogenesis remains unclear. Using gene-modified mice, we found that PTX3, highly conserved between mouse and humans, has a regulatory function in acute myocardial infarction. Thus, PTX3 is more than a marker in cardiovascular pathology.
Cardioprotective Function of the Long Pentraxin PTX3 in Acute Myocardial Infarction
Monica Salio, Stefano Chimenti, Noeleen De Angelis, Fabiola Molla, Virginia Maina, Manuela Nebuloni, Fabio Pasqualini, Roberto Latini, Cecilia Garlanda and Alberto Mantovani

_Circulation_. 2008;117:1055-1064; originally published online February 11, 2008; doi: 10.1161/CIRCULATIONAHA.107.749234

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/117/8/1055

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2008/03/03/CIRCULATIONAHA.107.749234.DC1

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

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

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