Innate Immune Molecular Connections Between Atherosclerosis and Statins

Can the Clinical Laboratory Venture Beyond C-Reactive Protein?

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Abstract

Innate immunity was originally envisioned as a nonspecific host response to microbial pathogens. However, in the past few years, immunologists have discovered toll-like receptors (TLRs) and their role in recognizing pathogen-associated molecular patterns. TLRs and other components of innate immunity now help to explain the pathologic mechanisms underlying many common diseases, including atherosclerosis. At this time, C-reactive protein is the most useful biomarker for evaluating the inflammatory aspect of atherogenesis. Yet, operating beneath this commonly assayed biomarker are other components of the immune response. These inflammatory components of atherosclerosis and their genetic variations provide important insights. Today, novel antiatherogenic therapies are emerging, and at the same time a molecular groundwork is being laid for the genetic testing of statin efficacy. However, the adoption of clinical testing will need to wait until new therapeutic choices enter medical practice.

Diverse Ligands Activate Innate Immunity via Toll-Like Receptors

Innate immunity operates like a hidden aquifer beneath the more easily visualized churnings of antibody diversity and T-cell mutations of adaptive immunity. To accomplish its protective function, innate immunity strongly relies on toll-like receptors (TLRs). For ancestral species and humans, TLRs recognize a diverse array of specific pathogen-associated molecular patterns and initiate pathways leading to production of cytokines and the activation of complement. Although not as specific as an antibody response, TLRs enable protective pathways that are rapidly turned on, connect with effector molecules that eliminate microbes, and pass a host response baton to adaptive immunity. For the clinical pathologist, this burgeoning field opens new avenues for understanding many diseases. Atherogenesis, with its dependence on immunogenetics and its interplay with the therapeutic effects of statins, provides an important connection to advances in basic scientific research.

The presence of an innate immune receptor was first seen through genetic work in the fruit fly. The toll gene in Drosophila first was recognized as being essential to dorsal ventral development and later was shown to be an integral part of fungus-induced antimicrobial peptide production. Shortly thereafter, a search for human homologs of the fly toll gene led to the discovery of TLR4. Following a separate trail, mapping of a lipopolysaccharide (LPS) hyporesponsive mouse strain independently yielded the discovery of the LPS receptor TLR4. To date, efforts in mining the human genome have led to the discovery of 10 TLRs.
TLRs have been elucidated. For example, TLR4 mainly mediates gram-negative bacterial host responses, TLR2 is activated by gram-positive bacteria and fungi, and viral RNAs function as ligands to TLR3. How do a limited number of TLRs offer a wide array of specificities? One possible scenario is that TLRs form homodimers and heterodimers in response to specific microbial challenges.

For example, TLR2 has been shown to heterodimerize with TLR1 and TLR6, which appear to respond specifically to ligands such as bacterial lipoprotein and peptidoglycan. In this manner, the immune response can establish a combinatorial repertoire achieving discrimination among a number of pathogen-associated molecular patterns found in nature.

In addition to TLRs forming complexes with a similar or different TLR, a number of cell membrane and soluble proteins also have been shown to enhance and refine the TLR response to ligands. In this manner, a number of molecular mechanisms lead to a wide spectrum of specificities. TLRs represent the sensing mechanism for important immune functions, and the resulting messages to the nucleus mediate signaling pathways. The best studied is the MyD88 pathway; however, separate pathways independent of MyD88 also are being described. Both pathways lead to controlling the production of important cytokines and cell-cycle control proteins. As a result of this new understanding, important therapeutic mechanisms now are explained partially by TLRs and their signaling pathways.

Such is the case for paclitaxel, the US Food and Drug Administration–approved cancer medication and drug used in coated coronary artery stents. Paclitaxel seems to mimic LPS, with its therapeutic effects dependent on TLR4. Both the MyD88 and MyD88-independent signaling pathways mediate this therapeutic effect. Ultimately, cells come under transcriptional controls initiated by paclitaxel and are eliminated by cell-cycle arrest and apoptosis.

For the purposes of practicing clinical pathologists, TLRs and their signaling pathways are important discoveries and deserve our attention for therapeutic intervention and possible clinical testing opportunities. Although the concept is speculative, atherosclerosis may provide the nexus where important innate immune components and the clinical laboratory first come together. Some authors with an interest in atherogenesis and C-reactive protein (CRP) might argue that this inflammatory marker already provides an important link.

### Disease Associations and the Importance of Innate Immunity

Disease associations with important innate immune molecular polymorphism are found readily in the medical literature. As of this time, clinical studies examining innate immunity molecule polymorphisms or expression are seen in asthma, infection, fatty liver disease, autoinflammatory disease, inflammatory bowel disease, and cardiovascular disease. For our purposes, atherosclerosis and cardiovascular disease provide an intriguing connection to TLRs and the immune response. Atherosclerosis of the coronary arteries is increasingly considered to be a chronic inflammatory process, in large part mediated by the innate immune response. Although it is unlikely that atherosclerosis is caused by a specific organism, studies point to bacterial and viral organisms as risk factors and modifiers of disease. Patients with chronic infections have a significantly higher risk of carotid atherosclerosis than subjects without chronic infections, and antibody response to multiple microorganisms is a risk factor for the presence and severity of coronary artery disease (CAD). Chlamydia pneumoniae is receiving considerable attention as a risk factor in different stages of the atherosclerotic process. But, most important, endotoxin has...
been the focus of numerous studies. In part, this is because the presence of endotoxin is not confined to sepsis, but also occurs in healthy individuals.32

**CRP: Our Best Marker for Assessing the Inflammatory Component of Atherosclerosis**

Before viewing the genetics of TLRs, we should examine the current means for evaluating the inflammatory component of atherosclerosis. High-sensitivity CRP provides an important independent prognostic marker for CAD and might be used as part of the therapeutic decision-making process according to the Third Report of the National Cholesterol Education Program.33 It is interesting that CRP levels also correlate with the magnitude and speed of the therapeutic response to statins.34

First discovered in the 1930s as a precipitin elicited by the C-polysaccharide extracted from pneumococci, CRP traditionally has been characterized as a nonspecific effector of innate immunity owing to its ability to activate the classic complement cascade and mediate phagocytosis.35 In response to infection, CRP levels increase from trace levels of 0.1 µg/mL to several hundred micrograms per milliliter. CRP production initiates with TLR activation and leads to powerful interleukins (ILs; particularly IL-6) and, ultimately, transcriptional activation of CRP genes within hepatocytes.36

Similar to other molecular participants in atherosclerosis, CRP levels may reflect a genetic component. A polymorphic GT repeat contributes to variation in baseline CRP in healthy people and in people with systemic lupus erythematosus.37 Another polymorphism, a C/G allele in exon 2, also has been described.38 Although this polymorphism was associated with differences in baseline CRP, its genetic variation was not associated with future vascular events. Although useful as a clinical marker for atherosclerosis, CRP’s precise function in the propagation of an atherosclerotic plaque is understood only partially. There is, however, some evidence that CRP is an active participant in plaque formation.39-42 Multiple in vitro effects have been studied, including the down-regulation of endothelial nitric oxide synthetase transcription41 and the stimulation of endothelin-1 and IL-6 release from endothelial cells.42 Both processes promote endothelial contraction and relaxation dysfunction, thereby setting the stage for atherogenesis.39,40

The most intriguing part of the CRP story emerges when patients with high CRP levels are studied in comparison with patients with low CRP levels. Muhlestein and colleagues31 stratified CRP by tertile in 3,055 postangiography patients who were treated with a statin. Statin use in patients with high CRP levels provides a larger and earlier survival benefit than statin use in patients with lower CRP levels. The patients in the lowest tertile of CRP showed a survival improvement after 2 years, whereas the patients in the highest CRP tertile showed a survival advantage after only 1 week.

Ridker and colleagues43 provide additional evidence for the anti-inflammatory effects of statin therapy. Their study showed that patients who have low CRP levels after statin therapy have better clinical outcomes than those with higher CRP levels, regardless of the resultant level of low-density lipoprotein (LDL) cholesterol.

Evidence for these so-called pleiotropic effects of statins is growing. The actions of statins are more complex than originally suspected, and studies have revealed multiple mechanisms for statins. Kwak et al44 described a number of mechanisms by which statins might influence vascular biology beyond cholesterol reduction. Although all statins lead to reduced cholesterol levels, the individual statins differ in their pleiotropic effects, with some seeming stronger for one mechanism of action, yet weaker in a different action. One proposed mechanism relies on the competitive inhibition of HMG coenzyme A reductase,45 the same biosynthetic pathway that leads to the decrease in hepatic cholesterol production, a compensatory increase in LDL receptors, and subsequent clearance of cholesterol from the circulation. In this manner, statins prevent the synthesis of isoprenoid molecules that serve as lipid attachments for a variety of intracellular signaling molecules.46 Other mechanisms support statins as immune-altering drugs. By using flow cytometric and immunohistochemical studies, statins have been shown to reduce major histocompatibility complex class II expression in human endothelial cells and monocytes.47 Also, a separate immunomodulating mechanism for statins has been proposed. Leukocyte function antigen-1 is inhibited selectively by statins via a novel binding site.48

Despite important pleiotropic effects, the current guidelines for statin therapy emphasize only target levels of LDL cholesterol, non–high-density lipoprotein cholesterol, or both.33 Recent clinical studies have raised the level of interest in using CRP for monitoring therapy. Because individual statins might have different effects on CRP levels and these levels are correlated only weakly with lipid levels, some cardiologists are suggesting CRP as a second means for measuring the effectiveness of statin therapy.49

**Genetic Variations in TLRs and Other Molecules Are Potentially Important in the Pathogenesis of Atherosclerosis and the Response to Statins**

The underlying genetic variations of TLRs lead to interesting scientific findings with clinical significance. For example, Arbour et al50 discovered 2 single nucleotide polymorphisms...
in the TLR4 gene that result in amino acid substitutions in the extracellular domain of the receptor. These receptor variants, Asp299Gly and Thr399Ile, result in an altered immunologic response to inhaled LPS. In a large clinical trial, the Asp299Gly TLR4 polymorphism has been shown to attenuate receptor signaling and diminish the inflammatory response to gram-negative pathogens.\(^5\)

An important clinical study sharpens the focus on TLR4. Boekholdt et al\(^5\) hypothesized that the TL4 Asp299Gly and the Thr399Ile polymorphisms would be associated with progression of coronary atherosclerosis, as measured by angiography. In addition, these investigators conjectured that these polymorphisms would alter the risk of cardiac events. Last, they asked whether carriers of the variant genes would respond differently to statin therapy than would noncarriers. These concepts were tested in patients with symptomatic CAD as part of the REgression GRowth Evaluation Statin Study (REGRESS).\(^5\) Briefly, the REGRESS was a randomized, placebo-controlled, multi-center study designed to test the effect of 2 years of treatment with 40 mg of pravastatin on the progression and regression of angiographically documented coronary atherosclerosis in 885 male patients. Patients had normal to moderately elevated serum cholesterol levels (155–310 mg/dL [4.00–8.02 mmol/L]) and were randomly assigned to receive 40 mg of pravastatin once daily or a matching placebo. In addition, the study was short, and only 1 statin was studied.

Despite these drawbacks, the TLR4 polymorphism data are striking.\(^5\) There was no significant difference between genetically defined subgroups with respect to baseline risk factors, previous treatment, or in-trial changes in lipid levels. Genotype was not associated with a study-wide progression of coronary atherosclerosis in 885 male patients. Patients had normal to moderately elevated serum cholesterol levels (155–310 mg/dL [4.00–8.02 mmol/L]) and were randomly assigned to receive 40 mg of pravastatin once daily or a matching placebo. In addition to the main angiographic study, a number of substudies were performed, including molecular genetic investigations.\(^5\) However, our enthusiasm for the REGRESS findings needs to be tempered by limitations inherent in this study. The clinical follow-up duration was short, and only 1 statin was studied.

The precise atherogenic contributions of TLRs to atherosclerosis are far from clear. However, some studies point to a TLR association with inflammatory activation in atherosclerotic lesions.\(^5\) Also, in support of a TLR contribution, TLR4 is present on endothelial cells, and evidence indicates that activation of endothelial cells and subsequent IL-6 release requires TLR4.\(^5\) To fully appreciate the importance of TLR4, it is useful to compare the genetic contributions of other molecules.\(^5\) Fortunately, the REGRESS provides numerous substudies that examine allelic variants of other proposed molecular contributors to atherogenesis.

Allelic variants are found in key molecules with different pathogenic associations to atherogenesis. For example, stromelysin-1, a matrix metalloproteinase-3, and fibrinogen contribute to atherogenesis. Stromelysin is thought to have a prominent role in destabilizing a plaque, whereas fibrinogen contributes to the acute thrombotic process that immediately leads to myocardial ischemia. Both molecules have allelic variations conferring altered risks of CAD.\(^5\) For our purposes, important differences are seen in statin responsiveness for both of these molecules’ allelic variations.

Allelic variations associated with altered statin efficacy are also found in lipid pathway enzymes. Their variant alleles affect atherosclerosis by altering serum lipid levels and the density of the lipid vesicle particles. In this lipid group are cholesteryl ester transfer protein (CETP), lipoprotein lipase, and hepatic lipase allelic variations. Taken together, these allelic variations affect the response to pravastatin. However, unlike variations seen in fibrinogen and stromelysin, their allelic variations showed baseline and in-trial differences in lipid levels or LDL cholesterol density.\(^5\) Our snapshot of the statin pharmacogenetics offers a few lessons. TLR4 and its genetic variants are associated with atherogenesis, offering interesting markers for

### Table 2
Incidence of Cardiovascular Events According to Toll-Like Receptor 4 (TLR4) Genotype and Treatment with Pravastatin\(^5\)*

<table>
<thead>
<tr>
<th></th>
<th>299Asp</th>
<th>299Gly+ (399Ile– and 399Ile+ Patients)</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>18.1</td>
<td>29.6 (8/27)</td>
<td>19.0 (62/326)</td>
<td>.10(^*)</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>11.5</td>
<td>2.0 (1/51)</td>
<td>10.9 (33/329)</td>
<td>.046(^*)</td>
</tr>
<tr>
<td>Total</td>
<td>14.9</td>
<td>11.45 (9/78)</td>
<td>11.45 (9/78)</td>
<td>.0007(^*)</td>
</tr>
<tr>
<td>(P)</td>
<td>.03(^*)</td>
<td>.0002(^*)</td>
<td>.025(^*)</td>
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</table>

\(^*\) Data are given as percentage (number affected/total number) for the incidence of cardiovascular events during 2-year follow-up.

\(^*\) \(P\) for log-rank test between genetic groups.

\(^*\) \(P\) for log-rank test between the treatment groups.

\(^*\) \(P\) for interaction between genotype and treatment by Cox regression model.
Table 3
Important Genetic Polymorphism Associated With Statin Efficacy

<table>
<thead>
<tr>
<th>Genetic Marker</th>
<th>Type</th>
<th>Distribution in the Population</th>
<th>Effect on Baseline or Posttherapy Lipid Levels</th>
<th>Effect on Study Endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolllike receptor-4</td>
<td>Asp299Gly and Thr399Ile</td>
<td>665 men genotyped:</td>
<td>No genetic marker–associated differences in baseline lipid data or postpravastatin lipid levels</td>
<td>Asp299Gly polymorphism carriers showed a reduction in clinical events; 29.6% of carrier placebo group had a clinical cardiac event, whereas only 2.0% of the pravastatin-treated carrier group had a clinical cardiac event (P &lt; .0002).</td>
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<tr>
<td></td>
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<td>wild-type, 88%;</td>
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<td></td>
<td></td>
<td>299Gly/399Ile, 11%;</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>299Gly/no 399Ile, 1.4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen data</td>
<td>–455G and –455A alleles</td>
<td>237 men genotyped:</td>
<td>No genetic marker–associated differences in baseline lipid data or postpravastatin lipid levels</td>
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<tr>
<td></td>
<td></td>
<td>455A/455A, 4%;</td>
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<tr>
<td></td>
<td></td>
<td>455G/455A, 34%;</td>
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<td></td>
<td></td>
<td>455G/455G, 62%</td>
<td></td>
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<tr>
<td>Stromelysin-1, a</td>
<td>5A and 6A alleles in the promoter</td>
<td>494 men genotyped:</td>
<td>No genetic marker–associated differences seen in baseline lipid data or postpravastatin lipid levels</td>
<td></td>
</tr>
<tr>
<td>connective tissue–</td>
<td>region</td>
<td>5A/6A, 50%;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>remodeling metal-</td>
<td></td>
<td>6A/6A, 26%;</td>
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<tr>
<td>lipoproteinase (data</td>
<td></td>
<td>5A/5A, 24%</td>
<td></td>
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<td>from a subpopulation</td>
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<td>of the REGRESS)54</td>
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<tr>
<td>CETP, a key molecular</td>
<td>B1 and B2 alleles;</td>
<td>807 men genotyped:</td>
<td>B1 allele associated with lower HDLC level (P &lt; .001) and higher CETP activity; baseline LDLC concentrations similar in all genotypes; all genotypes received same reduction in LDLC from pravastatin</td>
<td></td>
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<tr>
<td>determining the</td>
<td>B1 allele associated with a lower</td>
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<tr>
<td>metabolism of</td>
<td>HDLC than B2 allele</td>
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<td>HDLC (data from a</td>
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<tr>
<td>subpopulation of the</td>
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<td>REGRESS)55</td>
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</tr>
<tr>
<td>HL gene promoter</td>
<td>–514C and –514T; the C allele</td>
<td>49 men genotyped:</td>
<td>B1 allele associated with a decreased HDLC and a denser form of LDL-C. Patients with the C allele were at higher risk of progress of atherogenesis.</td>
<td></td>
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<tr>
<td>variant54</td>
<td>associated with higher HL activity and more dense and atherogenic LDL particles</td>
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| CHD, coronary heart disease; CETP, cholesteryl ester transfer protein; HDL-C, high-density lipoprotein cholesterol; HL, hepatic lipase; LDL-C, low-density lipoprotein cholesterol; MI, myocardial infarction; REGRESS, REgression GRowth Evaluation Statin Study; TIA, transient ischemic attack.

pravastatin effectiveness. Although the data are preliminary, the greatest genetic control seems to rest with a TLR, precisely at the point where the environment makes its first contact with the immune system. Oddly, though, the nonlipid effects of statins are not monitored typically in the clinical setting. From these data and other studies, a subtle concern arises. Results suggest that statin therapy improves lipid profiles in nearly all patients but selectively benefits patients with a high-risk proinflammatory genotype compared with patients with the wild-type genotype. This observation raises the possibility of determining an individual patient’s genotype as a useful means for optimizing the benefits of statin therapy. However, caution should be taken. Survival data need to be evaluated in prospectively genotyped patients receiving a number of different statins. Only after large clinical studies have been conducted can conclusions be drawn.

Economic Pressure and New Therapies May Drive Molecular Testing for Statin Efficacy

Economics may propel this issue. The expanding patient base that qualifies for statins and the cost of therapy eventually might lead to a new approach. In part, this is due to the United Kingdom’s Heart Protective Study, a 20,500-patient clinical trial that concluded that statins should be prescribed based on risk, not just cholesterol level. By following this approach, the number of patients benefiting from the drugs would more than triple. Collins, the principal investigator, predicted that statins would become the “new aspirin.” Unfortunately, unlike aspirin, statins carry a hefty price tag.

Similar studies also are expanding the indications for statins. The PROSPER trial examined pravastatin’s benefit in elderly patients (70-82 years). In a 3-year follow-up, mortality from CAD fell 24% in the pravastatin-treated group. Diabetic patients were examined in the Heart Protective
Study,71 and the investigators concluded that statin therapy should be considered routinely for all diabetic patients irrespective of initial cholesterol level.

As patient enrollment expands, the economic burden grows. As described in *JAMA*,69 Ireland’s bill for “statin therapy is doubling every 15 to 18 months, and the expenditure on statins has increased 22-fold since 1995, statins now account for 6% of all drug expenditures here,” according to Michael Barry, MD, director of the National Center for Pharmacoconomics, Dublin. In poorer countries, universal statin coverage would exhaust health care budgets. For example, in the Baltic states where expenditures for pharmaceuticals are set at 2% of the gross domestic product, statin therapy presents an insurmountable obstacle.73

To save precious health resources and achieve higher risk reductions, are we ready to rely on genotypes? Unfortunately, the answer is “no.” Maitland-van der Zee et al74 point out that there is not enough evidence to exclude patients from statin therapy solely on genotype. Further research in a large general population sample is needed to assess the importance of polymorphisms on the effectiveness of statins.

The current data fall short of what is needed. For example, although the genotypic *TLR4* event reduction data are compelling, the data on the CETP B2B2 variant did not hold up in a meta-analysis.75 However, considering the genotype data of the REGRESS and costs seen in Europe, the future might lead to important changes.67,68 That might be made possible by the availability of new single-step genotype methods for *TLR4*.76 New therapeutic choices for the treatment of coronary atherosclerosis will be needed to generate interest in the molecular genetics of atherogenesis. Fortunately, a number of inflammatory genes,78 AGI-107, one such antioxidant/anti-inflammatory drug, has been studied in the CART-1 (Canadian Antioxidant Restenosis Trial).79 In this randomized multicenter trial, researchers examined whether AGI-107 reduced restenosis assessed by intravascular ultrasound after percutaneous coronary intervention. AGI-107 lessened restenosis after intervention and also showed a significant reduction in plaque found in reference vessels not subjected to angioplasty. This medication has entered a phase 3 trial.

Despite promising research, until therapeutic choices are offered, examining the inflammatory genetic underpinnings of atherogenesis will remain largely an investigational interest.

**References**


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