

## Biomarkers in Cardiovascular Medicine

José L. Martín-Ventura,<sup>a</sup> Luis M. Blanco-Colio,<sup>a</sup> José Tuñón,<sup>b</sup> Begoña Muñoz-García,<sup>a</sup> Julio Madrigal-Matute,<sup>a</sup> Juan A. Moreno,<sup>a</sup> Melina Vega de Céniga,<sup>c</sup> and Jesús Egido<sup>a</sup>

<sup>a</sup>Laboratorio de Patología Vascular, Fundación Jiménez Díaz, Universidad Autónoma de Madrid, Madrid, Spain

<sup>b</sup>Departamento de Cardiología, Fundación Jiménez Díaz, Universidad Autónoma de Madrid, Madrid, Spain

<sup>c</sup>Departamento de Angiología y Cirugía Vasculard, Hospital de Galdakao-Usansolo, Galdácano, Vizcaya, Spain

Cardiovascular disease is the principal cause of death in developed countries. The underlying pathological process is arterial wall thickening due to the formation of atherosclerotic plaque, which is frequently complicated by thrombus, thereby giving rise to the possibility of acute coronary syndrome or stroke. One of the major challenges in cardiovascular medicine is to find a way of predicting the risk that an individual will suffer an acute thrombotic event.

During the last few decades, there has been considerable interest in finding diagnostic and prognostic biomarkers that can be detected in blood. Of these, C-reactive protein is the best known. Others, such as the soluble CD40 ligand, can be used to predict cardiovascular events. However, to date, no biomarker has been generally accepted for use in clinical practice. At present, there are a number of high-performance techniques, such as proteomics, that have the ability to detect multiple potential biomarkers. In the near future, these approaches may lead to the discovery of new biomarkers that, when used with imaging techniques, could help improve our ability to predict the occurrence of acute vascular events.

**Key words:** Biomarkers. Atherothrombosis. Proteomics.

### Biomarcadores en la medicina cardiovascular

Las enfermedades cardiovasculares son la primera causa de muerte en el mundo occidental. El proceso patológico que subyace a ellas es un engrosamiento de la pared arterial debido a la formación de placas ateroscleróticas, las cuales se complican frecuentemente con un trombo y pueden dar lugar a síndrome coronario agudo o accidente cerebrovascular. Uno de los mayores retos de la medicina cardiovascular es encontrar la manera de predecir el riesgo de un sujeto de sufrir un evento trombótico agudo.

En las últimas décadas, hay un gran interés en la búsqueda de biomarcadores diagnósticos y pronósticos que puedan ser identificados en sangre. Entre ellos, la proteína C reactiva es la más conocida. Otros, como el ligando de CD40 soluble, pueden predecir eventos cardiovasculares. En cambio, hasta el momento no hay un biomarcador aceptado en la práctica clínica. Actualmente, existen diversas técnicas de alto rendimiento como la proteómica, que permite la detección de múltiples biomarcadores potenciales. Estas aproximaciones pueden identificar en un futuro próximo nuevos biomarcadores que, junto con las técnicas de imagen, pueden ayudar a mejorar la predicción de eventos vasculares agudos.

**Palabras clave:** Biomarcadores. Aterotrombosis. Proteómica.

Section sponsored by the Dr Esteve laboratory

The authors of the present article would like to acknowledge the following sources of financial assistance: the Ministry of Science and Technology (SAF2007/63648, SAF2007-60896), CAM (S2006/GENE-0247), the Ministry of Health and Consumption, the Carlos III Health Institute, the RECAVA Network (RD06/0014/0035), the Ramón Areces Foundation, FCI 05/0451 CP04/00060, and the European Network // (HEALTH F2-2008-200647).

Correspondence: Dr. J. Egido.  
Servicio de Nefrología e Hipertensión. Laboratorio de Patología Vasculard.  
Fundación Jiménez Díaz.  
Avda. Reyes Católicos, 2. 28040 Madrid. España.  
E-mail: J.egido@fjd.es

### INTRODUCTION

Cardiovascular disease is the leading cause of death in the western world.<sup>1</sup> Among these diseases, atherosclerosis is the main cause of the enormous rates of morbidity and mortality. The pathological process that underlies this disease is arterial wall thickening due to the formation of atherosclerotic plaques.<sup>2</sup> Although these normally evolve gradually, atherosclerotic plaques may become complicated due to a thrombus and lead to a sudden obstruction of the vascular lumen. Depending on its location, this obstruction may lead to acute coronary syndrome (ACS) or stroke, and can cause sudden death or severe sequelae among the patients who develop

this. Although great progress has been made in the treatment of this disease, current medical knowledge is unable to effectively predict who is at risk of developing these problems. Thus, one of the greatest challenges of cardiovascular medicine is to find a way to predict who is at risk of experiencing an acute thrombotic event.

Cardiovascular risk factors have been used for many years to predict the risk of cardiovascular events in the general population, supplemented by other data, such as the ejection fraction. In patients with symptomatic atherosclerosis, as occurs in ischemic heart disease, techniques such as coronary angiography may also be employed to assess the extent of the disease. However, there continues to be a high incidence of unexpected acute ischemic events, both in the population with known atherosclerosis and in subjects classified as healthy but with the disease in its subclinical form. In this regard, one of the most active research fields in recent years is the use of magnetic resonance imaging (MRI) and multislice computed tomography. These techniques enable the non-invasive diagnosis of the presence and extent of atherosclerosis and may well lead to precise characterization of the size and composition of the lesions in the future. Another large research field in this area, which we will focus on, is the search for diagnostic and prognostic biomarkers that can be identified in blood.<sup>3</sup> The vascular wall releases molecules into the bloodstream that can reflect the pathological processes taking place there. On the other hand, blood itself is clearly involved in thrombus formation. Thus, in theory, the concentrations of the molecules involved in the different pathological processes present in atherosclerosis could be biomarkers. However, not all of these molecules are suited to this aim and should fulfill certain conditions. The characteristics of an ideal biomarker are shown in Table. Although most of the biomarkers studied up to now have been based on the possibility of being useful from the diagnostic/prognostic standpoint, it is worth recalling that ideally they would also provide a therapeutic target. Finally, although some have no diagnostic or therapeutic value, they can provide us with information on the origin and formation of atheromatous plaque (Figure 1).

### CARDIOVASCULAR BIOMARKERS

In this section we summarize the most frequently studied biomarkers in relation to the different mechanisms involved in development and rupture of atherosclerotic plaque, such as endothelial dysfunction, inflammation, oxidative stress, proteolysis, and thrombosis (Figure 2).

### Characteristics of a Biomarker

Specific	To a particular disease
Sensitive	Easily quantifiable
Predictive	Relevant to disease progression or treatment
Robust	Fast, simple, and cheap analysis
Stable	Equal concentrations at any time of day
Non-invasive	Samples easily acquired (blood, urine, etc)
Preclinical and clinical importance	Valid in animal/cell human models

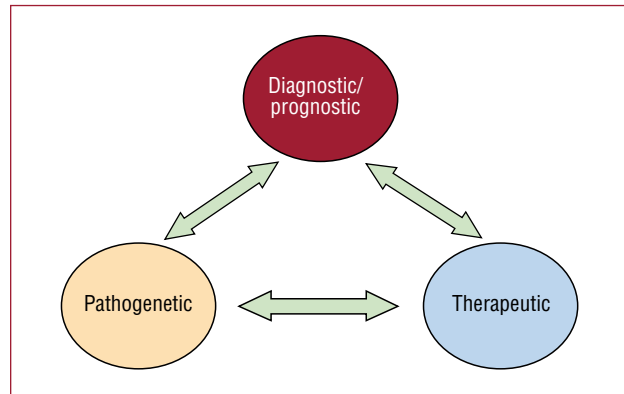


Figure 1. Types of biomarkers of cardiovascular disease.

### Endothelial Dysfunction

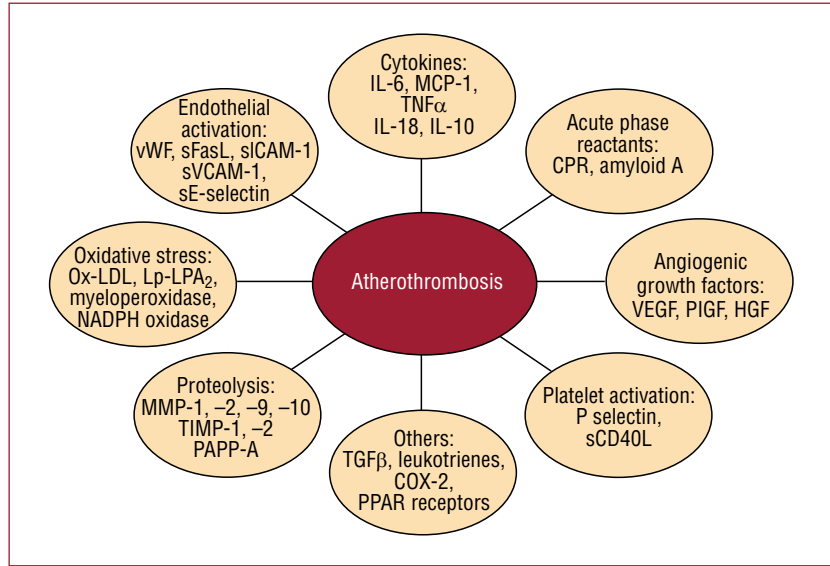
Cardiovascular risk factors and hemodynamic factors are among the causes of endothelial dysfunction, since it is known that the endothelium is damaged in places of increased blood turbulence. In particular, lipids play a special role, since their increased plasma concentrations can lead to their accumulation in the subendothelial space where, after undergoing various modifications, they stimulate adhesion molecule expression and the inflammatory process begins.

#### Adhesion Molecules

Adhesion molecules are crucial to cell recruitment toward the interior of the vascular wall. Given that their soluble forms can appear in plasma, various studies have associated their concentrations with the risk of cardiovascular events.

In healthy populations, the intercellular adhesion molecule-1 (ICAM-1) reaches greater concentrations in healthy subjects who will undergo acute myocardial infarction (AMI), whereas data suggest this is not the case regarding the vascular cell adhesion molecule-1 (VCAM-1).<sup>4,5</sup> The ARIC (Atherosclerosis in Risk Communities) study reported that ICAM-1 concentrations predicted

**Figure 2.** Circulating markers associated with atherosclerosis. COX-2 indicates cyclooxygenase 2; HGF, hepatocyte growth factor; IL, interleukin; Lp-PLA2, lipoprotein-associated phospholipase A2; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; ox-LDL, oxidized low-density lipoprotein; PAPP-A, pregnancy-associated plasma protein A; CRP, C-reactive protein; PIGF, placental growth factor; PPAR, peroxisome proliferator-activated receptor; sEselectin, soluble E-selectin; sFasL, soluble Fas ligand; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; TGF- $\beta$ , transforming growth factor- $\beta$ ; TIMP, tissue inhibitor of metalloproteinase; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; VEGF, vascular endothelium growth factor; vWF, von Willebrand factor.



coronary events and the development of carotid atherosclerosis, and also an association between this and soluble E-selectin concentrations.<sup>6</sup>

The Women’s Health Study showed that soluble P-selectin was a predictor of cardiovascular events.<sup>7</sup>

The Atherogene study showed that in populations with coronary heart disease the concentrations of E-selectin, ICAM-1 and VCAM-1 were greater in the patients who underwent cardiovascular events.<sup>8</sup> Mulvihill et al<sup>9</sup> determined that VCAM-1, together with C-reactive protein (CRP), was a predictor of future cardiovascular events in patients with ACS, whereas there was no correlation with ICAM-1, E-selectin, and P-selectin.

Finally, the results obtained by Malik et al<sup>10</sup> in the British Regional Heart Study are not very encouraging regarding the prognostic value of adhesion molecules. Of the 5661 men enrolled in the study, samples were analyzed from 643 men who developed coronary heart disease and 1278 who remained stable. In baseline conditions, there was evidence of coronary heart disease in 36% of those who had undergone events and in 20% of those who remained stable. The concentrations of ICAM-1, VCAM-1, E-selectin, and P-selectin did not add prognostic value to that provided by the classic risk factors.

Data obtained up to the present on the effect of treatment with lipid-lowering drugs on the plasma concentrations of different adhesion molecules are varied. Thus, treatment with fluvastatin (80 mg/d) reduced ICAM-1 and P-selectin plasma concentrations in 26 hypercholesterolemic patients.<sup>11</sup> However, these results have not been confirmed by other studies. Jilma et al<sup>12</sup> analyzed circulating ICAM-1, VCAM-1, and E-selectin concentrations in 75 hypercholesterolemic patients who had been

treated with 3 different statins for 3 months, and did not observe changes in plasma concentrations of the proteins analyzed. It should be pointed out that these studies were conducted in small populations; the results have recently been published of the AIM (Atorvastatin on Inflammatory Markers) study<sup>13</sup> which analyzed ICAM-1 plasma concentrations in 1078 subjects at high cardiovascular risk. It was observed that 3-month treatment with all the atorvastatin doses available (10-80 mg/d) decreased ICAM-1 concentrations.

## Inflammation

### Chemokines

Once leukocytes have adhered to the vascular wall, their entry into the interior is controlled by chemokines. The 2 most numerous are alpha and beta chemokines. Alpha chemokines are chemotactic for neutrophils or lymphocytes, and include the interleukins (IL). Beta chemokines attract monocytes and lymphocytes, in addition to basophils and eosinophils, but do not attract neutrophils. Monocyte chemoattractant protein-1 (MCP-1) belongs to this family.

### Interleukin 6

The value of IL-6 as predictor of risk was assessed in the prospective Health ABC cohort study.<sup>14</sup> In subjects without vascular disease, circulating IL-6 values were predictive of coronary heart disease, heart failure and stroke. Biasucci et al<sup>15</sup> demonstrated that patients with unstable angina who died, or presented AMI or refractory angina during hospitalization, at admission had higher IL-6

concentrations than in those who remained stable. In another study on unstable angina, which included 263 patients, IL-6 and CRP concentrations predicted the risk of coronary death during a 17-month follow-up period and were additive to the value provided by markers of myocardial damage.<sup>16</sup> The FRISC II (Fragmin and Fast Revascularisation During Instability in Coronary Artery Disease II trial) study,<sup>17</sup> randomized 3269 patients with ACS at admission to invasive treatment or a conservative strategy. The IL-6 values were independent predictors of mortality during 12-month follow-up. Furthermore, the patients who had high IL-6 concentrations were those who demonstrated improvement after invasive treatment, and thus IL-6 could be used to guide treatment in this population.

### *Monocyte Chemoattractant Protein-1*

This chemokine is the main one governing monocyte recruitment to tissues where there is an active inflammatory response, as is the case in atherosclerotic lesions. The diagnostic and prognostic value of soluble MCP-1 has been demonstrated in different studies. MCP-1 plasma concentrations have been associated with different cardiovascular risk factors, and with a greater risk of developing a cardiovascular event in the future.<sup>18,19</sup> The OPUS-TIMI 16 study analyzed its prognostic value in 2270 patients with non-ST-segment elevation acute coronary syndrome (NSTEMI), and it was observed that MCP-1 plasma concentrations predicted the risk of death or AMI at 10 months.

Our group has demonstrated that treatment with atorvastatin only or in combination with amlodipine reduces MCP-1 concentrations in patients with carotid atherosclerosis.<sup>20,21</sup> We also recently published the results of the AIM study, which found that all the atorvastatin doses available can reduce MCP-1 plasma concentrations after 3 months of treatment in patients at high cardiovascular risk.<sup>13</sup>

### *C-Reactive Protein*

Without doubt, CRP is the most well-known inflammatory marker.<sup>22,23</sup> In patients with unstable angina, CRP concentrations have been demonstrated as predictors of recurrent cardiac instability.<sup>24</sup> Similarly, CRP would appear to be useful in the diagnostic and prognostic management of peripheral vascular disease.<sup>25</sup> In patients with coronary heart disease, CRP has been associated with the risk of recurrent cardiovascular events.<sup>26,27</sup> On the other hand, some studies on patients with AMI have reported correlations between CRP concentrations and the size and extent of the necrosis, and with prognosis.<sup>28</sup> Various primary prevention studies have

demonstrated that baseline CRP concentrations can predict cardiovascular events.<sup>29-31</sup>

Regardless of the ability of CRP to predict risk in primary and secondary prevention, interest in CRP has increased since statins can reduce CRP concentrations independently of their lipid-lowering effect.<sup>32</sup> In this regard, the PROVE IT-TIMI22 (Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis in Myocardial Infarction 22) study<sup>33</sup> showed that the initial CRP concentrations obtained after treatment with statins were as relevant to predicting cardiovascular events as low-density lipoprotein cholesterol (LDL-C) concentrations. The results of the JUPITER study should be highlighted. This study analyzed the effect of treatment with rosuvastatin (20 mg/d) on 17 802 apparently healthy subjects who had LDL-C concentrations <130 mg/dL and CRP serum concentrations of 2 mg/L, with an average follow-up of 1.9 years.<sup>34</sup> The study showed that rosuvastatin significantly reduced the incidence of cardiovascular events. However, it is important to note that treatment with rosuvastatin reduced LDL-C concentrations to below 55 mg/dL and, as a result, the effect observed after treatment could be due to the drastic reduction found in LDL-C concentrations. In any case, great debate continues on its potential use in clinical practice.<sup>35,36</sup>

## **Oxidative Stress**

### *Lipoprotein-Associated Phospholipase A2*

Lipoprotein-associated phospholipase A2 (Lp-PLA2) is a calcium-independent lipase produced by leukocytes and is associated with circulating LDL and macrophages on atheromatous plaque. Together with CRP, it is the most thoroughly studied predictor of cardiovascular risk. More than 25 prospective epidemiological studies have been published on Lp-PLA2 both in primary and secondary prevention. These clinical trials have usually demonstrated strong correlations between circulating Lp-PLA2 concentrations and the increased risk of cardiovascular events, even after multivariable adjustment for traditional risk factors. Furthermore, Lp-PLA2 is a risk factor which is independent of and complementary to CRP.<sup>37-39</sup> These studies lend support to the recommendations of the American Heart Association that indicate that Lp-PLA2 could be used in clinical practice to fine tune the prediction of risk in subjects at intermediate cardiovascular risk.

Lipoprotein-associated phospholipase A2 has also created great interest as a therapeutic target in coronary heart disease. It is found in high concentrations in the lipid core of inflammatory

plaques and is produced by the inflammatory cells in the lesion or transported by LDL particles. It acts on oxidized phosphatidylcholine (found in the external part of oxidized LDL) to generate lysophosphatidylcholine and oxidized fatty acids. These 2 bioactive lipid products stimulate lipid core expansion and thinning of the fibrous cap. The selective inhibition of Lp-PLA2, through treatment with darapladib, inhibited the development of advanced coronary atherosclerotic lesions in an experimental model.<sup>40</sup> A study that included 330 patients with angiographically documented coronary heart disease found that the administration of darapladib for 12 months prevented necrotic core expansion, a key determinant of plaque vulnerability.<sup>41</sup> Although further studies are needed, these data indicate that Lp-PLA2 inhibition may offer a new therapeutic approach.

### Proteolysis

The imbalance between the synthesis and degradation of the extracellular matrix is a key event in the weakening and rupture of advanced atherosclerotic plaques. Although death by apoptosis of vascular smooth muscle cells seems to be the main mechanism involved in reducing the synthesis of matrix components, increased degradation has been associated with increases in the concentrations and activity of various proteolytic enzymes. Of these enzymes, the metalloproteinases (MMP) have been the most studied.

#### *Metalloproteinases*

Most of the risk factors for atherosclerotic disease have been associated with an increase in the concentrations of various circulating MMP, including, among others, hypertension<sup>42</sup> and diabetes mellitus.<sup>43</sup> Similarly, MMP-9 and TIMP-1 inhibitor concentrations are significantly increased in patients with carotid atherosclerosis<sup>44,45</sup> and in patients with coronary heart disease.<sup>46</sup> An increase in concentrations of both markers has also been detected in patients who have developed ACS.<sup>47</sup> Regarding the possible prognostic value of MMP, it has been observed in primary prevention that increases in circulating MMP-9 can predict cardiovascular events in healthy subjects.<sup>48</sup> Similarly, various studies in secondary prevention have associated elevated MMP-9 concentrations in patients with various cardiovascular disorders with greater cardiovascular mortality.<sup>49,50</sup>

Among the different therapeutic strategies used in atherosclerosis there are 2 which can modulate MMP concentrations: the administration of thrombolytics and of statins. Whereas thrombolytics can stimulate

MMP expression and collagen degradation,<sup>51</sup> statins reduce MMP-9 concentrations.<sup>52</sup>

### Thrombosis

The presence of these immuno-inflammatory-proteolytic processes in atherosclerotic plaque leads to destabilization, rupture and the consequent formation of thrombus, which forms the basis of the most severe clinical consequences of atherosclerosis. The plaque rupture process occurs in 70% of patients who present ACS. This normally involves a plaque that only slightly blocks the vessel, contains fat and, upon rupture, leads to the lipid core—rich in tissue factor—coming into contact with the bloodstream, causing the formation of a thrombus that impedes blood flow.

#### *CD40/CD40L*

Given the involvement of the CD40/CD40L system in atherothrombosis,<sup>53,54</sup> attempts have been made to analyze if determining its plasma values could provide prognostic information. An increase in soluble CD40L predicts a greater risk of cardiovascular events in healthy women.<sup>55</sup> However, most of the population who underwent cardiovascular events had similar concentrations to those who remained stable, the difference being due to a small subgroup that clearly had higher CD40L values. Thus, it is possible that in healthy women soluble CD40L can identify a group at special risk of vascular events, but not the majority.

It has been shown that patients with ACS express enhanced CD40L on platelets.<sup>56</sup> The CAPTURE study,<sup>57</sup> which assessed abciximab versus placebo, analyzed CD40L concentrations in patients with NSTEMI scheduled for angioplasty. In the first place, it was found that patients in the placebo group with high CD40L concentrations had an increased risk of death or non-fatal heart attack during the following 6 months. Secondly, the predictive value of CD40L was independent of troponin T concentrations—even when the values of this marker were elevated—since the CD40L values continued to have prognostic value. Finally, abciximab reduced the risk of events in the subjects with elevated soluble CD40L concentrations to the same as that found in the group with low concentrations, whereas the latter group did not benefit from the use of this IIb/IIIa receptor blocking agent. These findings could be related to the fact that soluble CD40L increases thrombus stability when binding to platelet IIb/IIIa receptors,<sup>58</sup> and thus patients with greater concentrations would particularly benefit from IIb/IIIa receptor blocking agents. Data from the OPUS-TIMI16 (Orbofiban Patients with Unstable coronary Syndromes-Thrombolysis In

Myocardial Infarction 16) study, also conducted in NSTEMI patients, confirmed the predictive value of CD40L independently of CRP and troponin I concentrations.<sup>59</sup>

Different works have analyzed the effect of lipid-lowering treatment using statins on CD40L plasma concentrations. It has been demonstrated that treatment with atorvastatin for 8 weeks reduces CD40L expression on blood platelets in hypercholesterolemic patients.<sup>60</sup> Furthermore, the ASAP (Atorvastatin versus Simvastatin on Atherosclerotic Progression) study showed that treatment with atorvastatin (80 mg/d) or simvastatin (40 mg/d) reduced CD40L plasma concentrations independently of the observed reductions in cholesterol concentrations.<sup>61</sup> The MIRACL (Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering) study, which recruited 2352 patients with ACS, found that treatment with atorvastatin (80 mg/d) for 16 weeks reduced CD40L concentrations and reduced the risk of a new vascular event in subjects who were in upper percentile (>90%).<sup>62</sup> Finally, the AIM study showed that treatment with all the doses of atorvastatin available reduced circulating CD40L concentrations in the subjects at high cardiovascular risk who were in the upper quartile.<sup>63</sup> All these studies indicate that lipid-lowering therapy with statins can reduce CD40L concentrations, probably independently of their lipid-lowering activity.

## NEW POTENTIAL BIOMARKERS

There are 2 possible approaches to the search for new biomarkers. The first is a classic approach based on the selection of proteins involved in the pathophysiology of atherothrombosis, like the examples reviewed in the first part of this article. In the second approach, which uses high-performance techniques such as proteomics, there is no need for prior knowledge of the proteins or of the function that they may perform in disease. Using this approach, we can compare a patient's fluids or tissues with those of a healthy subject and, via tracing, see which proteins are expressed differently in either sample. This generates lists of proteins potentially involved in this disease, among which we should select those whose function or properties indicate that they have the potential for being good biomarkers.

### The Classic Approach

An example of this approach is the study of the Fas/Fas ligand system, which belongs to the tumor necrosis factor receptor superfamily. Both Fas and Fas ligand (FasL) have soluble forms and, whereas soluble Fas (sFas) is generated by alternative splicing

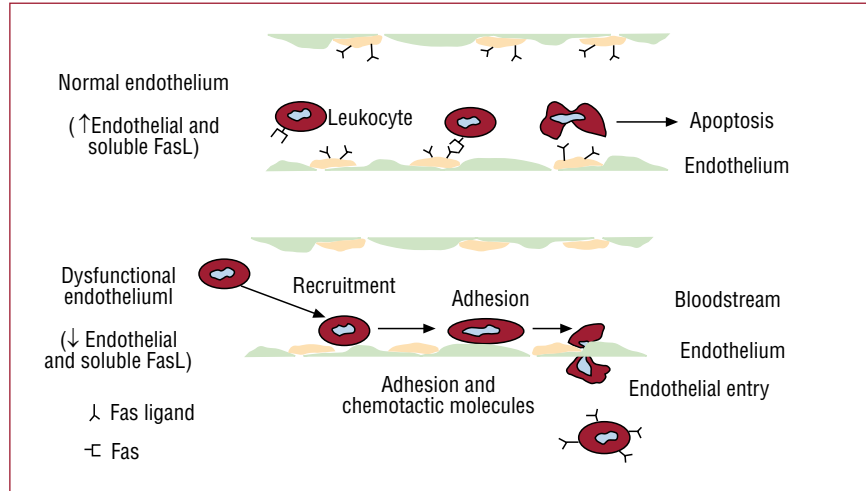
of a single gene,<sup>64,65</sup> the soluble Fas ligand (sFasL) is generated through the action of an MMP.<sup>66</sup>

The concentrations of sFasL are elevated in patients with heart failure, AMI, or unstable angina,<sup>67-69</sup> which are acute situations in which inflammatory cells are highly activated and could increase the production of this protein. In contrast, in chronic situations, it has been observed that patients with familial combined hyperlipidemia or carotid atherosclerosis present striking reductions in circulating sFasL concentrations.<sup>70</sup> The binding of FasL to its receptor leads to the activation of programmed cell death or apoptosis of the cell that expresses the receptor. It has been proposed that FasL expression in some tissues contributes to a state of immune privilege that prevents inflammatory cell infiltration, since these cells express its receptor and, as a result, would undergo apoptosis when coming into contact with the tissue. In this regard, it has been demonstrated that FasL is produced in endothelial cells in normal circumstances and its expression can negatively regulate cell extravasation.<sup>71</sup> Proinflammatory stimuli, such as tumor necrosis factor alpha (TNF $\alpha$ ), can reduce FasL expression in endothelial cells and facilitate the entry of inflammatory cells in the early stages of the development of the atherosclerotic lesion. Our hypothesis is that the endothelial dysfunction that occurs in these patients could be the reason for these findings, probably due to reduced endothelial synthesis or reduced release into the blood (Figure 3). To this end, we analyzed concentrations of sFasL in 110 patients with coronary heart disease in whom the vasodilator response to reactive hyperemia was assessed as a marker of endothelial function. Note that there is a linear relationship between sFasL concentrations and reactive hyperemia, but not with blood flow in response to nitroglycerin (an endothelium-independent response), which indicates that sFasL could be a marker of endothelial function in patients with coronary heart disease.<sup>72</sup>

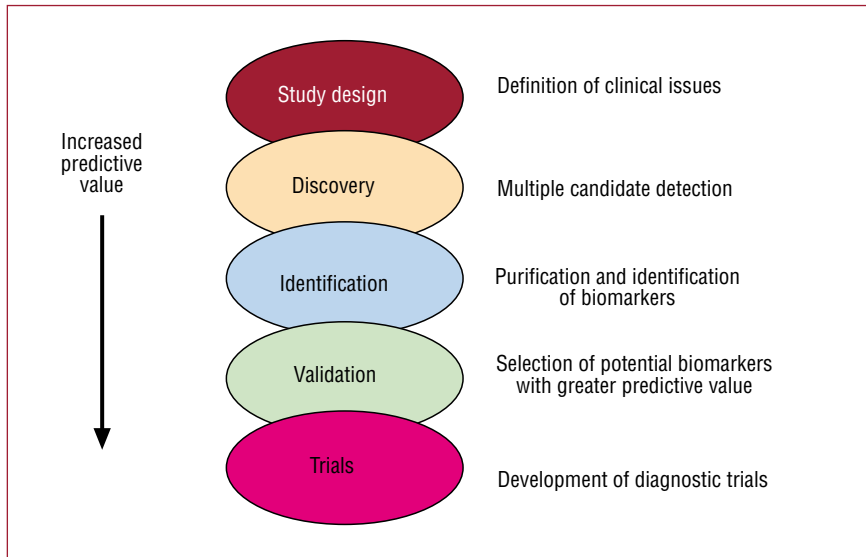
On the other hand, the AIM study assessed circulating sFas/sFasL concentrations in 1087 subjects at high cardiovascular risk. A reduction was observed in sFasL concentrations and an increase in sFas concentrations in patients at high cardiovascular risk, which could indicate that both proteins could be early markers of vascular injury. Unfortunately, the study was not designed to assess cardiovascular events due to the short follow-up time, which means that the predictive value of sFas and sFasL should be tested in future studies.<sup>73</sup>

The complex nature of the atherothrombotic process calls for the development of new technologies to help in the discovery of new mediators involved in this disease. Although genomics and proteomics have not been reviewed here due to limitations of

**Figure 3.** The Fas ligand (FasL), a potential biomarker of endothelial dysfunction. FasL produces and releases endothelial cells in normal circumstances and its expression can negatively regulate cell extravasation by inducing apoptosis in leukocytes. When the endothelium is dysfunctional, different proinflammatory stimuli can reduce FasL expression and release by endothelial cells, which facilitates the entry of inflammatory cells during the early stages of atherosclerotic lesion development.



**Figure 4.** How to identify a biomarker.



space, they could be vital tools for identifying the genes and proteins that confer greater predisposition to cardiovascular events. Within this approach, it is of importance to explicitly define clinical issues in order to obtain biomarkers specific to the disease in question (Figure 4).

**Proteomic Approach to the Search for New Biomarkers**

Two examples of new potential biomarkers obtained through proteomic techniques are detailed below.

*Heat Shock Proteins*

Heat shock proteins (HSP) are a family of proteins that are present in most cells. Heat shock proteins act as intra-cellular chaperones assisting in

establishing the correct conformation of proteins, as well as the translocation of oligomers, but they also aid in the elimination of irreversibly damaged proteins. On the other hand, they can be secreted and detected in plasma. In different cardiovascular diseases, it has been found that HSP expression can be modulated both in the lesion and in plasma.<sup>74,75</sup> Several studies have analyzed circulating quantities of different HSP. Thus, HSP60 can be a marker of atherosclerosis.<sup>76,77</sup> Among the hypotheses regarding the increase in circulating HSP60 in atherosclerosis, it has been proposed that it is potentially involved in the immunogenicity of certain bacteria<sup>78</sup> or in stress.<sup>79</sup> Heat shock protein 60 and HSP70 concentrations are increased in patients who have developed ACS, possibly in association with myocardial necrosis.<sup>80,81</sup> In contrast, HSP70 concentrations are reduced in patients with atherosclerosis.<sup>44</sup> Thus, a previous study analyzed the possible prognostic value of circulating HSP70 in

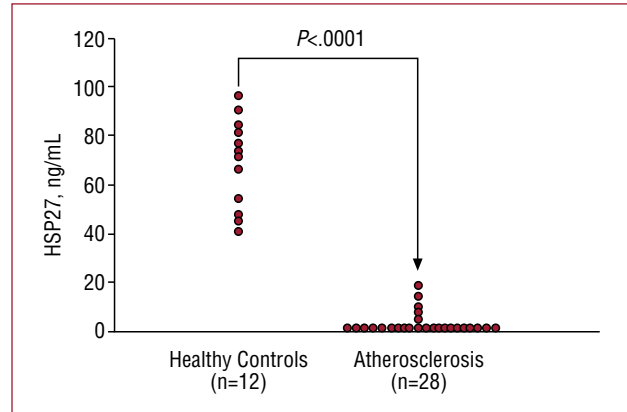
hypertensive patients, and an inverse correlation was found between HSP70 concentrations and carotid intima-media thickness.<sup>82</sup> The authors proposed that high HSP70 concentrations could indicate an antiatherogenic state within the blood vessels. Subsequently, it has been observed that the increase in HSP70 concentrations is associated with less risk of coronary heart disease and fewer affected vessels.<sup>83</sup>

A proteomic approach has identified HSP27 as a protein that is produced in great quantities by healthy arteries and whose production decreases to almost undetectable concentrations as the complexity of atherosclerotic plaque increases. Subsequently, a study was conducted which assessed HSP27 concentrations in a group of patients with carotid atherosclerosis and in healthy subjects of the same age and sex. Circulating HSP27 concentrations were significantly reduced in the experimental group compared to the control group<sup>84</sup> (Figure 5). Although these preliminary data should be validated in a broader group of patients, and in various stages of cardiovascular disease, they indicate that low HSP27 concentrations could serve as a diagnostic marker of advanced atherosclerotic disease. On the other hand, its possible prognostic value has only been tested in a study which compared baseline HSP27 concentrations among 225 women in the Women's Health Study who developed cardiovascular events over a 6-year follow-up period and among 225 women who did not develop them. This prospective study found that the baseline HSP27 concentrations were not associated with the development of cardiovascular events.<sup>85</sup>

#### *Tumor Necrosis Factor-Like Weak Inducer of Apoptosis*

The tumor necrosis factor-like weak inducer of apoptosis (TWEAK, Apo3L, TNFSF12) belongs to the TNF superfamily.<sup>86</sup> It is thought that this superfamily is a source of therapeutic targets that may be useful in the management of complex disease. These cytokines are involved in multiple biological responses, such as inflammation, the immune response and tissue repair.<sup>87</sup> The ligands of this family are expressed as type II transmembrane proteins that, in many cases, can be processed in secreted proteins of smaller size with biological activity.<sup>88,89</sup> Every ligand can bind to one or more members of the TNF superfamily, many of which are type I or type III transmembrane proteins.<sup>88,89</sup> Once the ligand binds to the receptor, different signaling cascades are activated that stimulate multiple biological responses.

Tumor necrosis factor-like weak inducer of apoptosis is widely expressed in different tissues, such as the pancreas, intestine, heart, brain, lung,



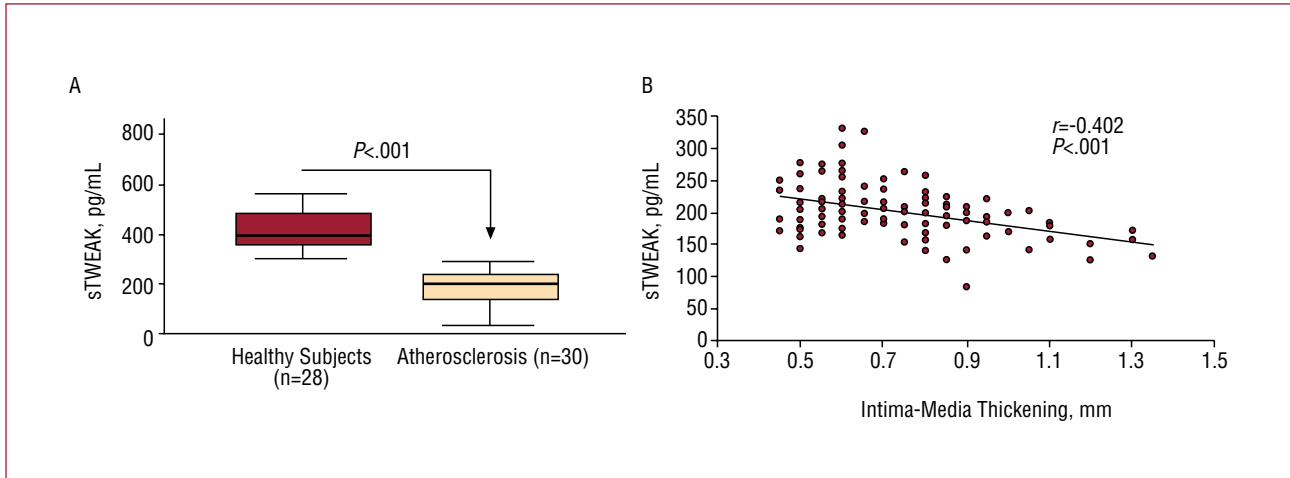
**Figure 5.** HSP27, a potential diagnostic biomarker of atherosclerosis. HSP27 plasma concentrations in patients with carotid atherosclerosis (n=28) and healthy controls (n=12). By permission of *Circulation*.<sup>84</sup>

ovary, and skeletal muscle, and to a lesser extent in the liver and kidney.<sup>86-90</sup> There is evidence that this protein could be involved in the pathogenesis of different diseases such as atherosclerosis, ictus, rheumatoid arthritis, autoimmune kidney damage, acute kidney damage, and cancer.<sup>91,92</sup> Depending on the cell type analyzed, TWEAK can stimulate proliferation,<sup>93</sup> survival,<sup>94</sup> migration,<sup>95</sup> cell growth,<sup>96</sup> and apoptosis.<sup>86</sup> Furthermore, it can stimulate<sup>91</sup> or inhibit<sup>97</sup> cell differentiation. Finally, TWEAK can induce the expression of multiple proinflammatory proteins.<sup>98,99</sup>

The SELDI-TOF technology has been used to identify soluble TWEAK (sTWEAK) as a potential marker of atherosclerosis and that is secreted in greater quantities by healthy arteries than by atherosclerotic plaques.<sup>100</sup> Quantification of sTWEAK in plasma showed that its concentrations are reduced in patients with carotid atherosclerosis compared to those in healthy subjects. Finally, sTWEAK demonstrated an inverse correlation with intima-media thickness in asymptomatic patients, which indicates that this protein could be a marker of subclinical atherosclerosis (Figure 6). These results were subsequently confirmed by Kralisch et al,<sup>101</sup> who found that patients with chronic kidney disease or with type 2 diabetes mellitus—2 disorders associated with high risk of cardiovascular disease—presented reduced circulating sTWEAK concentrations.

Furthermore, it has recently been shown that sTWEAK plasma concentrations predict total and cardiovascular mortality in patients undergoing hemodialysis.<sup>102</sup> These results were more evident when the sTWEAK plasma concentrations were concurrent with an inflammatory environment in the patient (increased IL-6 plasma concentrations).





**Figure 6.** TWEAK, a biomarker of subclinical atherosclerosis. The tumor necrosis factor-like weak inducer of apoptosis (TWEAK) plasma concentrations are reduced in patients with atherosclerosis (carotid stenosis >70%) (A) and are negatively correlated with carotid intima-media thickness in asymptomatic subjects (n=106) (B). With permission of *Arterioscler Thromb Vasc Biol.*<sup>100</sup>

All these results appear to indicate that sTWEAK may be a new diagnostic and prognostic biomarker of atherosclerosis.

## CONCLUSIONS

Predicting cardiovascular risk is one of the big challenges of modern medicine. Among the classic biomarkers reviewed, in addition to the CRP data, the best results are provided by the CD40 ligand, due to its independence from other variables and because these molecules participate in the pathophysiology of atherothrombosis. In this regard, soluble CD40L concentrations seem to be more effective in assessing risk in acute situations than in primary prevention. However, there is still a lack of standardization regarding determining concentrations and these clearly vary from one study to another. This is one of the factors that must be solved in order to address the use of CD40L as a risk marker in standard clinical practice.

The search for new biomarkers using proteomic techniques will enable the identification of new proteins that may play a key role in the development of the disease. Those proteins should have little variability and they should be able to be analyzed using standard techniques with low investments in time and cost. Finally, the use of a set of biomarkers (a multi-marker strategy) will provide more information on the degree of disease in individuals, as well as their prognosis and response to a treatment. The use of a multi-marker strategy in combination with non-invasive imaging could provide the key to the prevention of cardiovascular disease in the future.

## REFERENCES

- Murray CJ, López AD. Mortality by cause for eight regions of the world: global burden of disease study. *Lancet*. 1997;349:1269-76.
- Steinberg D. Atherogenesis in perspective: hypercholesterolemia and inflammation as partner in crime. *Nat Med*. 2002;11:1211-7.
- Vivanco F, Martín-Ventura JL, Duran MC, Barderas MG, Blanco-Colio L, Dardé VM, et al. Quest for novel cardiovascular biomarkers by proteomic analysis. *J Proteome Res*. 2005;4:1181-91.
- Ridker PM, Hennekens CH, Roitman-Johnson B, Stampfer MJ, Allen J. Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. *Lancet*. 1998;351:88-92.
- Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N Engl J Med*. 2000;342:836-43.
- Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM, et al. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. *Circulation*. 1997;96:4219-25.
- Ridker PM, Buring JE, Rifai N. Soluble P-selectin and the risk of future cardiovascular events. *Circulation*. 2001;103:491-5.
- Blankenberg S, Rupprecht HJ, Bickel C, Peetz D, Hafner G, Tiret L, et al. Circulating cell adhesion molecules and death in patients with coronary artery disease. *Circulation*. 2001;104:1336-42.
- Mulvihill NT, Foley JB, Murphy RT, Curtin R, Crean PA, Walsh M. Risk stratification in unstable angina and non-Q wave myocardial infarction using soluble cell adhesion molecules. *Heart*. 2001;85:623-7.
- Malik I, Danesh J, Whincup P, Bhatia V, Papacosta O, Walker M, et al. Soluble adhesion molecules and prediction of coronary heart disease: a prospective study and meta-analysis. *Lancet*. 2001;358:971-6.
- Romano M, Mezzetti A, Marulli C, Ciabattini G, Febo F, Di Lenno S, et al. Fluvastatin reduces soluble P-selectin and ICAM-1 levels in hypercholesterolemic patients: role of nitric oxide. *J Investing Med*. 2000;48:183-9.

12. Jilma B, Joukhadar C, Derhaschnig U, Rassoul F, Richter V, Wolzt M, et al. Levels of adhesion molecules do not decrease after 3 months of statin therapy in moderate hypercholesterolaemia. *Clin Sci*. 2003;104:189-93.
13. Blanco-Colio LM, Martín-Ventura JL, de Teresa E, Farsang C, Gaw A, Gensini G, et al. Elevated ICAM-1 and MCP-1 plasma levels in subjects at high cardiovascular risk are diminished by atorvastatin treatment. Atorvastatin on Inflammatory Markers study: a substudy of Achieve Cholesterol Targets Fast with Atorvastatin Stratified Titration. *Am Heart J*. 2007;153:881-8.
14. Cesari M, Penninx BW, Newman AB, Kritchevsky SB, Nicklas BJ, Sutton-Tyrrell K, et al. Inflammatory markers and onset of cardiovascular events: results from the Health ABC study. *Circulation*. 2003;108:2317-22.
15. Biasucci LM, Liuzzo G, Fantuzzi G, Caligiuri G, Rebuffi AG, Ginnetti F, et al. Increasing levels of interleukin (IL)-1Ra and IL-6 during the first two days of hospitalization in unstable angina are associated with increased risk of in-hospital coronary events. *Circulation*. 1999;99:2079-84.
16. Koukkunen H, Penttilä K, Kemppainen A, Halinen M, Penttilä I, Rantanen T, et al. C reactive protein, fibrinogen, interleukin-6 and tumour necrosis factor-alpha in the prognostic classification of unstable angina pectoris. *Ann Med*. 2001;33:37-47.
17. Lindmark E, Diderholm E, Wallentin L, Siegbahn A. Relationship between interleukin 6 and mortality in patients with unstable coronary artery disease: effects of an early invasive or noninvasive strategy. *JAMA*. 2001;286:2107-13.
18. Deo R, Khera A, McGuire DK, Murphy SA, Meo Neto J de P, Morrow DA, et al. Association among plasma levels of monocyte chemoattractant protein-1, traditional cardiovascular risk factors, and subclinical atherosclerosis. *J Am Coll Cardiol*. 2004;44:1812-8.
19. de Lemos JA, Morrow DA, Sabatine MS, Murphy SA, Gibson CM, Antman EM, et al. Association between plasma levels of monocyte chemoattractant protein-1 and long-term clinical outcomes in patients with acute coronary syndromes. *Circulation*. 2003;107:690-5.
20. Martín-Ventura JL, Muñoz-García B, Blanco-Colio LM, Martín-Conejero A, Madrigal-Matute J, Vega M, et al. Treatment with amlodipine and atorvastatin has additive effect on blood and plaque inflammation in hypertensive patients with carotid atherosclerosis. *Kidney Int Suppl*. 2008;S71-4.
21. Martín-Ventura JL, Blanco-Colio LM, Gómez-Hernández A, Muñoz-García B, Vega M, Serrano J, et al. Intensive treatment with atorvastatin reduces inflammation in mononuclear cells and human atherosclerotic lesions in one month. *Stroke*. 2005;36:1796-800.
22. Tsimikas S, Willerson JT, Ridker PM. C-reactive protein and other emerging blood biomarkers to optimize risk stratification of vulnerable patients. *J Am Coll Cardiol*. 2006;47 Suppl: C19-31.
23. Casas JP, Shah T, Hingorani AD, Danesh J, Pepys MB. C-reactive protein and coronary heart disease: a critical review. *J Intern Med*. 2008;264:295-314.
24. Biasucci LM, Liuzzo G, Grillo RL, Caligiuri G, Rebuffi AG, Buffon A, et al. Elevated levels of C-reactive protein at discharge in patients with unstable angina predict recurrent instability. *Circulation*. 1999;99:855-60.
25. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Plasma concentration of C-reactive protein and risk of developing peripheral vascular disease. *Circulation*. 1998;97:425-8.
26. Haverkate F, Thompson SG, Pyke SD, Gallimore JR, Pepys MB. Production of C-reactive protein and risk of coronary events in stable and unstable angina. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. *Lancet*. 1997;349:462-6.
27. Retterstol L, Eikvar L, Bohn M, Bakken A, Erikssen J, Berg K. C-reactive protein predicts death in patients with previous premature myocardial infarction—a 10-year follow-up study. *Atherosclerosis*. 2002;160:433-40.
28. Ueda S, Ikeda U, Yamamoto K, Takahashi M, Nishinaga M, Nago N, et al. C-reactive protein as a predictor of cardiac rupture after acute myocardial infarction. *Am Heart J*. 1996;131:857-60.
29. Ridker PM. Clinical application of C-reactive protein for cardiovascular disease detection and prevention. *Circulation*. 2003;107:363-9.
30. Koenig W, Sund M, Fröhlich M, Fischer HG, Löwel H, Döring A, et al. C-reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men: results from the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) Augsburg Cohort Study, 1984 to 1992. *Circulation*. 1999;99:237-42.
31. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N Engl J Med*. 2002;347:1557-65.
32. Ridker PM, Rifai N, Pfeffer MA, Sacks F, Braunwald E. Long-term effects of pravastatin on plasma concentration of C-reactive protein. The Cholesterol And Recurrent Events (CARE) Investigators. *Circulation*. 1999;100:230-5.
33. Ridker PM, Cannon CP, Morrow D, Rifai N, Rose LM, McCabe CH, et al. C-reactive protein levels and outcomes after statin therapy. *N Engl J Med*. 2005;352:20-8.
34. Ridker PM, Danielson E, Fonseca FAH, Genest J, Gotto AM, Kastelein JP, et al. Rosuvastatin to prevent vascular events in men and women with elevated c-reactive protein. *N Engl J Med*. 2008;359:2195-207.
35. Ridker PM. C-reactive protein and the prediction of cardiovascular events among those at intermediate risk: moving an inflammatory hypothesis toward consensus. *J Am Coll Cardiol*. 2007;49:2129-38.
36. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med*. 2004;350:1387-97.
37. Anderson JL. Lipoprotein-associated phospholipase A (2): An independent predictor of coronary artery disease events in primary and secondary prevention. *Am J Cardiol*. 2008;101:F23-33.
38. Davidson MH, Corson MA, Alberts MJ, Anderson JL, Gorelick PB, Jones PH, et al. Concensus panel recommendation for incorporating lipoprotein-associated phospholipase A2 testing into cardiovascular disease risk assessment guidelines. *J Am Coll Cardiol*. 2008;10:F51-7.
39. Lerman A, McConell JP. Lipoprotein-associated phospholipase A2: A risk marker or a risk factor. *Am J Cardiol*. 2008;101:F11-22.
40. Wilensky RL, Shi Y, Mohler ER 3rd, Hamamdziec D, Burgert ME, Li J, et al. Inhibition of lipoprotein-associated phospholipase A2 reduces complex coronary atherosclerotic plaque development. *Nat Med*. 2008;14:1059-66.
41. Serruys PW, García-García HM, Buszman P, Erne P, Verheye S, Aschermann M, et al. Effects of the direct lipoprotein-associated phospholipase A (2) inhibitor darapladib on human coronary atherosclerotic plaque. *Circulation*. 2008;118:1172-82.
42. Tayebjee MH, Nadar S, Blann AD, Gareth Beevers D, MacFadyen RJ, Lip GY. Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in hypertension and their relationship to cardiovascular risk and treatment: A substudy of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT). *Am J Hypertens*. 2004;17:764-9.
43. Sundstrom J, Evans JC, Benjamin EJ, Levy D, Larson MG, Sawyer DB, et al. Relations of plasma matrix metalloproteinase-9 to clinical cardiovascular risk factors

- and echocardiographic left ventricular measures. The Framingham Heart Study. *Circulation*. 2004;109:2850-6.
44. Martín-Ventura JL, Leclercq A, Blanco-Colio LM, Egido J, Rossignol P, Meilhac O, et al. Low plasma levels of HSP70 in patients with carotid atherosclerosis are associated with increased levels of proteolytic markers of neutrophil activation. *Atherosclerosis*. 2007;194:334-41.
  45. Beaudeux JL, Giral P, Bruckert E, Bernard M, Foglietti MJ, Chapman MJ. Serum matrix metalloproteinase-3 and tissue inhibitor of metalloproteinases-1 as potential markers of carotid atherosclerosis in infraclinical hyperlipidemia. *Atherosclerosis*. 2003;169:139-46.
  46. Noji Y, Kajinami K, Kawashiri MA, Todo Y, Horita T, Nohara A, et al. Circulating matrix metalloproteinases and their inhibitors in premature coronary atherosclerosis. *Clin Chem Lab Med*. 2001;39:380-4.
  47. Inokubo Y, Hanada H, Ishizaka H, Fukushi T, Kamada T, Okumura K. Plasma levels of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 are increased in the coronary circulation in patients with acute coronary syndrome. *Am Heart J*. 2001;141:211-7.
  48. Timms PM, Mannan N, Hitman GA, Noonan K, Mills PG, Syndercombe-Court D, et al. Circulating MMP9, vitamin D and variation in the TIMP-1 response with VDR genotype: mechanisms for inflammatory damage in chronic disorders? *Q J Med*. 2002;95:787-96.
  49. Blankenberg S, Rupprecht HJ, Poirier O, Bickel C, Smieja M, Hafner G, et al. for the AtheroGene Investigators. Plasma concentrations and genetic variation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease. *Circulation*. 2003;107:1579-85.
  50. Montaner J, Molina CA, Monasterio J, Abilleira S, Arenillas JF, Ribó M, et al. Matrix metalloproteinase-9 pretreatment level predicts intracranial hemorrhagic complications after thrombolysis in human stroke. *Circulation*. 2003;107:598-603.
  51. Host NB, Hansen SS, Jensen LT, Husum D, Nielsen JD. Thrombolytic therapy of acute myocardial infarction alters collagen metabolism. *Cardiology*. 1994;85:323-33.
  52. Koh KK, Son JW, Ahn JY, Jin DK, Kim HS, Choi YM, et al. Comparative effects of diet and statin on NO bioactivity and matrix metalloproteinases in hypercholesterolemic patients with coronary artery disease. *Arterioscler Thromb Vasc Biol*. 2002;22:e19-23.
  53. Mach F, Schönbeck U, Sukhova GK, Bourcier T, Bonnefoy JY, Pober JS, et al. Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for CD40-CD40 ligand signaling in atherosclerosis. *Proc Natl Acad Sci U S A*. 1997;94:1931-6.
  54. Schönbeck U, Libby P. CD40 signaling and plaque instability. *Circ Res*. 2001;89:1092-103.
  55. Schönbeck U, Varo N, Libby P, Buring J, Ridker PM. Soluble CD40L and cardiovascular risk in women. *Circulation*. 2001;104:2266-8.
  56. Garlich CD, Eskafi S, Raaz D, Schmidt A, Ludwig J, Herrmann M, et al. Patients with acute coronary syndromes express enhanced CD40 ligand/CD154 on platelets. *Heart*. 2001;86:649-55.
  57. Heeschen C, Dimmeler S, Hamm CW, van den Brand MJ, Boersma E, Zeiher AM, et al. Soluble CD40 ligand in acute coronary syndromes. *N Engl J Med*. 2003;348:1104-11.
  58. Andre P, Prasad KS, Denis CV, He M, Papalia JM, Hynes RO, et al. CD40L stabilizes arterial thrombi by a beta3 integrin-dependent mechanism. *Nat Med*. 2002;8:247-52.
  59. Varo N, de Lemos JA, Libby P, Morrow DA, Murphy SA, Nuzzo R, et al. Soluble CD40L: risk prediction after acute coronary syndromes. *Circulation*. 2003;108:1049-52.
  60. Hwang YS, Tsai WC, Lu YH, Lin CC, Chen YF. Effect of atorvastatin on the expression of CD40L and P-selectin in patients with hypercholesterolemia. *Am J Cardiol*. 2004;94:364-6.
  61. Semb AG, van Wissen S, Ueland T, Smilde T, Waehre T, Tripp MD, et al. Raised serum levels of soluble CD40 ligand in patients with familial hypercholesterolemia: downregulatory effect of statin therapy. *J Am Coll Cardiol*. 2003;41:275-9.
  62. Kinlay S, Schwartz GG, Olsson AG, Rifai N, Sasiela WJ, Szarek M, et al. Effect of atorvastatin on risk of recurrent cardiovascular events after an acute coronary syndrome associated with high soluble CD40 ligand in the myocardial ischemia reduction with aggressive cholesterol lowering (MIRACL) study. *Circulation*. 2004;110:386-91.
  63. Blanco-Colio LM, Martín-Ventura JL, de Teresa E, Farsang C, Gaw A, Gensini G, et al. Atorvastatin decreases elevated soluble CD40L in subjects at high cardiovascular risk. Atorvastatin on inflammatory markers study: a substudy of ACTFAST. *Kidney Int*. 2008;74:S60-3.
  64. Cheng J, Zhou T, Liu C, Shapiro JC, Braeur MJ, Kiefer MC, et al. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. *Science*. 1994;263:1759-62.
  65. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, et al. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell*. 1991;66:233-43.
  66. Okumura K, Yagita H. Metalloproteinase-mediated release of human Fas ligand. *J Exp Med*. 1995;182:1777-83.
  67. Toyozaki T, Hiroe M, Saito T, Iijima Y, Takano H, Hiroshima K, et al. Levels of soluble Fas in patients with myocarditis, heart failure of unknown origin, and healthy volunteers. *Am J Cardiol*. 1998;81:798-800.
  68. Yamaguchi S, Yamaoka M, Okuyama M, Nitobe J, Fukui A, Shirakabe M, et al. Elevated circulating levels and cardiac secretion of soluble Fas ligand in patients with congestive heart failure. *Am J Cardiol*. 1999;83:1500-3.
  69. Shimizu M, Fukuo K, Nagata S, Sahara T, Okuro M, Fujii K, et al. Increased plasma levels of the soluble form of Fas ligand in patients with acute myocardial infarction and unstable angina pectoris. *J Am Coll Cardiol*. 2002;39:585-90.
  70. Blanco-Colio LM, Martín-Ventura JL, Sol JM, Díaz C, Hernández G, Egido J. Decreased circulating Fas ligand in patients with familial combined hyperlipidemia or carotid atherosclerosis: normalization by atorvastatin. *J Am Coll Cardiol*. 2004;43:1188-94.
  71. Sata M, Walsh K. TNF- $\alpha$  regulation of Fas ligand expression on the vascular endothelium modulates leukocyte extravasation. *Nat Med*. 1998;4:415-20.
  72. Blanco-Colio LM, Martín-Ventura JL, Tuñón J, García-Camarero T, Berrazueta JR, Egido J. Soluble Fas ligand plasma levels are associated with forearm reactive hyperemia in subjects with coronary artery disease: a novel biomarker of endothelial function? *Atherosclerosis*. 2008;201:407-12.
  73. Blanco-Colio LM, Martín-Ventura JL, de Teresa E, Farsang C, Gaw A, Gensini G, et al. Increased soluble Fas plasma levels in subjects at high cardiovascular risk: Atorvastatin on Inflammatory Markers (AIM) study, a substudy of ACTFAST. *Arterioscler Thromb Vasc Biol*. 2007;27:168-74.
  74. Xu Q. Role of heat shock proteins in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2002;22:1547-59.
  75. Pockley AG. Heat shock proteins, inflammation, and cardiovascular disease. *Circulation*. 2002;105:1012-7.
  76. Pockley AG, Wu R, Lemne C, Kiessling R, de Faire U, Frostegard J. Circulating HSP60 is associated with early cardiovascular disease. *Hypertension*. 2000;36:303-7.
  77. Xu Q, Schett G, Perschinka H, Mayr M, Egger G, Oberhollenzer F, et al. Serum soluble HSP60 is elevated in subjects with atherosclerosis in a general population. *Circulation*. 2000;102:14-20.
  78. Rothenbacher D, Hoffmeister A, Bode G, Miller M, Koenig W, Brenner H. Helicobacter pylori heat shock protein 60 and risk of coronary heart disease: a case control study

- with focus on markers of systemic inflammation and lipids. *Atherosclerosis*. 2001;156:193-9.
79. Lewthwaite J, Owen N, Coates A, Henderson B, Steptoe A. Circulating human heat shock protein 60 in the plasma of British civil servants: relationship to physiological and psychosocial stress. *Circulation*. 2002;106:196-201.
  80. Zhang X, He M, Cheng L, Chen Y, Zhou L, Zeng H, et al. Elevated heat shock protein 60 levels are associated with higher risk of coronary heart disease in Chinese. *Circulation*. 2008;118:2687-93.
  81. Dybdahl B, Slørdahl SA, Waage A, Kierulf P, Espevik T, Sundan A. Myocardial ischaemia and the inflammatory response: release of heat shock protein 70 after myocardial infarction. *Heart*. 2005;91:299-304.
  82. Pockley AG, Georgiades A, Thulin T, de Faire U, Frostegard J. Serum HSP70 levels predict the development of atherosclerosis in subjects with established hypertension. *Hypertension*. 2003;42:235-8.
  83. Zhu J, Quyyumi AA, Wu H, Csako G, Rott D, Zalles-Ganley A, et al. Increased serum levels of heat shock protein 70 are associated with low risk of coronary artery disease. *Arterioscler Thromb Vasc Biol*. 2003;23:1055-9.
  84. Martín-Ventura JL, Duran MC, Blanco-Colio LM, Meilhac O, Leclercq A, Michel JB, et al. Identification by a differential proteomic approach of heat shock protein 27 as a potential marker of atherosclerosis. *Circulation*. 2004;110:2216-9.
  85. Kardys I, Rifai N, Meilhac O, Michel JB, Martín-Ventura JL, Buring JE, et al. Plasma concentration of heat shock protein 27 and risk of cardiovascular disease: a prospective, nested case-control study. *Clin Chem*. 2008;54:139-46.
  86. Chicheportiche Y, Bourdon PR, Xu H, Hsu Y, Scott H, Hession C, et al. TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. *J Biol Chem*. 1997;272:32401-10.
  87. Foster D, Parrish-Novak J, Fox B, Xu W. Cytokine-receptor pairing: accelerating discovery of cytokine function. *Nature Rev Drug Discov*. 2004;3:160-70.
  88. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell*. 2001;104:487-501.
  89. Bodmer J, Schneider P, Tschopp J. The molecular architecture of the TNF superfamily. *Trends Biochem Sci*. 2002;27:19-26.
  90. Marsters SA, Sheridan JP, Pitti RM, Brush J, Goddard A, Ashekenazi A. Identification of a ligand for the death-domain containing receptor Apo3. *Curr Biol*. 1998;8:525-8.
  91. Zheng TS, Burkly LC. No end in site: TWEAK/Fn14 activation and autoimmunity associated end-organ pathologies. *J Leukocyte Biology*. 2008 [In press].
  92. Winkles JA. The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting. *Nat Rev Drug Discov*. 2008;7:173-8.
  93. Donohue PJ, Richards CM, Brown SA, Hanscom HN, Buschman J, Thangada S, et al. TWEAK is an endothelial cell growth and chemotactic factor that also potentiates FGF-2 and VEGF-A mitogenic activity. *Arterioscler Thromb Vasc Biol*. 2003;23:594-600.
  94. Jakubowski A, Browning B, Lukashev M, Sizing I, Thompson JS, Benjamin CD, et al. Dual role for TWEAK in angiogenic regulation. *J Cell Sci*. 2002;115:267-74.
  95. Tran NL, McDonough WS, Donohue PJ, Winkles JA, Berens TJ, Ross KR, et al. The human Fn14 receptor gene is up-regulated in migrating glioma cells in vitro and overexpressed in advanced glial tumors. *Am J Pathol*. 2003;162:1313-21.
  96. Wiley SR, Winkles JA. TWEAK, a member of the TNF superfamily, is a multifunctional cytokine that binds the TweakR/Fn14 receptor. *Cytokine Growth Factor Rev*. 2003;14:241-9.
  97. Xu H, Okamoto A, Ichikawa J, Ando T, Tasaka K, Masuyama K, et al. TWEAK/Fn14 interaction stimulates human bronchial epithelial cells to produce IL-8 and GM-CSF. *Biochem Biophys Res Commun*. 2004;318:422-7.
  98. Kim SH, Kang YJ, Kim WJ, Woo DK, Lee Y, Kim DI, et al. TWEAK can induce pro-inflammatory cytokines and matrix metalloproteinase-9 in macrophages. *Circ J*. 2004;68:396-9.
  99. Saitoh T, Nakayama M, Nakano H, Yagita H, Yamamoto N, Yamaoka S. TWEAK induces NF-kappaB2 p100 processing and long lasting NF-kappaB activation. *J Biol Chem*. 2003;278:36005-12.
  100. Blanco-Colio LM, Martín-Ventura JL, Muñoz-García B, Meilhac O, Michel JB, Páramo J, et al. Identification of soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) as a possible biomarker of subclinical atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2007;27:916-22.
  101. Kralisch S, Ziegelmeier M, Bachmann A, Seeger J, Lössner U, Blüher M, et al. Serum levels of the atherosclerosis biomarker sTWEAK are decreased in type 2 diabetes and end-stage renal disease. *Atherosclerosis*. 2008;199:440-4.
  102. Carrero JJ, Ortiz A, Qureshi AR, Martín-Ventura JL, Bárány P, Heimbürger O, et al. Additive effects of soluble TWEAK and inflammation on mortality in hemodialysis patients. *Clin J Am Soc Nephrol*. 2009;4:110-8.