DEPARTAMENT DE MEDICINA

PROGNOSTIC VALUE OF CIRCULATING PREGNANCY-ASSOCIATED PLASMA PROTEIN-A (PAPP-A) AND PROFORM OF EOSINOPHIL MAJOR BASIC PROTEIN (PRO-MBP) LEVELS IN PATIENTS WITH CHRONIC STABLE ANGINA PECTORIS.

LUCIANO CONSUEGRA-SÁNCHEZ

UNIVERSITAT DE VALÈNCIA
Servei de Publicacions
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Aquesta Tesi Doctoral va ser presentada a València el dia 20 de gener de 2010 davant un tribunal format per:

- Dr. Cándido Martín Luengo
- Dr. José Millet Roig
- Dr. Jose María Cruz Fernández
- Dr. Alfredo Bardají Ruiz
- Dr. Francisco Javier Chorro Gascó

Va ser dirigida per:
Dr. Juan Sanchis Forés

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Luciano Consuegra-Sánchez

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Departament de Medicina

"Prognostic Value of Circulating Pregnancy-Associated Plasma Protein–A (PAPP-A) and Proform of Eosinophil Major Basic Protein (pro-MBP) Levels in Patients with Chronic Stable Angina Pectoris"

"Valor Pronóstico de los Niveles de Proteína Plasmática Asociada a Embarazo A circulante (PAPP-A) y Proforma de la Proteína Eosinofílica Básica Mayor (pro-MBP) en Pacientes con Angina Crónica Estable"

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INDEX.

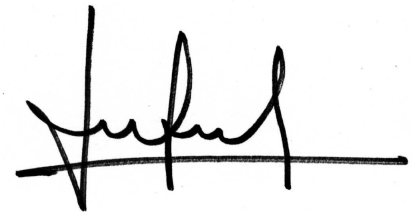
El **Dr. Juan Sanchis Forés**, Jefe Clínico de Cardiología del Hospital Clínico de Valencia.

CERTIFICA:

Que D. Luciano Consuegra Sánchez, con Grado de Licenciado en Medicina y Cirugía, ha realizado bajo mi dirección el trabajo para la elaboración de su Tesis Doctoral titulada**:** "Prognostic Value of Circulating Pregnancy-Associated Plasma Protein–A (PAPP-A) and Proform of Eosinophil Major Basic Protein (pro-MBP) Levels in Patients with Chronic Stable Angina Pectoris".

Tras su redacción, la presente memoria ha sido revisada por mí, encontrándola conforme para ser presentada y aspirar al grado de Doctor en Medicina ("Doctor Europeus") ante el tribunal designado.

Para que conste, en cumplimiento de las disposiciones vigentes, expido el presente documento en Valencia a 1 de Septiembre de 2009.



Fdo. Dr. Juan Sanchis Forés.

UNIVERSITÀ E AZIENDA OSPEDALIERA DI PERUGIA

Cardiologia

e

Fisiopatologia Cardiovascolare

Direttore
Prof. Giuseppe Ambrosio

To whom it may concern
October 14, 2008

RE: Luciano Consuegra Sánchez, MD

This letter is to support Dr. Luciano Consuegra Sánchez' application for the degree of *European Doctor*.

I have read his thesis "Prognostic Value of Circulating Pregnancy-Associated Plasma Protein–A (PAPP-A) and Proform of Eosinophil Major Basic Protein (pro-MBP) Levels in Patients with Chronic Stable Angina Pectoris". The thesis concerns the measurement of concentration of a biomarker, pregnancy-associated plasma protein A, in human blood, and its association with coronary artery disease progression in patients. It is the results of obviously intense work, performed with state of the art methodologies in collaboration with investigators who have long standing interest and solid reputation in the field of biomarker assay and cardiovascular disease.

In my opinion, this is an original and interesting piece of work, the results of which have potentially relevant implications with respect to prognostic stratification of patients with coronary artery disease, and to management of this frequent and clinically important conditions. Results from this endeavor have been presented at major international cardiology meetings, and published in peer-reviewd cardiology journals. I have no reservations in recommending the thesis for the award of *European Doctor.*

Please, do not hesitate in contacting me if I can be of further help with this application.
Sincerely,

Giuseppe Ambrosio, M.D., Ph.D., FESC, FACC, FAHA
Director
Division of Cardiology
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St George's
University of London

Medicine, Biomedical Sciences, Health and Social Care Sciences

14 October 2008

To whom it may concern

Cranmer Terrace
London SW17 ORE
www.sgul.ac.uk

Luciano Consuegra Sánchez

I write in support of the application for the degree of European MD by Dr Luciano Consuegra Sánchez. The thesis concerns the measurement of a biomarker, pregnancy-associated plasma protein A, and its association with coronary artery disease progression. I have evaluated this thesis and have concluded that it is an original piece of work, the results of which have potential in monitoring coronary artery disease and its management. Publications and presentations have resulted from the work.

I have no hesitation in recommending the thesis for the award of European MD.

Yours faithfully

David W Holt
BSc. PhD, DSc (Med), CSci, EurClin Chem, FESC, FRCPath
Professor of Bioanalytics

Professor David W Holt – Director
Analytical Unit
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3. MENTIONS.

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(1) This investigation was presented in part in *United States* at the 56th Annual Scientific Session of the American College of Cardiology, New Orleans, March 2007 (*Consuegra-Sanchez L, Petrovic I, Cosin-Sales J, Holt DW, Christiansen M, Kaski JC. Prognostic Value of Circulating Pregnancy-Associated Plasma Protein–A (PAPP-A) and Proform of Eosinophil Major Basic Protein (pro-MBP) Levels in Patients with Chronic Stable Angina Pectoris. J Am Coll Cardiol 2007;49(supplement 2);9:B1-B60*).

1:00 p.m.

905-217

Circulating Pregnancy-associated Plasma Protein-A Levels And Long-term Prognosis In Patients With Chronic Stable Angina Pectoris

LUCIANO CONSUEGRA-SANCHEZ, IVANA PETROVIC, JUAN COSIN-SALES, DAVID W HOLT, JUAN C KASKI, JUAN C KASKI, LONDON, United Kingdom

Background: Pregnancy-associated plasma protein-A (PAPP-A) levels are increased in patients with acute coronary syndromes and in chronic stable angina (CSA), patients with complex coronary artery stenoses and extensive coronary artery disease. PAPP-A is a marker of clinical outcome in patients with ACS but little is known about the prognostic value of PAPP-A in "real-life" CSA. Only a single report in a small number of selected patients has been published to date.
Methods: We assessed whether PAPP-A levels predict long-term all-cause mortality in 663 consecutive patients with CSA (169 women [25.5 %]; mean age 62.9 ± 9.7 years) undergoing routine diagnostic coronary angiography who were followed for a median of 8.8 years (interquartile range 3 - 10.6 years). Clinical, angiographic (coronary vessel disease and Sullivan extension score), and biochemical variables were assessed in all patients at study entry.
Results: 106 patients (16 %) died during follow-up. After adjusting for confounders we found, on a Cox proportional hazards model, that increased PAPP-A concentration (> 4.8 mIU/L) was an independent predictor of the occurrence of all-cause mortality (HR 1.791, 95% CI 1.038-3.088, p = 0.036). Analysis of PAPP-A quartiles in relation to survival showed the existence of a threshold effect whereby patients in the lowest quartile (PAPP-A ≤ 4.6 mIU/L) (group 1) had better clinical outcome than patients in quartiles 2, 3 and 4 (group 2). No differences in mortality were found among patients in quartiles 2 to 4. Compared to group 1, group 2 showed a higher prevalence of elderly people (p = 0.002), male gender (p < 0.001) and hypertension (p = 0.054), as well a higher number of diseased vessels (p < 0.001) and higher Sullivan extension score (p < 0.001).
Conclusions: High PAPP-A levels (cut off point 4.8 mIU/L) predicted all-cause mortality during long-term follow-up in "real-life" patients with CSA. Thus PAPP-A measurements may be a useful marker of long-term cardiovascular risk in stable angina patients

(2) This investigation was presented in part in *Europe* at the 14th International Congress on Cardiovascular Pharmacotherapy, November 29th – 2nd December, 2007. Antalya, Turkey.

The present research was further awarded in that meeting: "*Young Investigator Award*" to Dr. Luciano Consuegra Sánchez.


ISCP International Society of Cardiovascular Pharmacotherapy

14th INTERNATIONAL CONGRESS ON CARDIOVASCULAR PHARMACOTHERAPY

Young Investigators' Award 2007

The International Society of Cardiovascular Pharmacotherapy is pleased to give this award to the winners of the best four presented abstracts.

Sanchez Luciano Consuegra

Circulating Pregnancy-Associated Plasma Protein-A Levels and Long-Term Prognosis in Patients with Chronic Stable Angina Pectoris

Antalya, Turkey

29 November - 2 December 2007

Awarded to: ISCP/OP 31

Date: 1 December 2007

Ali Oto, MD
President, ISCP 2007

A. Bayes de Luna, MD
President, ISCP

(3) The present research was published in *the following indexed & peer-reviewed* journals:

3.1 Consuegra-Sánchez L, Petrovic I, Cosin-Sales J, Holt DW, Christiansen M, Kaski JC. Prognostic Value of Circulating Pregnancy-Associated Plasma Protein–A (PAPP-A) and Proform of Eosinophil Major Basic Protein (pro-MBP) Levels in Patients with Chronic Stable Angina Pectoris. Clin Chim Acta 2008; 391:18-23.

3.2 Sanchis J, Bosch X, Bodí V, Bellera N, Núñez J, Benito B, Ordóñez J, Consuegra L, Heras M, Llàcer A. Combination of clinical risk profile, early exercise testing and circulating biomarkers for evaluation of patients with acute chest pain without ST-segment deviation or troponin elevation. Heart 2008;94:311-315.

3.3 Consuegra-Sánchez L, Fredericks S, Kaski JC. Pregnancy associated plasma protein-A (PAPP-A) and cardiovascular risk. Atherosclerosis 2009;203:346-52.

3.4 Consuegra-Sánchez L, Fredericks S, Kaski JC. Pregnancy associated plasma protein A: Has this biomarker crossed the

boundary from research to clinical practice? Drug News Perspect 2009 (accepted for publication, in press).

(4) The present research was supported by:

4.1 Beca de Investigación de la Fundación de Investigación del Hospital Clínico de Valencia (concedida 15 de Septiembre de 2005).

4.2 Ayuda para Estancia Corta en Centro Extranjero de la Sociedad Española de Cardiología (concedida 15 de Mayo de 2005).

4.3 Beca de Investigación Pfizer/Sociedad Española de Cardiología 2004 al trabajo: Estratificación del riesgo de los pacientes con dolor torácico y troponina normal. Valor de los nuevos marcadores serológicos (concedida 20 de Octubre de 2004).

4. ABREVIATIONS AND ACRONYMS.

IA = Injurious agents

LDL = Low density lipoprotein

PG = Proteoglycans

CSPG = Chondroitin Sulfate Proteoglycans

ECM = Extracellular matrix

SMC = Smooth muscle cells

EC = Endothelial cells

VCAM-1 = Vascular cell adhesion molecule 1

MMP-9 = Monocyte matrix metalloproteinase 9

IL = Interleukin

IFN = Interferon

HDL = High density lipoprotein

PAI-1 = Plasminogen activator inhibitor 1

CRP = C-reactive protein

CSA = Chronic stable angina

CAD = Coronary artery disease

ECG = Electrocardiogram

LVEF = Left ventricular ejection fraction

ACS = Acute coronary syndrome

PAPP-A = Pregnancy associated plasma protein A

ROC = Receiver operator characteristic

hs-CRP = High sensitivity C-reactive protein

Pro-MBP = Proform of eosinophil major basic protein

IGF-1 = Insulin-like growth factor 1

HR = Hazard ratio

CI = Confidence interval

c-Tn I/T = Cardiac troponin I/T

CK-MB = Creatine-kinase MB isoenzyme

OR = Odds ratio

MI = Myocardial infarction

SD = Standard deviation

IGFBP = Insulin-like growth factor binding protein

5. ABSTRACT.

Background: The search for markers to improve risk prediction for individuals at risk of developing serious cardiovascular events is ongoing. New markers of coronary artery disease progression have been identified in recent years, among which, circulating levels of pregnancy-associated plasma protein-A (PAPP-A) offer an interesting profile. PAPP-A may play a role in the development of atherosclerotic lesions and represent also a marker of atheromatous plaque instability and extent of cardiovascular disease. PAPP-A has been shown to be a marker of adverse outcome in the acute coronary syndrome.

The proform of eosinophil major basic protein (pro-MBP) is the endogenous inhibitor of the proteolytic activity of PAPP-A. PAPP-A levels and PAPP-A/pro-MBP ratio are increased in chronic stable angina (CSA) patients with complex coronary artery stenoses. Little is known however, about the long-term prognostic value of PAPP-A and pro-MBP in “real-life” CSA patients. We sought to assess whether

PAPP-A, pro-MBP and PAPP-A/pro-MBP levels predict long-term all-cause mortality in patients with CSA.

Methods: We recruited 663 consecutive patients (169 women [25.5 %]; mean age 62.9 ± 9.7 years) undergoing routine diagnostic coronary angiography. Samples for PAPP-A and pro-MBP were taken at study entry. Patients were followed for a median of 8.8 years (interquartile range 3 - 10.6 years).

Results: One hundred and six patients (16 %) died during follow-up. On a Cox proportional hazards model, increased PAPP-A concentration (> 4.8 mIU/L) was an independent predictor of the occurrence of all-cause mortality (HR 1.953, 95% CI 1.135-3.360, p = 0.016). Neither pro-MBP nor PAPP-A/pro-MBP ratio were markers of all-cause mortality (p = 0.45 and 0.54, respectively).

Conclusions: High PAPP-A levels (> 4.8 mIU/L) showed an association with all-cause mortality during long-term follow-up in patients with CSA.

Keywords: PAPP-A, pro-MBP, chronic stable angina, prognosis.

6. RESUMEN.

Antecedentes: La búsqueda de marcadores para mejorar la predicción de individuos en riesgo de desarrollar eventos cardiovasculares está en marcha. Recientemente se han identificado nuevos marcadores de progresión de la enfermedad coronaria, entre los cuales, la proteína plasmática asociada a embarazo tipo A (PAPP-A) presenta un perfil interesante. PAPP-A podría desempeñar un papel en el desarrollo de las lesiones ateroscleróticas, así como representar un marcador de inestabilidad de placa ateromatosa y extensión de la enfermedad aterosclerótica. Además PAPP-A es un marcador de eventos adversos en el contexto del síndrome coronario agudo.

La proforma de la proteína mayor básica eosinofílica (pro-MBP) es un inhibidor endógeno de la actividad proteolítica de PAPP-A. Los niveles de PAPP-A y del cociente PAPP-A/pro-MBP están aumentados en pacientes angina crónica estable que presentan lesiones coronarias complejas en la angiografía. Se desconoce el valor pronóstico a largo

plazo de los niveles de PAPP-A y pro-MBP en pacientes con angina crónica estable de la "práctica real".

Se pretendió en este estudio evaluar si los niveles de PAPP-A, pro-MBP y del cociente PAPP-A/pro-MBP predicen la mortalidad por cualquier causa a largo plazo en pacientes con angina crónica estable.

Métodos: Reclutamos 663 pacientes consecutivos (169 mujeres [25.5 %]; edad media 62.9 años ± 9.7 años) con angina crónica estable remitidos para angiografía coronaria diagnóstica. Se tomaron muestras para medir PAPP-A y pro-MBP al inicio del estudio. Los pacientes fueron seguidos por una mediana de tiempo de 8.8 años (rango intercuartílico 3 – 10.6 años).

Resultados: Ciento seis (16 %) pacientes murieron durante el seguimiento. La concentración de PAPP-A (> 4.8 mIU/L) fué un predictor independiente de la mortalidad por cualquier causa (HR 1.953, 95% CI 1.135-3.360, p = 0.016) en un modelo de riesgos proporcionales de Cox. Ni pro-MBP ni el cociente PAPP-A/pro-MBP fueron

marcadores de mortalidad por cualquier causa (p = 0.45 and 0.54, respectivamente).

Conclusiones: En el presente estudio, los niveles altos de PAPP-A superiores a 4.8 mIU/L se asociaron con la muerte por cualquier causa a largo plazo en pacientes con angina crónica estable.

Palabras clave: Proteína plasmática asociada a embarazo tipo A, proforma de la proteína mayor básica eosinofílica, angina crónica estable, pronóstico.

7. THE INFLAMMATORY HYPOTHESIS.

7.1. The "response-to-injury" concept (Fig. 1).

The response-to-injury hypothesis states that the initial event in the pathogenesis of atherosclerosis is injury to the endothelium (1). A variety of injurious agents (IA) produce an inflammatory response in which leucocytes, primarily monocytes, migrate to the area of injury (2). The result is retention and oxidation of lipoproteins and transformation of monocytes into macrophages that ingest lipid, particularly oxidized low density lipoproteins (LDL). These form the fatty streak that is an early objective sign of atherosclerosis (2). Important considerations in this theory are the precise nature of the IA, and the sequence of events that lead to the retention of lipid. Several studies in experimental animals have demonstrated that lipid retention occurs before the monocytes migrate into the intima (3), showing that the monocyte is not the cause of the lipid retention (4,5).

Previous studies demonstrated that the initial lesion in atherosclerosis is asymmetrical intimal thickening, the result of increased production of

sulfate-containing proteoglycans (PGs) - primarily Chondroitin Sulfate Proteoglycans (CSPG) and other forms of extracellular matrix (ECM) - by resident intimal smooth muscle cells (SMCs) in a focal area of the arterial wall (6–9). The IA, directly or indirectly, enters the arterial wall from the circulating blood, and then either stimulates or enters the resident SMC, the principal source of vascular PGs (10), to produce increased amounts of PGs and ECM. Walton (8) showed this mucoid thickening of the intima occurs before lipid infiltration and is composed primarily of collagen, PGs and ECM. Thus, although lipid accumulation in the artery wall is considered an early event in atherosclerosis, lipid retention is not the initiating event, and the fatty streak is not the first sign of atherosclerotic injury (11,12). This initial intimal thickening is not characterized by hypercellularity or proliferation of SMCs (13), as is seen in other types of vascular injury (14), but rather by relative acellularity, apparently due to the increased amounts of PGs and ECM without associated SMC proliferation (15). The relative acellularity noted in these early lesions is not believed to be due to massive cell death of resident intimal cells (16). Increased production of PG and

ECM, without an increase in the number of SMCs, is an unusual response to injury, suggesting a specific type of IA and/or a specific type of injury or effect on the SMC (17,18).

Whether the increase in PGs and ECM is a pathologic response and, therefore, to be prevented, or is a physiologic defensive, protective, or reparative response to the IA is not known (6,10). The fact that these intimal thickenings develop very early after wall injury and before lipid accumulation suggest this is a protective, healing, or defensive response (1,6). This view is supported by the knowledge that CSPG is required and is the predominant PG in normal wound repair (9). However, if this is a physiologic defence, it fails badly because the IA agent is not halted, proliferation of PGs and ECM continues, and resolution, healing, and stabilization do not occur. The disease continues to progress.

The ECM is a visco-elastic material containing primarily CSPG, a biochemically active scaffold that regulates arterial permeability, filtration, transport of plasma constituents, and regulation of wall metabolism and function (10). The increased amount of PGs produced by the SMC in response to various growth factors associated with

atherosclerotic injury have much longer side chains and form larger aggregates than do the PGs normally found in the artery wall (10,13,19). Thus, there is not only an increase in the PGs and ECM produced, but a change in the structure of the PGs in the areas of atherosclerotic injury. This change in PG structure is believed to alter the metabolic properties and biochemical function of the PGs and ECM, resulting in a disturbance in the transfer of substrates through the zone of injury, particularly alteration of interactions with lipoproteins (10,13,19,20). These structural and functional changes in the PGs as well as their turnover rate, are directly related to the rate of retention of lipid in the interstices of the ECM (8,9). The alteration in structure and the increased production of PGs suggest a pathologic component of the disease process, produced and altered, not as a physiologic defence (2,8), but for the specific purpose of retaining lipid, particularly LDL.

7.2. The "Intimal thickenings".

Stary et al. (21), believe many asymmetric intimal thickenings, termed Adaptive Intimal Thickening, reflect a physiologic adaptive response to

hemodynamic stress. They found this thickening at points of arterial bifurcation in infant human beings and animals. The authors point out that those physiologic thickenings may also be the site of atherosclerotic plaques. It may be difficult to distinguish thickenings that are physiologic adaptations from those that are pathologic, particularly in the early stages of atherosclerosis. These adaptive intimal thickenings are rich is PGs (21). Evidence of lipid retention, then, is a primary feature that distinguishes physiologic thickening from pathologic atherosclerosis (21). The presence of intimal thickening at points of bifurcation supports the view that these lesions are an adaptation to hemodynamic stresses, but the occurrence of the same lesions in areas without bifurcations, or areas of low or relatively low hemodynamic stress, suggests other factors are also involved. These other additional factors may be acting independently or in conjunction with hemodynamic stresses to transform adaptive intimal thickening into atherosclerotic lesions. The IA causing atherosclerosis appears to enter at a focal point in the artery wall. In some way it stimulates the resident intimal SMC to produce increased amounts of an abnormal form of

PGs, resulting in asymmetric intimal thickening and lipid retention. Asymmetric intimal thickenings are ubiquitous throughout the coronary tree because atherosclerosis is multicentric in origin and the IA, present in circulating blood, may enter the wall at any vulnerable point. The production of an abnormal form of PGs appears to be a pathologic component of the disease process, produced specifically to retain lipid. The IA appears to establish a locus or focus of injury and then spreads in all directions from this central focus, to contiguous areas within the intimal layer. Lipid-laden SMC are an early, but not the earliest sign of atherosclerosis. Degeneration, necrosis and calcification of plaque tissue can occur very early in plaque development. The defensive responses, whatever they may be, appear to be unable to halt, sequester, or neutralize the IA or to effect healing and resolution of the injured area.

7.3. From "fatty streak" to "overt atherosclerotic plaque" (Fig. **2).**

Fatty streaks do not cause symptoms and may either progress to more complex lesions or involute. Fatty streaks have focal increases in the content of lipoproteins within regions of the intima, where they

associate with components of the extracellular matrix such as proteoglycans, slowing their egress. This retention sequesters lipoproteins within the intima, isolating them from plasma antioxidants, thus favoring their oxidative modification (22–24). Oxidatively-modified LDL particles comprise an incompletely defined mixture, because both the lipid and protein moieties can undergo oxidative modification. Constituents of such modified lipoprotein particles can induce a local inflammatory response (25). Endothelial cells (ECs) normally resist leukocyte adhesion. Proinflammatory stimuli, including a diet high in saturated fat, hypercholesterolemia, obesity, hyperglycemia, insulin resistance, hypertension, and smoking, trigger the endothelial expression of adhesion molecules such as P-selectin and vascular cell adhesion molecule-1 (VCAM-1), which mediate the attachment of circulating monocytes and lymphocytes (26–28). Interestingly, atherosclerotic lesions often form at bifurcations of arteries, regions characterized by disturbed blood flow, which reduces the activity of endothelial atheroprotective molecules such as nitric oxide and favors regional VCAM-1 expression (29). Chemoattractant

factors, which include monocyte chemoattractant protein-1 produced by vascular wall cells in response to modified lipoproteins, direct the migration and diapedesis of adherent monocytes (30, 31).

Monocytic cells directly interacting with human ECs increase monocyte matrix metalloproteinase 9 (MMP-9) production several fold, allowing for the subsequent infiltration of leukocytes through the endothelial layer and its associated basement membrane (33). Within the intima, monocytes mature into macrophages under the influence of macrophage colony stimulating factor, which is overexpressed in the inflamed intima (34,35). Macrophage colony-stimulating factor stimulation also increases macrophage expression of scavenger receptors, members of the pattern-recognition receptor superfamily, which engulf modified lipoproteins through receptor-mediated endocytosis. Accumulation of cholesteryl esters in the cytoplasm converts macrophages into foam cells, i.e., lipid-laden macrophages characteristic of early-stage atherosclerosis. In parallel, macrophages proliferate and amplify the inflammatory response through the secretion of numerous growth factors and cytokines, including tumor necrosis factor and interleukin

(IL)-1. Recent evidence supports selective recruitment of a proinflammatory subset of monocytes to nascent atheroma in mice (36, 37). These observations point to a previously unappreciated layer of complexity in the inflammatory aspects of early atherogenesis. T cells, representing the adaptive arm of the immune response, also play a critical role in atherogenesis, entering lesions in response to the chemokineinducible protein-10, monokine induced by interferon (IFN), and IFN-inducible T cell chemoattractant (38). The CD4 subtype, which recognizes antigens presented as fragments bound to major histocompatibility complex class II molecules, predominates in the lesion. Interestingly, human lesions contain CD4 T cells reactive to the disease-related antigens associated with oxidized LDL (39). The atherosclerotic lesion contains cytokines that promote a T-helper 1 response, inducing activated T cells to differentiate into T-helper 1 effector cells (40). These cells amplify the local inflammatory activity by producing proinflammatory cytokines such as IFN and CD40 ligand (CD40L, CD154), which contribute importantly to plaque progression. Adiponectin, a product of adipose tissue, has insulinsensitizing,

antiatherogenic, and antiinflammatory properties (41). An important autocrine/paracrine factor in adipose tissue, it modulates the differentiation of preadipocytes and favors the formation of mature adipocytes. Curiously, adiponectin concentrations are lower in obese than lean individuals. This adipokine also functions as an endocrine factor, influencing whole-body metabolism via effects on target organs. Adiponectin exerts multiple biologic effects pivotal to cardiovascular biology, including increasing insulin sensitivity, reducing visceral adipose mass, reducing plasma triglycerides, and increasing high-density lipoprotein (HDL) cholesterol (42). Adiponectin alters the concentrations and activity of enzymes responsible for the catabolism of triglyceride-rich lipoproteins and HDL, such as lipoprotein lipase and hepatic lipase. It thus influences atherosclerosis by affecting the balance of atherogenic and antiatherogenic lipoproteins in plasma (43). Adiponectin also directly affects the function of endothelial cells, reducing VCAM-1 expression, and macrophages, decreasing the expression of scavenger receptors and the production of tumor necrosis factor (41, 44).

7.4. The "advanced atherosclerotic plaque".

Macrophages and T cells infiltrate atherosclerotic lesions and localize particularly in the shoulder region, where the atheroma grows. Whereas foam cell accumulation characterizes fatty streaks, deposition of fibrous tissue defines the more advanced atherosclerotic lesion. SMC synthesize the bulk of the extracellular matrix that characterizes this phase of plaque evolution (45). In response to platelet derived growth factor released by activated macrophages and ECs, and silent plaque disruptions that lead to clinically unapparent mural thrombi, SMCs migrate from the tunica media into the intima via degradation of the extracellular matrix mediated by MMP-9 and other proteinases (46). In the intima, SMCs proliferate under the influence of various growth factors and secrete extracellular matrix proteins. This process causes the lesion to evolve from a lipid-rich plaque to a fibrotic and, ultimately, a calcified plaque that may create a stenosis. Human atheromata express IL-18 and increased concentrations of its receptor subunits, IL-18R (47). IL-18 occurs predominantly as the mature 18-Kd form and colocalizes with mononuclear phagocytes while ECs, SMCs, and

macrophages all express IL 18R. Importantly, IL-18 signaling evokes essential effectors involved in atherogenesis, e.g., adhesion molecules (VCAM-1), chemokines (IL-8), cytokines (IL- 6), and matrix metalloproteinases (MMP-1/-9/-13). In addition, IL-18, particularly in combination with IL-12, is a proximal inducer and regulator of the expression of IFN, a major proinflammatory cytokine, during atherogenesis. Interestingly, IL-18 induces IFN expression not only in T cells (48), but also in macrophages and, surprisingly, even in SMCs, thus activating in a paracrine mode several proinflammatory pathways operating during atherogenesis (47). Neovascularization arising from the artery's vasa vasorum contributes to lesion progression in many ways (49). It provides another portal for leukocyte entry into established atherosclerotic lesions (50). In addition, these fragile neovessels can favor focal intraplaque haemorrhage that provides a mechanism for the discontinuous increments seen in plaque growth. Local haemorrhage within the plaque in turn generates thrombin, which activates ECs, monocytes/ macrophages, SMCs, and platelets (51). These cells respond to thrombin by producing a broad array of inflammatory mediators,

including CD40L, RANTES (regulated on activation, normal T cell expressed and secreted), and macrophage migration inhibitory factor. These molecules further promote lesion formation and favor the thrombotic complications of atherosclerosis (52). Platelets also play a central role in the biology of atherosclerosis by producing inflammatory mediators such as CD40L, myeloid-related protein-8/14, and platelet-derived growth factor, as well as directing leukocyte incorporation into plaques through platelet mediated leukocyte adhesion. These results reveal the synergism between inflammation and thrombosis in the pathobiology of atherothrombosis (51). CD40L plays an important role in this phase of atherogenesis. All the main cell types involved in atherosclerosis, including ECs, macrophages, T cells, SMCs, and platelets, express this proinflammatory cytokine as well as its receptor, CD40 (53). CD40 ligation triggers the expression of adhesion molecules and the secretion of numerous cytokines and MMPs involved in extracellular matrix degradation (54–56). Importantly, CD40L has a prothrombotic effect, inducing EC (57), macrophage (54), and SMC (58) expression of tissue factor, which initiates the coagulation cascade.

Accordingly, inhibition of CD40 signaling reduces experimental atherosclerosis development (59) as well as the evolution of established atherosclerosis (60).

7.5. Plaque rupture & the Acute Coronary Event.

Plaque rupture and the ensuing thrombosis commonly cause the most dreaded acute complications of atherosclerosis (Fig. **3**). In many cases, the culprit lesion of acute coronary artery thrombosis does not produce a critical arterial narrowing, rendering its a priori identification using standard angiographic methods uncertain (61). Indeed, it now appears that inflammatory activation, rather than the degree of stenosis, renders the plaque rupture prone and precipitates thrombosis and resulting tissue ischemia (62). Advanced complex atheroma exhibit a paucity of SMCs at sites of rupture and abundant macrophages, key histological characteristics of plaques that have ruptured and caused fatal coronary thrombosis. Inflammation can interfere with the integrity of the interstitial collagen of the fibrous cap by stimulating the destruction of existing collagen fibers and by blocking the creation of new collagen

(63). IFN, secreted by activated T cells, inhibits collagen production by SMCs. T lymphocytes can also contribute to the control of collagenolysis. CD40L as well as IL-1 produced by T cells induce macrophages to release interstitial collagenases, including MMP-1, -8, and -13 (64). The shoulder region of plaques as well as areas of foam cell accumulation contain MMP-9, a member of the gelatinase class of the metalloproteinase family (65). Interestingly, retroviral overexpression of an active form of MMP-9 in macrophages induces morphologic appearances interpreted as plaque disruption (66). Human plaque analysis has revealed that MMP-9 is catalytically active and may thus contribute to the dysregulation of extracellular matrix that leads to plaque rupture during the complication of atherothrombosis (65). Further evidence suggests that local overexpression of MMP-9 promotes intravascular thrombus formation through increased tissue factor expression and tissue factor–mediated activation of the coagulation cascade (67). These data support an important role for MMP-9 in several stages of atherosclerosis. Acute coronary syndromes most often result from a physical disruption of the fibrous cap, either

frank cap fracture or superficial endothelial erosion, allowing the blood to make contact with the thrombogenic material in the lipid core or the subendothelial region of the intima (62). This contact initiates the formation of a thrombus, which can lead to a sudden and dramatic obstruction of blood flow through the affected artery. If the thrombus is non occlusive or transient, it may either be clinically silent or cause symptoms characteristic of an acute coronary syndrome. Importantly, with relation to the propensity of a given plaque disruption to lead to a sustained and occlusive thrombus, the fluid phase of blood, most notably circulating plasminogen activator inhibitor 1 (PAI-1) and fibrinogen concentrations, may determine the fate of a given plaque disruption (68, 69). Indeed, impaired fibrinolysis can result from an imbalance between clot-dissolving enzymes and their endogenous inhibitors, primarily PAI-1 (70). PAI-1 belongs to the serine protease inhibitor superfamily (serpins) and originates from several sites, including the endothelium, liver, and adipose tissue (71). Experimental work using transgenic mice that overexpress a stable form of human PAI-1 demonstrates an association of chronically increased

concentrations of PAI-1 with age-dependent coronary arterial thrombosis (72).

In summary, inflammation participates pivotally in all stages of atherosclerosis (73–79), from lesion initiation to progression and destabilization. In addition, inflammation regulates both the "solid-state", thrombotic potential in the plaque itself and the prothrombotic and antifibrinolytic capacity of blood in the fluid phase. The ominous presence of inflammation in atherosclerosis has prompted the evaluation of certain key inflammatory factors in cardiovascular risk prediction.

7.6. The "inflammatory hypothesis".

About a decade ago, the prevailing wisdom was that conventional risk factors explained only about half of the risk for a myocardial infarction or stroke. However, most studies did not assess some of the newer lipid markers (such as the apolipoproteins) or measures of abdominal obesity, diet, or psychosocial factors and moreover did not try to quantify the population-attributable risk. Consequently, efforts to identify novel risk factors were undertaken to improve cardiovascular risk prediction.

The hypothesis that inflammation is a central contributor to atherothrombosis has stimulated sustained efforts to characterize the specific molecules and pathways that may be involved and to identify biomarkers in humans that enable detection of underlying inflammatory activation to improve cardiovascular risk prediction. This intriguing concept stimulated intense interest in further investigating the ability of inflammatory markers to add information for cardiovascular risk stratification beyond that obtained from traditional risk factors.

Recent investigations of atherosclerosis have focused on inflammation, providing new insight into mechanisms of disease. Further, inflammatory pathways involved in both innate and adaptive immune responses appear to transduce many of the traditional and emerging risk factors for atherosclerosis (Fig. **4**). Inflammatory cytokines involved in vascular inflammation stimulate the generation of endothelial adhesion molecules, proteases, and other mediators, which may enter the circulation in soluble form. These primary cytokines also induce production of the messenger cytokine interleukin-6, which stimulates the liver to increase production of acute-phase reactants such as C-

reactive protein (CRP). In addition, platelets and adipose tissue can generate inflammatory mediators relevant to atherothrombosis. Despite the irreplaceable utility of plasma lipid profiles in assessment of atherosclerotic risk, these profiles provide an incomplete picture. Indeed, many cardiovascular events occur in individuals with plasma cholesterol concentrations below the National Cholesterol Education Program thresholds of 200 mg/dL for total cholesterol and 130 mg/dL for LDL cholesterol (boundaries in general population). The concept of the involvement of inflammation in atherosclerosis has spurred the discovery and adoption of inflammatory biomarkers for cardiovascular risk prediction. C-reactive protein is currently the best validated inflammatory biomarker; in addition, brain natriuretic peptide, soluble CD40 ligand, adiponectin, interleukin 18, interleukin 10, pregnancy associated plasma protein A, neopterin, cystatin C and matrix metalloproteinase 9 may provide additional information for cardiovascular risk stratification and prediction.

7.7. Biomarkers *vs.* "mediators" of disease: *C-reactive protein as an example.*

The distinction between biomarkers and mediators of disease has proven quite confusing. A particular analyte may participate clearly in a pathogenic pathway but not serve as an effective biomarker. Soluble VCAM-1, for example, does not predict the risk of future myocardial infarction in apparently healthy men (79). However, research has repeatedly and unequivocally demonstrated the essential role of VCAM-1 in experimental atherosclerotic lesion initiation and progression (26, 27, 81–82).

On the other hand, a useful biomarker may not mediate pathogenic processes associated with disease. In the case of CRP, the bulk of current evidence supports its utility as a biomarker of risk, not only in apparently healthy populations but also in risk stratification of individuals with established disease. Yet, the role of CRP as a mediator rests on a less secure foundation; notably, many in vitro studies with CRP have used extraordinarily high concentrations of the molecule, causing concern about endotoxin contamination or preservatives in CRP

preparations that might have spurious effects on cells. Experimental results suggest that CRP displays a direct proinflammatory effect on endothelial cells (83) and mediates LDL uptake by macrophages (84). In vivo data, both in animals and humans, suggest that CRP may promote processes involved in the pathogenesis of atherothrombosis, including dysregulation of fibrinolysis by increasing the expression and activity of PAI-1 (85). Recent studies have also shown that CRP originates not only in the liver, but also from other tissues, including SMCs from normal coronary arteries (86-89) and diseased coronary artery bypass grafts (90) as well as coronary artery endothelial cells (88-91), which may provide an explanation for potential local actions of CRP.

8. CHRONIC STABLE ANGINA (CSA).

8.1. Definition and importance of risk stratification.

Stable angina is a clinical syndrome characterized by discomfort in the chest, jaw, shoulder, back, or arms, typically elicited by exertion or emotional stress and relieved by rest or nitroglycerin. Less typically, discomfort may occur in the epigastric area. It is usual to confine the term to the cases in which the syndrome can be attributed to myocardial ischaemia, although essentially similar symptoms can be caused by disorders of the oesophagus, lungs, or chest wall (92). Although the most common cause of myocardial ischaemia is atherosclerotic coronary artery disease (CAD), demonstrable myocardial ischaemia may be induced by other cardiac conditions (92).

The long-term prognosis of stable angina is variable, and the range of treatment options has expanded considerably from simple symptomatic control to potent and often expensive strategies to improve prognosis. When discussing risk stratification in stable angina, risk refers primarily to the risk of death. The process of risk stratification serves a dual

purpose, to facilitate an informed response to queries regarding prognosis from patients themselves, employers, insurers, non-cardiology specialists considering treatment options for comorbid conditions and others and secondly to assist in choosing appropriate treatment (92). For certain management options, particularly revascularization and/or intensified pharmacological therapy, prognostic benefit is only apparent in high risk subgroups, with limited if any benefit in those whose prognosis is already good. This mandates identification of those patients at highest risk, and therefore most likely to benefit from more aggressive treatment, early in the assessment of stable angina.

Information on the prognosis associated with chronic stable angina is derived from long-term prospective population based studies, clinical trials of anti-anginal therapy, and observational registries, with selection bias, an important factor to consider when evaluating and comparing the available data. Irrespective of the origin of the data, the individual's prognosis can vary considerably, up to 10-fold, depending on baseline

clinical, functional and anatomical factors, emphasizing the importance of careful risk stratification (92).

8.2. Risk stratification.

8.2.1. Echocardiography.

The estimation of ventricular function is extremely important in risk stratification (92). Further, this technique is useful to detect or rule out the possibility of other disorders such as valvular heart disease or hypertrophic cardiomyopathy (93) as a cause of symptoms and to evaluate ventricular function (94) For purely diagnostic purposes, echo is useful in patients with clinically detected murmurs, history and electrocardiogram (ECG) changes compatible with hypertrophic cardiomyopathy or previous myocardial infarction and symptoms or signs of heart failure.

One of the strongest predictors of long-term survival is probably the left ventricular ejection fraction (LVEF). In patients with stable angina as LVEF declines, mortality increases. A resting LVEF of 35% is associated with an annual mortality rate 3% per year (95).

8.2.2. Stress testing.

The exercise ECG has been extensively validated as an important tool in risk stratification in symptomatic patients with known or suspected coronary disease. Prognostic information obtained from stress testing relates not only to the detection of ischaemia as a simple binary response, but also the ischaemic threshold, the extent and severity of ischaemia (for imaging techniques), and functional capacity (for exercise testing). Stress testing alone is insufficient to assess risk of future events. Risk stratification with the exercise test should be a part of a process that includes readily accessible data from clinical examination and should not take place in isolation. Thus the stress test is performed to provide additional information regarding the patient's risk status.

8.2.3. Coronary arteriography.

Despite the recognized limitations of coronary arteriography to identify vulnerable plaques which are likely to lead to acute coronary events, the extent, severity of luminal obstruction, and location of coronary disease

on coronary arteriography have been convincingly demonstrated to be important prognostic indicators in patients with angina (96).

Coronary arteriography is generally undertaken as part of a series of tests to establish a diagnosis and ascertain treatment options. Coronary arteriography holds a fundamental position in the investigation of patients with stable angina, providing reliable anatomical information to identify the presence or absence of coronary lumen stenosis, define therapeutic options (suitability of medical treatment or myocardial revascularization) and determine prognosis.

8.3. CONCLUSIONS.

The revolution in the understanding of the pathophysiology of atherosclerosis has focused attention on inflammation and provided new insight into mechanism of disease. The clinical application of the concept that inflammation participates in atherosclerosis has stimulated the adoption of biomarkers of inflammation in risk prediction and other applications, as noted above. Contemplation of the clinical use of biomarkers in the context of atherosclerotic cardiovascular disease requires considerable care. Evaluation of the utility of a biomarker

requires a clear understanding of the question being asked. Is the task to risk-stratify apparently well or diseased populations? Should the biomarker be measured serially as a target of therapy? Should the biomarker be used as a guide for therapy in addition to the traditional accepted risk factors? Each of these 3 questions requires different types of clinical validation. The example of inflammation in atherosclerosis illustrates rapid translation of basic science understanding to the clinic. Further studies, both in progress and on the horizon, will help evaluate the role of novel and emerging biomarkers in the clinical management of atherosclerosis and targeting of therapies. Although the circulating concentrations of several inflammatory mediators correlate with increased cardiovascular risk, few are ready for clinical practice. CRP attracts particular attention and has stood the test of time, although not all experts agree on its utility. As a downstream biomarker, CRP provides functional integration of overall upstream cytokine activation. With the exception of CRP, however, none of the established and emerging novel biomarkers for cardiovascular risk have demonstrated additive value to the Framingham risk score, and few have available

commercial assays that achieve adequate levels of standardization and accuracy for clinical use.

CSA is an important clinical condition that warrants a good clinical risk stratification. The evaluation of LVEF and the extent of atherosclerotic coronary disease are essential in this process. However, further improvement in the ability to identify individuals at risk to develop adverse events is needed.

9. OVERVIEW OF THE VALUE OF PREGNANCY ASSOCIATED PLASMA PROTEIN-A (PAPP-A) AND CARDIOVASCULAR RISK.

Consuegra-Sánchez L, Fredericks S, Kaski JC. Pregnancy associated plasma protein-A (PAPP-A) and cardiovascular risk. Atherosclerosis. 2009;203:346-52 (97).

Consuegra-Sánchez L, Fredericks S, Kaski JC. Pregnancy associated plasma protein A: Has this biomarker crossed the boundary from research to clinical practice? Drug News Perspect. 2009 (accepted for publication, in press, 98)

Coronary artery disease progression leading to acute coronary syndrome (ACS) is a rather unpredictable phenomenon and is often associated to atheromatous plaque disruption or endothelial erosion (99). Plaques prone to disruption and rapid progression are known as "vulnerable" or "complex" plaques (100). Vulnerable plaques progress faster than smooth uncomplicated coronary stenoses and lead to the development of ACS (101-104) (Fig. **5**). Stratification of patients regarding cardiovascular risk is largely dependant on relatively crude scores. Conventional risk factors, clinical characteristics, electrocardiographic findings and serum markers of necrosis are widely used in clinical

practice but these are able to single out only a proportion of those individuals who are at a high risk of developing acute coronary events. Consequently the search for markers that may help to improve risk prediction is ongoing. Substantial research efforts have been directed towards the identification of vulnerable plaques including the development of sophisticated imaging techniques. These methods, however, are expensive and some of them invasive thus limiting their use in daily practise. The fact that both atherogenesis and CAD progression are directly related to inflammatory responses, immune activation, and activation of the coagulation and fibrinolytic cascades, has generated interest in the potential role of soluble circulating "biomarkers" as markers of cardiovascular risk. As abovementioned, these serum biomarkers can be an expression of the underlying pathogenic process and/or play an active mechanistic role in the development and progression of the disease. New markers of CAD progression have been identified in recent years, among which, circulating levels of pregnancy-associated plasma protein-A (PAPP-A, pappalysin-1, EC 3.4.24.79) appear to offer an interesting profile.

PAPP-A is a high molecular-mass zinc-binding metalloproteinase mainly produced by the placental syncytiotrophoblast during pregnancy, but also by fibroblasts, osteoblasts and vascular smooth muscle cells (105,106).

Data from animal and human studies suggest that PAPP-A could play a role in the development of atherosclerotic lesions (107-108). In CSA patients, high PAPP-A levels correlate with the extent of angiographic CAD (109) and the presence of vulnerable coronary artery stenoses (110). Moreover, PAPP-A levels are increased in patients with ACS and represent a marker of adverse events in ACS patients (table **1**) (111-113).

PAPP-A and stable atherosclerotic disease.

*9.1. Coronary artery disease***.**

Previous findings from our group showed that PAPP-A levels correlate with the extent of angiographic CAD. Cosin-Sales *et al* (109) (Fig. **6**) showed that high levels of PAPP-A identified patients with CSA and multivessel disease (6.45 ± 2.58 mIU/L) compared to those with single-

vessel disease and subjects without CAD, who had lower circulating PAPP-A concentrations (5.49 ± 1.54 mIU/L; $p < 0.001$ and 4.62 ± 1.17 mIU/L; $p < 0.001$), respectively. Interestingly these differences remained statistically significant after multivariable adjustment. Receiver operator characteristic (ROC) curve analysis for the presence of CAD in these patients showed that the area under the curve was 0.75 (95% CI, 0.72-0.78) for PAPP-A and 0.45 (95% CI 0.41-0.49) for high sensitivity C-reactive protein (hs-CRP).

The proform of eosinophil major basic protein (pro-MBP) is an endogenous inhibitor of PAPP-A enzymatic activity. PAPP-A specifically degrades insulin-like growth factor (IGF) binding proteins (114-115). In 396 patients with CSA, Cosin-Sales *et al* (110) demonstrated that the PAPP-A/proMBP ratio was an independent predictor of the number of complex stenoses (standardized $\beta = 0.10$, $p = 0.026$) in a fully adjusted model.

9.2. Carotid and peripheral arterial diseases.

Circulating PAPP-A has been shown to be a marker of echogenicity of atheromatous carotid plaques in hyperlipidemic patients (116). In an elegant study, Sangiorgi *et al* (117) reported an association between PAPP-A circulating levels, carotid plaque morphology and vascular complications. A significant inverse correlation between atheromatous plaque cap thickness and PAPP-A levels was found ($r = - 0.62$, $p = 0.01$), whereas a positive correlation was observed between plaque inflammation (inflammatory cells/mm^2) and PAPP-A levels ($r = 0.70$, $p = 0.01$). In this study transcription of PAPP-A messenger ribonucleic acid (mRNA) was found to be 2-4 fold higher in symptomatic patients i.e those developing a stroke or a transient ischemic attack, compared to control individuals and patients without events. PAPP-A has been also suggested to be a predictor of ankle-brachial index ($r = -0.294$, $p = 0.0039$) and intima-media wall thickness in common carotid arteries (partial coefficient = 0.414, $p = 0.002$) in type 2 diabetes mellitus with hypercholesterolemia (118). Moreover, it has been reported that PAPP-A may be a marker for the presence of peripheral arterial disease in

elderly patients. In these patients it has been suggested that PAPP-A concentrations are indicative of the atherosclerotic burden (119).

9.3. Prognostic value in patients with stable atherosclerotic disease (table **1**).

In a small study in selected patients with stable angina pectoris, Elesber *et al* (120) showed that PAPP-A, considered as a continuous (log-2 transformed) variable, correlated with the occurrence of death and ACS (adjusted HR = 3.56, 95% CI 1.27-10, p = 0.015), but not with the need for revascularization (120).

9.4. PAPP-A and lipids.

The relationship between PAPP-A levels and statin treatment is important in relation to the potential ability of PAPP-A to predict patient outcome. Cosin-Sales *et al* (108) reported that CSA patients receiving treatment with statins had lower PAPP-A levels (5.5 ± 1.5 vs. 6.1 ± 2.4 mIU/L; p = 0.02) than those on no statin treatment. However, prospective studies in patients with hypercholesterolemia showed that

atorvastatin did not significantly reduce PAPP-A concentration in plasma (121). In myocardial infarction (MI) survivors, Aarsetøy *et al* (122) interestingly reported that patients receiving n-3 fatty acids and corn oil for 1 year showed higher levels of circulating PAPP-A.

PAPP-A and the acute coronary syndrome.

9.5. Diagnostic value.

Bayes-Genis *et al* (108) showed that PAPP-A levels were higher in patients with ACS (blood samples taken 8.4 ± 3.0 [mean ± standard deviation, SD] hours in the MI group, 9.4 ± 3.9 hours in the UA group, from last ischemic episode) compared to those with stable angina pectoris. They reported a sensitivity for PAPP-A (threshold value of 10 mIU/L) of 89.2 % and a specificity of 81.3 % in discriminating ACS from CSA and control (healthy) subjects. Interestingly, they showed that PAPP-A concentrations correlated with free IGF-1 levels but not with markers of myocardial damage such as creatin-kinase MB (CK-MB) isoenzyme and cardiac troponin (c-Tn). This latter finding, however, differs from data reported by Khosravi *et al* (105) who

observed a significant correlation between PAPP-A and troponin levels in a subset of troponin-T positive patients with ACS (those with troponin T levels up to 2.9 ug/L). These authors, like Bayes-Genis previously, also found significantly higher concentrations of PAPP-A in patients with ACS (time interval between symptoms onset and blood sample not reported) compared to control subjects (105).

In patients presenting to the emergency department with acute chest pain suggestive of ACS (time interval between symptoms onset and blood sample not reported), Elesber *et al* (123) reported that PAPP-A levels were predictive of a final diagnosis of ACS in a multivariable model (Odds ratio [OR] = 2.093; 95% CI 1.037-4.224; p= 0.039). Laterza *et al* (124) recruited 346 patients with symptoms suggestive of ACS (blood samples taken 9 hours on average after symptoms onset), 33 of whom suffered adverse events (3 deaths, 14 acute MI, and 23 revascularization procedures) over a 30 day follow-up period. They reported that on analysis of the ROC curves, cTnT was a better predictor of 30-day events than PAPP-A. Our group recently (125) evaluated the value of PAPP-A, NT-proBNP and CRP in combination

with clinical data and exercise testing for the evaluation of 422 patients with chest pain without ST-segment deviation or troponin elevation. By univariate unadjusted ROC analysis, PAPP-A showed a border-line significant association with the occurrence of all-cause death or myocardial infarction (p = 0.07) and adverse cardiac events (all-cause death, myocardial infarction or coronary revascularization; p = 0.04) during a 60 weeks (median) follow-up. However -in a Cox multivariable model- PAPP-A was not an independent predictor of either study endpoints. Contrary to the findings of previous investigations, a small study by Dominguez-Rodriguez *et al* (126) in patients with ST-elevation ACS found no differences in PAPP-A concentrations in patients (n = 80) compared to control subjects (n = 80), in samples taken (mean ± SD) 6.3 ± 2.8 hours after the onset of symptoms. The small sample, absence of a sample-size calculation and the case-control nature of the study might have accounted for the probable type 2 error. Thus, PAPP-A levels –when determined within the first twelve hours- may identify patients in the process of plaque

instability, when it might be most beneficial to receive intensive treatment for the ACS.

9.6. Kinetics of PAPP-A (98).

Qin *et al* (113) reported that increases in PAPP-A levels above the reference range are highly variable in ACS patients, in that they may be present early at 2 hours or late at 30 hours after onset of chest pain. Lund *et al* (127) suggested that PAPP-A release patterns in ST-segment elevation ACS might be influenced by the occurrence and timing of reperfusion.

Recently, Iversen *et al* (128) published results concerning the releasing pattern of PAPP-A after the occurrence of an ST- elevation ACS in 354 patients. For this study, that in turn was specifically designed with the purpose of assessing the PAPP-A release, samples were taken at admission (median time from symptoms onset to admission 7.5 hours) and every 6 to 8 hours until biomarkers of necrosis were consistently decreasing. The peak concentrations of circulating PAPP-A (Fig. **7**) were found 5.77 hours (95% CI 4.95-6.58) after the onset of symptoms

and significantly earlier than cTnT or CKMB peaks. In patients who undergo percutaneous coronary intervention (PCI), a single substantial PAPP-A increase shortly after the procedure followed by a return to normal levels (128). This finding is in accordance with other studies showing a similar pattern (129).

In patients with a non-ST segment elevation acute MI, it has been reported that PAPP-A concentrations significantly correlate with ST-depression, multivessel disease, typical angina at presentation and other "high risk" conditions (130).

*9.7. Prognostic value in ACS***.** (table **1**) *Negative troponin patients.* In a cohort of 200 patients with suspected ACS and negative cardiac troponin, Lund et al (111) interestingly reported that highest (assessed within the first 24 hours after admission) PAPP-A levels (> 2.9 mIU/L) were independently associated with an adverse outcome at 6 months follow-up (i.e. cardiac events and need for revascularization). Moreover, a single admission PAPP-A level > 2.9 mIU/L also showed a significant

association with the study endpoint (Relative risk = 2.3, 95 % CI 1.1-5, p = 0.003).

Positive troponin patients. In patients with ACS (comprising 205 patients with acute MI and 342 with unstable angina) and documented CAD recruited in the large CAPTURE (Chimeric c7E3 AntiPlatelet Therapy in Unstable angina REfractory to standard treatment trial) study (112), Heeschen *et al* showed that patients with higher PAPP-A values (>12.6 mIU/L) were at increased risk of death or MI during the 6 month follow-up (multivariate OR = 2.44, 95% CI 1.43-4.15, p = 0.001). Importantly this independent association of PAPP-A with adverse clinical outcome was also observed in patients with negative cTnT values (multivariate OR = 2.72, 95% CI 1.25-5.89, p = 0.009). Of interest a significant interaction between PAPP-A and the anti-inflammatory cytokine interleukin (IL) 10 was found whereby the association of PAPP-A with the composite endpoint (death and nonfatal MI) was limited to patients with circulating IL-10 concentrations <3.5 ng/mL. The authors therefore concluded that the balance between pro-inflammatory and anti-inflammatory cytokines determines the course of

the disease in those patients with IL-10 concentrations <3.5 ng/mL, who in turn, had a higher rate of revascularization procedures during follow-up. In a small cohort of patients with ST-segment elevation acute MI (n = 62), Lund *et al* (127) found that PAPP-A > 10 mIU/L was a significant predictor of 12-month risk of cardiovascular death or non-fatal MI.

9.8. PAPP-A and plaque instability: Cause or consequence?

Figure **8** summarizes the evidences supporting the role of PAPP-A in cardiovascular disease.

It has been suggested that PAPP-A is implicated in repair mechanisms directly contributing to plaque stabilization, thus having a protective role (131-132). In contrast PAPP-A may have a more deleterious role by breaking down extra-cellular matrix via its metalloproteinase character. These paradoxical functions can only be understood through understanding the pathophysiology. During pregnancy PAPP-A is synthesized in the trophoblastic cells of the placenta and released to the circulation. Although the role of PAPP-A in pregnancy is still not well

understood, it seems to play a role in the placental development (133). PAPP-A produced during pregnancy, however, differs from that present in eroded and ruptured atherosclerotic plaques in that the latter is not complexed with its endogenous inhibitor, the pro-MBP. This "free" PAPP-A shows metalloproteolytic activity directed towards insulin-like growth factor binding protein 4 and 5, and leads to the release of bound IGF (114,115). Given its proteolytic potential and the associations found between high PAPP-A concentrations and complex atherosclerotic plaques and impaired clinical outcome, it may be speculated that PAPP-A may contribute to the degradation of the plaque extracellular matrix. However, beyond the degradation of IGF-1 binding proteins, no other proteolytic action has been so far demonstrated for PAPP-A (134). Furthermore, recent data suggest that PAPP-A is not synthesized in macrophages but rather binds to the membrane of these cells, with only 50 % being internalized (135). It has been shown that whilst membrane-bound PAPP-A has proteolytic activity, internalized PAPP-A has little or no protease activity (135). In vitro studies showed that IGF may induce macrophage activation, chemotaxis, LDL-

cholesterol uptake by macrophages and release of proinflammatory cytokines, thus suggesting a pro-atherogenic action of IGF-1 (136,137). However more recent clinical and experimental evidences suggest that IGF-1 may protect against ischaemic heart disease preserving endothelial function, promoting plaque stability and exerting anti-inflammatory and anti-oxidant effects (131, 132). If IGF is "cardioprotective", it should -at least theoretically- follow that elevated PAPP-A levels should also be cardioprotective (134). The question thus emerges as to whether PAPP-A causes plaque instability or plaque instability and myocardial ischaemia are stimuli for PAPP-A secretion, which may contribute to plaque stability and healing. It has been suggested that PAPP-A suppresses inflammation (138). The relatively poor correlation between PAPP-A and markers of necrosis in ACS patients, suggests that increased PAPP-A levels cannot be attributed to myocardial necrosis (139). Recently Park *at al* examined the association of four single nucleotide polymorphisms within the *PAPP-A* gene in relation to acute MI. In a case-control study containing 170 acute MI patients and 170 age-matched controls the authors found the *PAPP-A*

IVS6+95 C allele to be an independent predictor of increased risk of acute MI (heterozygote- OR=1.89 [95% CI: 1.14-3.16]; p=0.015) (140). This polymorphism is located in an intron that is believed to be closely related with other unknown gene variants in the exon portion. The functional significance (i.e. circulating PAPP-A levels, enzymatic activity) of this and other PAPP-A polymorphism is not known so far (140). These data taken together indicate that more work is required to define the true role of PAPP-A in plaque instability; the reverse causality hypothesis requires further investigation.

9.9. Biochemical considerations.

The identification of PAPP-A in atherosclerotic plaques by immunohistochemical techniques (108,117,141) has been central in forging a link between PAPP-A and cardiovascular disease. Unfortunately, the use of different assays by different researchers has lead to some controversy regarding the importance of this potential marker of plaque instability (26,142,143). Moreover there has been uncertainty as to which is the most appropriate molecular form of the

PAPP-A protein to measure (142). To a substantial extent these disparities relate to the method of quantitative analysis. Current research has been based on immunoassay systems which depend on two antibodies (a capture and a detection antibody) used to recognise specific epitopes on the PAPP-A antigen (Fig. **9**). In circulation PAPP-A typically exists as a disulfide-bound heterotetrameric complex with the proMBP (144), present at low concentrations in non-ACS individuals and at higher concentrations in pregnant women. However, the PAPP-A immunoassays extensively used for cardiovascular research have been based mainly on assays designed to recognise the PAPP-A/proMPB complexed form of the molecule (108,111,112).

Historically PAPP-A assays used for diagnostics purposes in pregnancy, were first used to identify foetal Down's syndrome (145), and were then adapted for use in other disease areas. Qin *et al* reported that, theoretically, the free form of PAPP-A (disassociated from proMBP) is the only relevant molecular form in patients with ACS (142,146), as it is released from unstable atherosclerotic plaques. Recently two high sensitive assays have become commercially available for the detection

of PAPP-A: one assay manufactured by DRG Co. (Germany) failed to detect any difference in serum PAPP-A concentrations between healthy controls (n = 80) and patients with ST-segment elevation myocardial infarction (n = 80) (126), whilst the other manufactured by Diagnostic System Laboratories (DSL, Webster, TX) has been used in several positive investigations of vascular disease (119,123,124,147).

Although it has been suggested that the most appropriate method of analysis would be based on the specific detection of the free PAPP-A molecule (142), several of the early reports on ACS and serum PAPP-A concentrations were performed using an in-house assay which employed a polyclonal capture antibody, rather than the more specific monoclonal pairs used in commercial assays (108-110,120).

The direct measurement of free PAPP-A would be the ideal method (142). One approach has been to perform two assays in conjunction, one that specifically detects the PAPP-A/proMBP complex and one that detects total PAPP-A. The amount of free PAPP-A can then be calculated as the difference in concentration determined by the two assays (Fig. **9**). This method has been presented in a report by Wittfooth

et al (148) who described two point-of-care assays performed on an auto-analyser; All-In-One! Immunoanalyzer (Aio!; Innotrac Diagnostics). Another approach -that has been successfully employed for other metalloproteinases- may be to analyse the enzymatic activity of PAPP-A that is attributed to free-PAPP-A.Total PAPP-A assays have proved their worth in cardiovascular research in the clinical setting. However, the development of assays for PAPP-A activity and free PAPP-A, have the potential to be useful tools for investigating the pathophysiology of PAPP-A at the molecular and cellular level.

9.10. Statistical considerations.

Despite of the existence of encouraging literature reporting "independent associations" between PAPP-A (and other biomarkers) and events during follow-up in painstakingly adjusted models, its clinical applicability is still poor. Besides the biochemical concern with PAPP-A assays, one of the aspects that might -at least partially- account for this situation is the controversy regarding statistical methods for the evaluation of "clinical impact" of a biomarker in a particular scenario.

Pepe *et al* (149) indicated that the global evaluation of a biomarker should comprise not only the assessment of independent associations but also the analysis of the "incremental value or predictive ability" (so-called "discrimination") by mean of the study of the area under the ROC curve or *c* statistic. This means that a biomarker should increase sensitivity and /or specificity -to identify individuals at higher risk- as compared to a standard risk score to improve the C-statistic. However, this approach has pitfalls since standard methods do not exist for deriving ROC curves for time-to-event data. Cook *et al* (150) recently reported that the C statistic might not be optimal in assessing models that predict future risk and that the solely reliance on this parameter may be insufficient. Several traditional and emerging risk factors may have little effect on the C statistic but substantially shift future cardiovascular risk for an individual patient. In this regard many accepted risk factors (i.e. hypertension) have only marginal impact on C statistic but are clinically relevant as stratification variables (150,151). Model calibration is a measure of how well the predicted risk fits with the real (observed) risk and is becoming important as an indicator of the

accuracy of a model (151). Moreover it has been suggested that a useful method to evaluate a biomarker may consist in the evaluation of the number of subjects that eventually would be reclassified (in terms of risk category) with the use of a biomarker compared to a classical model of risk prediction (152). To date, beyond "independent associations", no studies have been reported that demonstrate a significant improvement in discrimination, calibration or reclassification ability with the use of PAPP-A.

9.11. General Conclusions.

There is evidence suggesting that PAPP-A could play a role in the development of atherosclerotic lesions (Fig. **8**) and may represent a marker of atheromatous plaque instability and extent of cardiovascular disease. Studies have shown that PAPP-A levels are increased in patients with ACS and represent a marker of adverse events in this group (table **1**). Whether PAPP-A causes vascular wall damage or –on the contrary- has a protective vascular effect, as suggested by some authors, needs to be further investigated in *ad hoc* studies. Taken the

available data together PAPP-A appears to provide complementary information to that provided by markers of myocardial necrosis. Assays measuring total PAPP-A have proved their worth in clinical research. However, the development of assays to measure PAPP-A activity and free PAPP-A levels, may offer potential for the investigation of the true role of PAPP-A in the pathophysiology of cardiovascular disease. Studies are necessary also at the molecular and cellular levels to better characterize this protein and its changes in the disease process.

10. ORIGINAL RESEARCH.

Consuegra-Sanchez L, Petrovic I, Cosin-Sales J, Holt DW, Christiansen M, Kaski JC. Prognostic Value of Circulating Pregnancy-Associated Plasma Protein–A (PAPP-A) and Proform of Eosinophil Major Basic Protein (pro-MBP) Levels in Patients with Chronic Stable Angina Pectoris. Clin Chim Acta 2008; 391:18-23 (153).

10.1. Hypothesis. Rationale and objectives.

The concept that inflammation is pivotal in atherothrombosis has boosted sustained efforts to identify biomarkers that might improve cardiovascular risk prediction. PAPP-A has become a promising biomarker in cardiovascular medicine (105-125, 127-140) in recent years. However, in CSA patients, data regarding the value of PAPP-A as a risk marker of adverse events are sparse (120). Moreover, in addition to statistical associations, no studies -to date- have evaluated the contribution of PAPP-A in the ability to identify individuals at risk –so-called discrimination- or the precision in terms of risk –calibration-.

The a priori *objectives* of this study were:

1. We primarily sought to assess whether PAPP-A levels -evaluated during routine angiography- are a marker of all-cause

mortality during long term follow-up in a non-selected population of CSA patients.

2. Beyond the "statistical association", the study was also aimed at assessing whether the determination of this biomarker might complement and improve the "discrimination" and "calibration", when compared to the traditional (clinical) model.
3. As third objective, we evaluated whether the determination of pro-MBP levels and the ratio PAPP-A/pro-MBP is a marker of all-cause mortality in a subgroup of the total sample.
4. Finally, we aimed at assessing the clinical independent predictors of adverse outcome in CSA patients.

We consequently hypothesized that (1) the determination of PAPP-A - as a readily available marker obtained by mean of a simple blood test- might be associated with an adverse outcome in CSA patients (2) PAPP-A might improve the sensitivity and/or specificity in the identification of individuals at risk, compared to that obtained with traditional or clinical risk factors and (3) PAPP-A might improve the precision in terms of risk estimation, compared to a traditional model.

10.2. METHODS.

10.2.1. Patients. Inclusion and exclusion criteria.

This was a prospective study in 663 consecutive patients with diagnosis of CSA (recruited from 1st, November 1994 to 15th, August 1997) undergoing routine diagnostic coronary angiography in St. George's Hospital, London.

CSA was defined as typical chest pain during exercise, relieved by rest and/or sublingual nitrates, with symptoms unchanged for at least 6 months before study entry.

The following variables were collected at study entry: age, gender, body mass index, systolic and diastolic blood pressure, Canadian Cardiovascular Society functional class, family history of CAD, smoking, diabetes mellitus, hyperlipidemia, hypertension, previous myocardial infarction, prior percutaneous coronary angioplasty, total cholesterol, HDL-cholesterol, LDL-cholesterol, creatinine, triglycerides, C-reactive protein, number of diseased vessels, Sullivan's extension score, LVEF, revascularization at index hospitalisation, treatment at discharge (aspirin, betablocker, lipid lowering drugs, angiotensin-

converting enzyme inhibitors, calcium channel blockers) and all-cause deaths.

We did not include patients with any of the following: <12 weeks ACS, life threatening arrhythmias, acute or chronic liver disease, renal failure, and chronic inflammatory and/or immunological conditions. Follow-up information on mortality was obtained by a periodic review of the patients' clinical notes, postal questionnaires sent to the patients' general practitioners, and telephone contact with patients and/or their families.

10.2.2. Study endpoint. Follow-up. Variables' definitions.

The primary study endpoint was all-cause mortality. We limited our analysis to all cause mortality as this information could be obtained accurately during the long term follow-up.

Follow-up data were available in 624 patients (94 %). Follow-up information on mortality was obtained –as abovementioned- by a periodic review of the patients' clinical notes, postal questionnaires sent to the patients' general practitioners, and telephone contact with patients

and/or their families. The study protocol, which is in accordance with the current revision of the Helsinki Declaration, was approved by the Local Research Ethics Committee, and all patients gave written informed consent before study entry.

Arterial hypertension was defined in the presence of a systolic blood pressure of ≥140mmHg and/or diastolic blood pressure of ≥90mmHg measured on at least two separate occasions. Hyperlipidemia was defined as a documented total cholesterol value >5.4 mmol/L. Smokers were defined as those currently smoking tobacco. Patients were considered to have diabetes mellitus if they were receiving active treatment with insulin or oral hypoglycemic agents. For patients on dietary treatment alone, documentation of abnormal fasting blood glucose or glucose tolerance tests according to World Health Organization criteria were required for the diagnosis of diabetes.

10.2.3. Angiographic analyses.

As described previously (109, 110), during coronary angiography (Judkins technique) images of the coronary tree were obtained in routine

standardized projections (Philips Integris 3000 system, Philips, Holland). In every coronary angiogram, we assessed the number of major coronary arteries showing ≥ 75 % lumen diameter reduction ("vessel score") and quantified the extent of angiographic coronary atherosclerosis using the Sullivan scoring system (154). The Sullivan "extension score" refers to the proportion of the coronary tree showing angiographically detectable atheroma. The observed proportion of atheroma in each vessel is multiplied by a factor that varies according to the artery involved: left main stem, 5; left anterior descending coronary artery, 20; main diagonal branch, 10; first septal perforator, 5; left circumflex artery, 20; obtuse marginal and posterolateral vessels, 10; right coronary artery, 20; and main posterior descending branch, 10. When the major lateral wall branch was a large obtuse marginal or intermediate, this was given a factor of 20, and left circumflex artery, a factor of 10. When a vessel was occluded and the distal bed was not fully visualized by collateral flow, the proportion of vessel not visualized was given the mean extent score of the remaining vessels. The scores for each vessel or branch were added to give a total score out

of 100, representing the percentage of the coronary lumenal surface involved by atheroma. Interobserver agreement (calculated as intraclass correlation coefficient) regarding extension score in the present study was 0.97.

10.2.4. Biochemical measurements.

Fasting blood samples were obtained from every patient at the time of coronary angiography. Blood was drawn and centrifuged immediately and the serum was then aliquoted and stored at -80ºC. PAPP-A levels were determined using a biotin-tyramide-amplified enzyme immunoassay with a limit of detection of 0.03 mIU/L. Intra-assay and inter-assay coefficients of variation were 10 and 15%, respectively. PAPP-A polyclonal antibodies were used for capture and a combination of monoclonal antibodies were used for detection. The assay detects and quantifies total PAPP-A (*free* PAPP-A plus the PAPP-A/proMBP complex) and was calibrated against the World Health Organization's International Reference Standard 78/610.

CRP measurements were performed on the COBAS Integra (Roche Diagnostics Limited, Lewes, East Sussex, UK) using the CRP-Latex assay in both the high sensitivity application (analytical range 0.2-12mg/L) and the normal application (analytical range 2-160 mg/L). Analytical precision of the high-sensitivity CRP-latex assay was 7.6% at a level of 1.02 mg/L, 3.3% at 1.79 mg/L, and 1.3% at a level of 4.36 mg/L. Samples outside the analytical range of the high sensitivity CRP-Latex assay were analysed by the CRP-Latex in the normal application. Analytical precision of the normal CRP-latex assay was 2.4% at a level of 29.5 mg/L and 1.3% at a level of 113 mg/L. LVEF was assessed in 563 patients (79 %).

Total pro-MBP levels were assessed in 385 patients with an immunoassay developed at the Statens Serum Institute, Copenhagen. Within the calibrator range used, the interassay variation coefficient was < 5% (155).

10.2.5. Statistical analysis.

In this study involving 663 patients, the statistical power was 95 % to detect a difference of 12 percent units (4^{th} quartile vs 1^{st} quartile) assuming an expected event rate of 8 % in subjects in the first PAPP-A quartile -based on previous pilot work from our group- and a type I error of 0.05. Differences in variables between groups were assessed using the Chi-Square test or Fisher's exact test for categorical variables and Student *t* test or the Mann-Whitney test for continuous variables. The Spearman two way test was used to assess the relation between two quantitative variables with non-normal distribution. Long-term mortality was analyzed by the Kaplan-Meier method, and differences were compared with log-rank (Cox-Mantel) test. We tested the proportional hazard assumption with visual plots. Variables included in the multivariable analysis (covariates) were those that showed a significant association both with the study endpoint and higher values of PAPP-A (Group 2 = PAPP-A > 4.6 mIU/L) on univariate analysis ($p < .05$), variables that showed a trend ($p < .10$) towards an association, and variables considered to be of clinical relevance (gender, type 2 diabetes

mellitus, revascularization at index hospitalization and CRP). In patients in whom ejection fraction was not measured, the value was imputed with the sample median to include all subjects in the model. The backward stepwise likelihood ratio was used to derive the final model for which significance levels of 0.1 and 0.05 were chosen to exclude and include terms, respectively. The HR and their 95% CI were calculated with a Cox multivariable regression model and bootstrapping. χ^2 statistic analysis was performed to assess the importance of each covariate in the final multivariable model. We used a multivariate fractional polynomial approach to test the probability of a threshold effect. Calibration of the multivariable model with and without the incorporation of the biomarker PAPP-A was assessed with Hosmer-Lemeshow test. Logarithmic transformation was performed to normalize the distribution of CRP. The Harrell's C-statistic (equivalent to the area under the Receiver Operating Characteristic curve) was calculated for the model. Tests were considered to be statistically significant if $p < 0.05$. Statistical analyses were performed with SPSS 13.0 (SPSS Inc., Chicago, IL).

10.3. RESULTS.

10.3.1. Baseline Characteristics.

Six-hundred and sixty-three CSA patients (mean age 62.9 ± 9.7 years, 494 (74.5 %) male) were recruited. Patients were followed for a median of 8.8 years (interquartile range 3 - 10.6 years). Table **2** shows demographic, clinical, angiographic and biochemical data of all patients included in the study. PAPP-A levels were *a priori* stratified in quartiles (1^{st} quartile ≤ 4.6 mIU/L; 2^{nd} quartile 4.6-5.6 mIU/L; 3^{rd} quartile 5.6-6.8 mIU/L; 4^{th} quartile ≥ 6.8 mIU/L). Analysis of PAPP-A quartiles in relation to survival with Kaplan-Meier curves (Fig. **10**) showed little, if any, difference in the cumulative mortality rate between patients in quartiles 2, 3 and 4 suggesting the presence of a threshold effect between patients in the 1^{st} quartile and those in the remaining quartiles (log rank = 9.39, p = 0.002). Consequently, patients were subdivided into two groups according to the 25^{th} percentile, i.e. Group 1 comprised patients in the 1^{st} PAPP-A quartile, and Group 2, patients belonging to the remaining 3 quartiles.

Median (25th and 75th percentiles) PAPP-A was 5.6 mIU/L (4.6-6.8). PAPP-A levels ranged from 1.2 mIU/L to 17.20 mIU/L. Median CRP concentration (25th and 75th percentiles) was 2.3 mg/L (1.1-4.62) and CRP levels ranged from 1.1 mg/L to 4.62 mg/L. Median (25th and 75th percentiles) pro-MBP was 1938 mIU/L (1484-2516). Pro-MBP levels ranged from 484 mIU/L to 8696 mIU/L. CRP levels did not correlate with PAPP-A or pro-MBP levels ($p = 0.66$ and 0.38, respectively). Circulating PAPP-A levels, however, correlated significantly with pro-MBP levels ($r = 0.485$, $p < 0.001$). Table **2** shows the univariable comparison between group 1 and 2. Compared to patients in group 1, group 2 patients were older ($p < 0.01$), with a predominance of males ($p < 0.01$). Moreover , more patients in group 2 had systemic hypertension ($p = 0.05$), a higher number of coronary arteries with ≥ 75 % stenosis (vessel score) ($p < 0.01$), a higher Sullivan extension score ($p < 0.01$), higher creatinine levels ($p < 0.01$) and lower LVEF ($p < 0.01$). Importantly, cumulative all-cause mortality was lower (8.5 %) in patients in the 1st PAPP-A quartile compared to those in the 2nd, 3rd and 4th quartiles (20 %) ($p < 0.001$). This suggested the presence of a

threshold effect, which we specifically tested using a multivariate fractional polynomial model that identified a PAPP-A threshold at 4.8 mIU/L (Percentile 30)(Fig. **11**).

10.3.2. Events during follow-up and univariate analysis.

During follow-up, 106 patients (16 %) died. On univariate analysis age ($p < 0.01$), Canadian Cardiovascular Society Functional Class ($p = 0.04$), smoking ($p = 0.02$), systemic hypertension ($p = 0.02$), previous MI ($p = 0.01$), creatinine levels ($p < 0.01$), CRP ($p < 0.01$), PAPP-A ($p < 0.01$), vessel score ($p < 0.01$), Sullivan extension score ($p < 0.01$) and LVEF ($p < 0.01$) correlated with the occurrence of the endpoint (Table **3**).

Coronary revascularization performed during index hospitalization, however, was associated with a reduction in the occurrence of the study endpoint ($p = 0.05$).

10.3.3. Multivariable analysis.

We further tested the proportional hazard assumption with visual plots and appeared valid for all analysis (Fig. **12**).

An increased PAPP-A concentration > 4.8 mIU/L was independently associated with all-cause mortality (HR 1.953, 95% CI 1.135-3.36, p = 0.016), after adjusting for age, gender, hypertension, hyperlipidaemia, creatinine, vessel score, Sullivan extension score, LVEF, type 2 diabetes mellitus, revascularization at index hospitalization and CRP. No significant interaction was found for gender regarding the association of circulating PAPP-A levels and the study endpoint (p for interaction = 0.79).

PAPP-A levels (> 4.8 mIU/L) remained an independent predictor of the study endpoint (HR 1.911, 95% CI 1.112-3.285, p = 0.019) even after entering treatment at discharge (aspirin, beta-blockers, lipid-lowering drugs and angiotensin-converter enzyme inhibitors) in addition to the full model.

Of importance χ^2 analysis showed that PAPP-A > 4.8 mIU/L (χ^2 = 6.683) was the strongest predictor after age (χ^2 = 17.052) and CRP (χ^2 =

14.066) followed by revascularization at index hospitalization (χ^2 = 5.099), vessel score (χ^2 = 5.059), creatinine (χ^2 = 4.141) and LVEF (χ^2 = 3.181) and in the final model.

In our study the independent predictors of all-cause mortality are shown in table **4**. Beyond PAPP-A, age (years) (HR = 1.051, p < 0.001), revascularization at index hospitalization (HR = 0.513, p = 0.038), vessel score (HR = 1.290, p = 0.026), creatinine (μmol/L) (HR = 1.009, p = 0.010) and CRP (mg/L) (HR = 1.414, p < 0.001) were independent predictors of all-cause death. LVEF (percent units) was borderline significant (HR = 0.986, p = 0.065).

10.3.4. Calibration. Discrimination. Bootstrapping. Additive value.

Model calibration -as assessed with Hosmer-Lemeshow test- did not either significantly change with and without the inclusion of PAPP-A to the full clinical model (Table **5**).

The analysis of the Receiver Operating Characteristic Curve (Fig. **13A**) of PAPP-A in relation to survival revealed that the optimal PAPP-A cut-point (4.8 mIU/L) showed a high sensitivity (0.84) albeit low specificity

(0.33). We also compared two multivariate models, with and without the inclusion of PAPP-A; the Harrell's C-index before incorporation of PAPP-A (complete model included age, gender, hypertension, hyperlipidaemia, creatinine, vessel score, Sullivan extension score, LVEF, type 2 diabetes mellitus, revascularization at index hospitalization and CRP) was 0.74, which increased to 0.75 after including PAPP-A in the model (Fig. **13B**). To remain on the cautious side we further applied a bootstrapping method to estimate standard errors and confidence intervals till 4000 replications. This method interestingly showed that the association between PAPP-A levels and all-cause death was consistently significant till 100 replications and borderline-significant afterwards (see table **6**).

We also explored to what extent the biomarker PAPP-A might add to the predictive value of CRP in patients presenting with CSA (Fig. **14**). The test for interaction was positive (interaction p value = 0.01). Interestingly when CRP levels were below the median, the contribution of PAPP-A levels was neutral; however, when CRP levels were elevated above the median, those patients with higher PAPP-A levels

(above the median) represented an even higher risk subgroup of adverse events. These individuals revealed a hazard ratio of 2.09 (95% CI: 1.08–4.04) (Fig. **14**) compared to those with CRP and PAPP-A below the median (reference category, HR = 1).

10.3.5. Prognostic value of Pro-MBP and PAPP-A/pro-MBP ratio.

Pro-MBP levels (mIU/L), considered as a continuous variable, did not differ significantly in survivors, compared with patients who died during follow-up (1896 [1466-2481] vs 1982 [1447-2811], p = 0.45). A trend towards a higher PAPP-A/pro-MBP ratio was observed in patients who died compared to survivors (2.92 [2.24-3.69] x 10^{-3} vs 2.80 [2.24-3.51] x 10^{-3} vs, p = 0.54).

Analysis of pro-MBP quartiles and PAPP-A/proMBP ratio quartiles (Figs. **15, panels A & B**) did not show difference in the cumulative mortality rate between patients in the different quartiles (log rank = 0.99, p = 0.8 and log rank = 2.18, p = 0.55, respectively).

10.4. DISCUSSION.

Our study showed an independent association between circulating PAPP-A levels and the occurrence of all-cause death in CSA patients during long term follow up. Our findings in a larger group of patients undergoing diagnostic coronary arteriography confirm and expand recent observations by Elesber et al (120) in a small group of selected patients with CSA. The present study was not aimed at dissecting the mechanisms whereby PAPP-A is a predictor of mortality. However, findings in previous studies from our group that PAPP-A levels correlate with the extent and severity of angiographic coronary artery disease (109) and with the occurrence of angiographically complex stenoses (110) in stable angina patients, provide clues in this regard. The metalloproteolytic activity of PAPP-A is directed towards insulin-like growth factor binding protein 4 and 5 (IGFBP 4-5), leading to the release of insulin growth factor-1 (IGF-1) (114). *In vitro* studies have shown that IGF-1 can induce macrophage activation, LDL-cholesterol uptake and the release of proinflammatory cytokines by macrophages (137) thus suggesting a pro-atherogenic action of IGF-1. However more

recent studies suggest that IGF-1 may be protective against ischaemic heart disease by preserving endothelial function, promoting plaque stability and exerting anti-inflammatory/anti-oxidant effects (134,138). Pro-MBP functions as an inhibitor of the proteolytic activity of PAPP-A and, as such, might potentially affect IGF-1 actions (156). We have shown previously that PAPP-A/pro-MBP ratio and PAPP-A levels are both higher in CSA patients with complex coronary lesions (110). Complex stenoses have prognostic importance in CSA patients, as these lesions progress faster than smooth coronary stenoses and lead to ACS (103). In the present study, patients who died had higher PAPP-A levels than those who survived and the prognostic information provided by PAPP-A was independent from that provided by CRP. However, the PAPP-A/pro-MBP ratio was not significantly different in patients who died during follow up compared to survivors (*adjusted* HR = 1.011, 95 % CI 0.82 – 1.247, p = 0.91). The lack of prognostic value of PAPP-A/pro-MBP was likely due low statistical power given the relatively small number of patients in this subgroup. Moreover, the pro-MBP assay used in the present study was aimed at measuring total pro-MBP

i.e. *free* pro-MBP and pro-MBP complexed with angiotensinogen, complement C3dg and PAPP-A (155). This might have diluted the potential value of pro-MBP and PAPP-A/pro-MBP ratio as markers of all-cause mortality. Also, the use of one time point measurement, as opposed to an average value over a period of time, to predict mortality over the long-term follow-up might have underestimated our results due to the regression dilution phenomenon (157). Further studies at a molecular level are necessary to elucidate the relationship between pro-MBP and atherogenesis (155).

In our study, contrary to Elesber et al's findings (120), PAPP-A concentration entered in the statistical model as a continuous variable (log base-e transformed) was not a significant predictor of all-cause mortality (*adjusted* HR 1.634, 95% CI 0.818-3.264, p = 0.164). Elesber et al assessed a relatively small (n = 103) and highly selected patient group, excluded patients with <50% coronary stenoses, and included a substantial number (39%) of patients without angina with a positive stress test result. These differences may have accounted for the different findings in our study. We do not have an explanation for the puzzling

finding of a PAPP-A threshold, so further investigation for this phenomenon is required.

Of importance, whilst increased PAPP-A levels have been shown to be a marker of atherogenesis and coronary artery disease activity, it has been suggested that the circulating form of PAPP-A that accounts for the increased concentration observed in ACS patients differs from that seen in pregnant women, or normal non-pregnant individuals (142). In fact, ACS-related PAPP-A is believed to originate from ruptured atherosclerotic plaques and is not complexed with pro-MBP (free PAPP-A) (142). Non-complexed PAPP-A retains its proteolytic activity. In a recent study, Wittfooth et al showed that circulating PAPP-A in non-ACS individuals consisted predominantly of complexed PAPP-A/pro-MBP, and free PAPP-A concentrations were negligible (148). It is therefore likely that free PAPP-A may be a more specific marker of risk of adverse events than complexed or "total" PAPP-A, as suggested by Qin et al (142), but this needs to be formally assessed in *ad hoc* studies.

In our study we found that CRP was both independently associated and the second most powerful predictor of all-cause death in CSA patients. This finding is in agreement with previous observations of our group and others. Arroyo-Espliguero [158] recently observed that hs-CRP (OR 2.2 [1.3–3.6] CI 95%; *P* = 0.002) was an independent predictor of NYHA functional classes III–IV irrespective of LVEF and angiographic severity of CAD. Authors identified a CRP value of 3.2 mg/L that showed a moderate-to-high sensitivity (72%) and specificity (75%), but excellent negative predictive value (96%) for detecting an impaired functional class. More importantly, our group [159-160] reported that CRP levels predict cardiac adverse events in patients with stable CAD, regardless of the presence or absence of flow limiting coronary lesions and revascularization. In the large prospective study by Zebrack [161], CRP correlated significantly with several measures of the extent and severity of CAD, but the degree of correlation was low, suggesting that other factors are more important in determining CRP levels. Moreover, the multivariate adjustment resulted in little change in the HR (CRP levels & adverse events), arising the concept that C-reactive protein

retains predictive value and contributes independently and additively to risk prediction with respect to extent/severity CAD.

However, the predictive value of CRP in CSA might not be universal. Schnabel [162], on behalf of the *Atherogene Investigators,* reported that hs-CRP is a weaker predictor compared to IL-18, myeloperoxidase, or soluble CD40 ligand, after full adjustment. Authors stated that their data data do not support the hypothesis that the additional assessment of hs-CRP leads to better risk stratification compared with Nt-proBNP alone.

Current guidelines on the management of CSA patients [92] remain reluctant concerning the use of biomarkers in daily practice. The fact that they might fluctuate over time, abscense of information about whether they can change current management strategies or treatments, the problem of the cost and availability have severely limited their use. However, biomarkers might have a role in selected patients for decision making and further research is welcome in this regard.

Recently, an American Heart Association Scientific Statement concerning the criteria for evaluation of novel markers of cardiovascular risk has been published by Hlatky and colleagues [163]. The document

provides a practical framework for assessing the value of novel markers of risk by using a 6-phase protocol. In this regard, PAPP-A has "passed" the *proof of concept*, as values differ –as shown above- in subjects with adverse outcome compared to those without (phase 1). PAPP-A has been also proven to be independently associated with future adverse clinical outcomes in several prospective cohort studies in different clinical scenarios (phase 2). Conversely, however, PAPP-A has not been shown to improve risk prediction over and above that of traditional risk factors, since no significant changes in AUC after addition of PAPP-A has been demonstrated (phase 3). No studies –to date- have assessed the incremental value of PAPP-A with reclassification methodology. Finally, the clinical utility of PAPP-A regarding its ability to change patient management and whether the use of this circulating marker is able to improve clinical outcome have not been demonstrated so far (phases 4,5 & 6).

10.5. FINAL CONCLUSSIONS.

1. Our study illustrates an association between PAPP-A levels and all-cause mortality during long-term follow-up in CSA patients undergoing diagnostic coronary arteriography.

2. In our study PAPP-A was the strongest predictor of all-cause mortality, after age and CRP, followed by revascularization at index hospitalization, vessel score, creatinine and LVEF.

3. Of importance, we showed for the first time the existence of a threshold effect regarding PAPP-A levels that may be useful in the prediction of clinical outcome.

4. However, the determination of PAPP-A in CSA patients did not improve the discrimination ability –as assessed with Harrell's C statistic- or the calibration –as evaluated with Hosmer-Lemeshow test- when compared to the model that included traditional risk factors.

5. The determination of pro-MBP circulating levels and the ratio PAPP-A/proMBP did not appear to be associated with an adverse outcome (all-cause mortality) in CSA patients.

6. In our study the independent predictors of all-cause death were age, CRP levels, revascularization at index hospitalization, vessel score, creatinine levels and PAPP-A (>4.8 mIU/L) levels.

10.6. CONCLUSIONES FINALES.

1. El presente estudio ilustra la existencia de una asociación independiente entre los niveles de PAPP-A y la muerte por cualquier causa a largo plazo, en pacientes con angina crónica estable remitidos para coronariografía diagnóstica, con motivo de estratificación de riesgo.

2. En este estudio PAPP-A fue el predictor más potente de muerte por cualquier causa tras la edad y los niveles de PCR, seguido por necesidad de revascularización durante la hospitalización, el número de vasos afectos, la creatinina y la fracción de eyección del ventrículo izquierdo.

3. De importancia y por primera vez, se muestra la existencia de un "efecto umbral" para los niveles de PAPP-A que podría ser útil en la práctica clínica en la predicción de eventos.

4. Sin embargo, la determinación de los niveles de PAPP-A no mejoró el poder discriminativo ni la calibración respecto del modelo que incluyó a los factores de riesgo tradicionalmente usados en estratificación en este grupo de pacientes.

5. La determinación de los niveles circulantes de pro-MBP o de la razón PAPP-A/proMBP no pareció asociarse con un peor desenlace en el seguimiento en el presente estudio.

6. En el presente estudio se identificaron como predictores de muerte por cualquier causa la edad, los niveles de CRP, revascularización en la hospitalización inicial, el número de vasos significativamente afectos (vessel score), niveles de creatinina y los niveles altos de PAPP-A (> 4.8 mIU/L).

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10.9. TABLES.

Table 1. Evidences supporting a role for PAPP-A as a marker of cardiovascular risk.

Authors	Condition	n	PAPP-A	Time of sample*	Type of assay	Endpoint	OR/ HR	95 % CI	P
Laterza *et al* (124)	**Symptoms suggestive of ACS†**	346	Continuous	9 hours	*US*-DSL ELISA	30-day death, MI and revascularization	ND ‡	ND	ND
Elesber *et al* (123)	**Acute chest pain****	59	Continuous	NR	*US-DSL* ELISA	Final diagnosis of ACS	2.09	1.03-4.2	.039
Heeschen *et al* (112)	**NSTEACS**	547	> 12.6 mIU/L	8.7 ± 4.9 hours	Roche Elecsys	6-month death and non fatal MI	2.33	1.3-4.2	.005
Lund *et al* (111)	**Suspected ACS**	136	≥ 2.9 mIU/L	NR	POC Assay	6-month cardiovascular mortality, non-fatal MI or revascularization	4.6	1.8-11.8	.002
Lund *et al* (126)	**STEMI**	62	> 10 mIU/L	150 (90-300) min.[a]	POC Assay	12-month cardiovascular mortality or non-fatal MI.	ND[b]	ND	.049
Elesber *et al* (120)	**Chronic Stable Angina**	103	Continuous	-	BTA ELISA	Future death[c]	5.29	1.2-22	.023
						Future death and ACS[c]	3.56	1.2-10	.015

ACS = Acute coronary syndrome; BTA ELISA = Biotin-tyramide-amplified enzyme immunoassay; min = minutes; ND = Not Determined; NR = Not reported; NSTEACS = Non-ST-segment elevation acute coronary syndrome; POC Assay = Point-of-care time-resolved immunofluorometric assay; Roche Elecsys = Elecsys 2010 assay system, Roche Diagnostics; STEMI = ST-segment elevation myocardial infarction; *US*-DSL ELISA = *Ultrasensitive* Diagnostic Systems Laboratory ELISA.

* Time from symptoms onset to first blood sampling.
† Chest pain, epigastric pain, unexplained shortness of breath or syncope.

‡ A cut-off point of 0.22 mIU/L was reported to show a sensitivity of 66.7 % (95 % CI 48.2-82) and specificity 51.1 % (95 % CI 45.4-56.8).
** Intermediate to high risk of ACS.
[a] Median and interquartile range.
[b] Cumulative risk was 15% if PAPP-A levels <3 mIU/L, 20% if PAPP-A 3-10 mIU/L and 45% if PAPP-A > 10mIU/L (data coming from an unadjusted Kaplan-Meier method).
[c] Median follow-up was 4.9 and interquartile range 1.1-5.9 (years).

Table 2. Baseline characteristics of patients in the lowest quartile of PAPP-A levels (Group 1, ≤4.6 mIU/L) vs. remaining quartiles (Group 2, >4.6 mIU/L).

Variable	Total Cohort (n = 663)	Group 1 PAPP-A ≤4.6 mIU/L* (n=175)	Group 2 PAPP-A >4.6 mIU/L§ (n=488)	P
Age, mean ± SD	62.9 ± 9.7	60.9 ± 8.9	63.7 ± 9.9	< 0.01
Male, n (%)	494 (74)	103 (58.9)	391 (80.1)	< 0.01
BMI, median (Q1, Q3)	27 (24-30)	27 (24-31)	27 (24-29)	0.31
Systolic blood pressure, (mmHg), mean ± SD	134 ± 20	132 ± 20	134 ± 20	0.12
Diastolic blood pressure, (mmHg), mean ± SD	80 ± 11	80 ± 10	80 ± 11	0.35
High CCS class, n (%) #	127 (19)	35 (20)	92 (19)	0.72
Cardiovascular risk factors				
Family history of CAD, n (%)	348 (52)	104 (59)	244 (50)	0.03
Current smoking, n (%)	191 (29)	42 (24)	149 (30.5)	0.11
Diabetes mellitus, n (%)	41 (6)	10 (5.7)	31 (6.4)	0.76
Hyperlipidemia, n (%)	273 (41)	84 (48)	189 (38.8)	0.03
Hypertension, n (%)	209 (31)	45 (25.7)	164 (33.6)	0.05
Previous MI, n (%)	250 (38)	63 (36)	187 (38.3)	0.58
Prior PTCA, n (%)	53 (8)	10 (5.7)	43 (8.8)	0.19
Biochemistry				
Total cholesterol, (mmol/L), mean ± SD	5.85 ± 1.16	5.92 ± 1.31	5.82 ± 1.09	0.44
HDL-cholesterol, (mmol/L), median (Q1,Q3)	1.22 ± 0.89	1.16 ± 0.65	1.26 ± 1	0.67
LDL-cholesterol, (mmol/L), mean ± SD	3.78 ± 1.60	3.86 ± 1.52	3.74 ± 1.65	0.55
Creatinine, (µmol/L), median Q1, Q3)	82 (82-82)	78 (73-85)	88 (75-102)	< 0.01

Triglycerides,(mmol/L), median (Q1, Q3)	1.5 (1-2.1)	1.5 (1.1-2.2)	1.4 (1-2)	0.32
C-reactive protein, (mg/L), median (Q1, Q3)	2.3 (1.1-4.6)	2.6 (1-4.6)	2.2 (1.1-4.7)	0.84
Cardiac catheterization				
Number of vessels **, median (Q1, Q3)	2 (1-2)	1 (0-2)	2 (1-2)	< 0.01
Total extension score, median (Q1, Q3) †	17 (8-28)	12 (4-25)	18 (10-29)	< 0.01
LVEF, (%) mean ± SD	65.7 ± 10.9	67.9 ± 8.7	64.9 ± 11.4	< 0.01
Revascularization at index hospitalization,n (%)	116 (17.5)	30 (14.7)	86 (18.7)	0.21
Treatment at discharge				
Aspirin at discharge, n (%)	530 (80)	136 (78)	394 (81)	0.39
Betablocker at discharge, n (%)	353 (53)	98 (56)	255 (52)	0.39
Lipid lowering drugs at discharge, n (%)	96 (14)	31 (18)	65 (13)	0.15
ACE inhibitors at discharge, n (%)	122 (18)	31 (18)	91 (18)	0.78
CCB at discharge, n (%)	263 (40)	68 (39)	195 (40)	0.79
All-cause death, n (%)	106 (16)	14 (8.5)	92 (20)	< 0.01

* Range 1.2-4.6 mIU/L ; § Range 4.61-17.2 mIU/L.
# stratified by the median (CCS > 2), ** "vessel score" = number of vessels with ≥ 75 % reduction in lumen diameter , † Sullivan extension score.
Continuous variables are presented as mean values ± standard deviation (SD) or median (interquartile range Q1-Q3) (p values for Mann-Whitney test). Categorical variables are presented as percentage.
ACE = Angiotensin converting enzyme; BMI = Body Mass Index; CAD = Coronary artery disease; CCB = Calcium channel blocker; CCS = Canadian Cardiovascular Society; HDL = High density lipoprotein; LDL = Low density lipoprotein; LVEF = Left ventricular ejection fraction; MI = Myocardial infarction; PTCA = Percutaneous transluminal coronary angioplasty; SD = Standard deviation.
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Table 3. Baseline characteristics of patients with and without events.

Variable	Events (n=106)	No events (n=518)	P
Age, mean ± SD	68.2 ± 8.3	62.1 ± 9.4	< 0.01
Male, n (%)	82 (77)	390 (75)	0.65
BMI, median (Q1, Q3)	27 (24-29)	27 (24-30)	0.37
Systolic blood pressure, (mmHg), mean ± SD	139 ± 20	133 ± 20	< 0.01
Diastolic blood pressure, (mmHg), mean ± SD	81 ± 10	80 ± 11	0.25
High CCS class, n (%) *	28 (26)	93 (18)	0.04
Cardiovascular risk factors			
Family history of CAD, n (%)	50 (47)	275 (53)	0.26
Current smoking, n (%)	41 (39)	141 (27)	0.02
Diabetes mellitus, n (%)	10 (9)	27 (5)	0.09
Hyperlipidemia, n (%)	32 (30)	228 (44)	< 0.01
Hypertension, n (%)	44 (41)	154 (30)	0.02
Previous MI, n (%)	51 (48)	185 (36)	0.01
Prior PTCA, n (%)	9 (8)	42 (8)	0.89
Biochemistry			
Total cholesterol, (mmol/L), mean ± SD	6.12 ± 1.41	5.81 ± 1.08	0.10
HDL-cholesterol, (mmol/L), median (Q1,Q3)	0.9 (0.7-1.2)	1.1 (0.8-1.3)	0.12
LDL-cholesterol, (mmol/L), mean ± SD	4.20 ± 2.12	3.66 ± 1.49	0.13
Creatinine, (µmol/L) mean ± SD	89 ± 30	83 ± 11	< 0.01
Triglycerides, (mmol/L), median (Q1, Q3)	1.4 (1.1-2.1)	1.5 (1-2.1)	0.90

C-reactive protein, (mg/L), median (Q1, Q3)	3 (2.1-6.7)	2.1 (1-4.2)	< 0.01
PAPP-A (mIU/L), median (Q1-Q3)	5.9 (5.1-7.3)	5.5 (4.5-6.7)	< 0.01
Cardiac catheterization			
Number of vessels [§], median (Q1, Q3)	2 (1-3)	1 (1-2)	< 0.01
Total extension score, median (Q1, Q3) [#]	21 (12-33)	17 (8-27)	< 0.01
LVEF, (%), mean ± SD	61.2 ± 13.9	66.5 ± 10.1	< 0.01
Revascularization at index hospitalization,n (%)	12 (11.3)	99 (19.1)	0.05
Treatment at discharge			
Aspirin at discharge, n (%)	78 (73)	420 (81)	0.08
Betablocker at discharge, n (%)	58 (55)	276 (53)	0.79
Lipid lowering drugs at discharge, n (%)	14 (13)	77 (15)	0.66
ACE inhibitors at discharge, n (%)	27 (25)	90 (17)	0.05
CCB at discharge, n (%)	50 (47)	207 (40)	0.17

[*] stratified by the median (CCS > 2), [§] "vessel score" = number of vessels with ≥ 75 % reduction in lumen diameter, [#] Sullivan extension score.

Continuous variables are presented as mean values ± standard deviation (SD) or median (interquartile range Q1-Q3) (p values for Mann-Whitney test). Categorical variables are presented as percentage.ACE = Angiotensin converting enzyme; BMI = Body Mass Index; CAD = Coronary artery disease; CCB = Calcium channel blocker; CCS = Canadian Cardiovascular Society; HDL = High density lipoprotein; LDL = Low density lipoprotein; LVEF = Left ventricular ejection fraction; MI = Myocardial infarction; PTCA = Percutaneous transluminal coronary angioplasty; SD = Standard deviation. Reproduced with permission from Consuegra-Sanchez L et al. Clin Chim Acta 2008;391:18-23 (ref. 153).

Table 4. Independent predictors of adverse outcome in patients with CSA (multivariable Cox model).

	HR[#]	95% CI	p value
PAPP-A*	1.953	1.135-3.360	0.016
Age (years)	1.051	1.026-1.077	<0.001
CRP (mg/L)	1.414	1.183-1.690	<0.001
Revascularization at index hospitalization	0.513	0.273-0.963	0.038
Vessel Score (points)	1.290	1.031-1.615	0.026
Creatinine (μmol/L)	1.009	1.002-1.016	0.010
LVEF (percent units)	0.986	0.971-1.001	0.065

CI = Confidence interval; CRP = C-Reactive protein; HR = Hazard ratio; LVEF = Left-ventricular ejection fraction.
* PAPP-A > 4.8 mIU/L. # multivariately adjusted (age, gender, hypertension, hyperlipidaemia, creatinine, vessel score, Sullivan extension score, LVEF, type 2 diabetes mellitus, revascularization at index hospitalization and CRP).

Table 5. Model Calibration (Hosmer-Lemeshow test).

	Chi-squ: re value	P v ılue
Without PAPP-A	2.705	0.951
With I APP-A	5.393	0.715

Table 6. Bootstrapping method.

Replic tions	H R*	95% CI
0	1.95	1.13-3.36
2	1.95	1.04-3.04
5	1.95	0.99-3.21
7	1.95	1.02-3.11
10	1.95	1.02-3.10
15	1.95	0.97-3.27
50	1.95	0.96-3.30
10 0	1.95	0.98-3.25
30 0	1.95	0.97-3.29
40 0	1.95	0.97-3.29

* adjusted by age, gender, hypertension, hyperlipidaemia, creatinine, vessel score, Sullivan extension score, LVEF, type 2 diabetes mellitus, revascularization at index hospitalization and CRP.
CI = Confidence interval; HR = Hazard ratio (PAPP-A > 4.8 mIU/L).

10.10. FIGURES.

Figure 1.

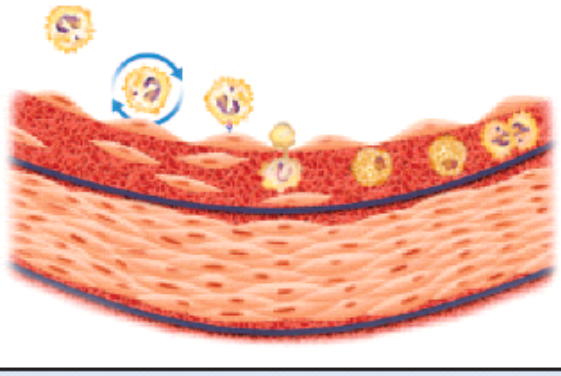


Fig. 1. Initiation of atherosclerosis.
The diagram shows a cross-section through a muscular artery depicting a classic trilaminar structure. The intima of normal arteries is composed of a single layer of endothelial cells overlying a subendothelial matrix that contains occasional resident smooth muscle cells. The underlying tunica media, separated from the intima by the internal elastic lamina, contains multiple layers of vascular smooth muscle cells. The adventitia, the outermost layer of the blood vessel, separated from the media by the external elastic lamina, is not depicted in this diagram. Circulating leukocytes adhere poorly to the normal endothelium under normal conditions. When the endothelium becomes inflamed, however, it expresses adhesion molecules that bind cognate ligands on leukocytes. Selectins mediate a loose rolling interaction of leukocytes with the inflammatorily activated endothelial cells. Integrins mediate firm attachment. Chemokines expressed within atheroma provide a chemotactic stimulus to the adherent leukocytes, directing their diapedesis and migration into the intima, where they take residence and divide. These steps are depicted in a left-to-right chronological sequence. Reprinted with permission from **(32)**

Figure 2.

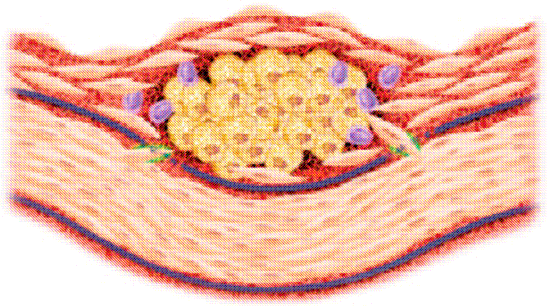


Fig. 2. Progression of atherosclerosis.
Macrophages augment the expression of scavenger receptors in response to inflammatory mediators, transforming them into lipid-laden foam cells following the endocytosis of modified lipoprotein particles. Macrophage-derived foam cells drive lesion progression by secreting proinflammatory cytokines. T lymphocytes join macrophages in the intima and direct adaptive immune responses. These leukocytes, as well as endothelial cells, secrete additional cytokines and growth factors that promote the migration and proliferation of SMCs. In response to inflammatory stimulation, vascular SMCs express specialized enzymes that can degrade elastin and collagen, allowing their penetration into the expanding lesion. Reprinted with permission from **(32)**

Figure 3.

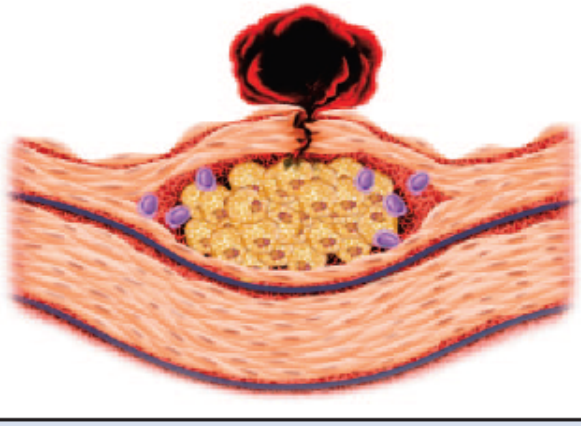


Fig. 3. Thrombotic complication of atherosclerosis. Ultimately, inflammatory mediators can inhibit collagen synthesis and evoke the expression of collagenases by macrophage foam cells within the intima. This imbalance diminishes the collagen content of the fibrous cap, rendering it weak and rupture-prone. In parallel, crosstalk between T lymphocytes and other cell types present within lesions heightens the expression of the potent procoagulant tissue factor. Thus, when the fibrous cap ruptures, as illustrated in this diagram, tissue factor induced by inflammatory signaling triggers the thrombus that causes most acute complications of atherosclerosis. Clinically, this may translate into an acute coronary syndrome. Reprinted with permission from **(32)**

Figure 4. Inflammation links classic risk factors to altered cellular behavior within the arterial wall and secretion of inflammatory markers in the circulation. Primary proinflammatory risk factors elicit the expression of primary proinflammatory cytokines that can be released directly into the blood. Cytokines orchestrate the production of adhesion molecules, matrix metalloproteinases, and reactive oxygen species that may also be released from lesions. Reprinted with permission from ref. 80.

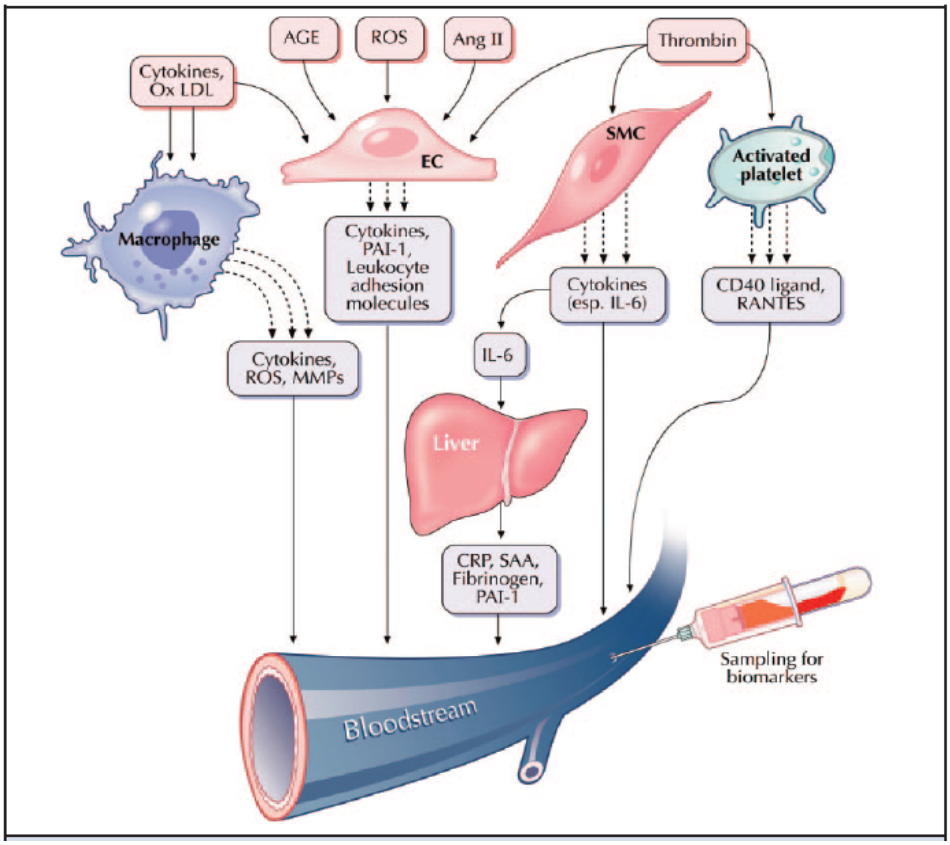


Figure 5. The plot shows that complex stenoses progress significantly more than smooth stenoses (11.6 ± 17.5% vs 3.9 ± 10.2%, change from baseline; Coronary arteriography was repeated at 8 ± 3 months' follow-up). From Kaski JC et al. Circulation. 1995;92:2058-65. Reproduced with permission.

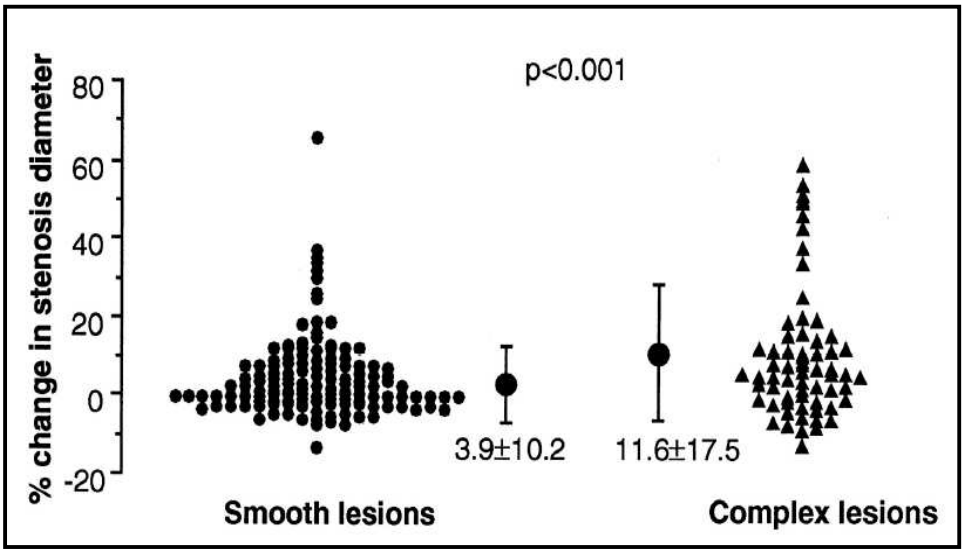


Figure 6. Error bars showing mean (95% CI) PAPP-A levels in patients without coronary disease (no CAD), patients with single vessel disease (single-VD) and those with multi-VD. From Cosin-Sales J et al. Eur Heart J. 2005;26:2093-8. Reproduced with permission.

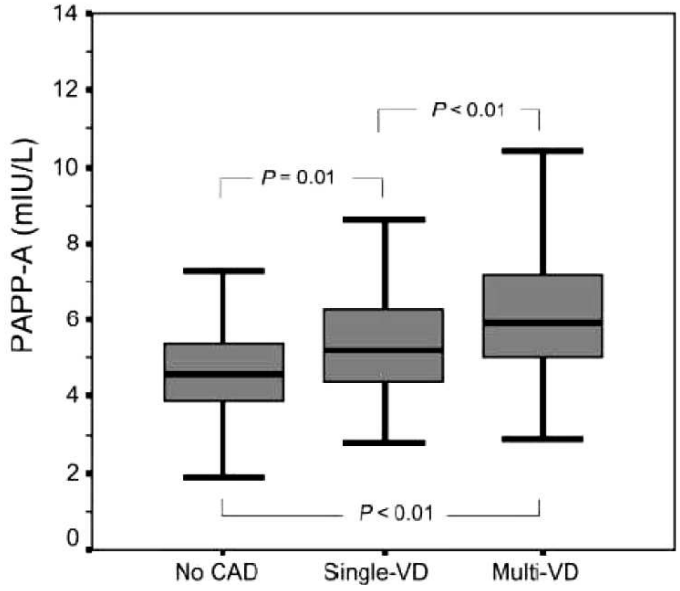
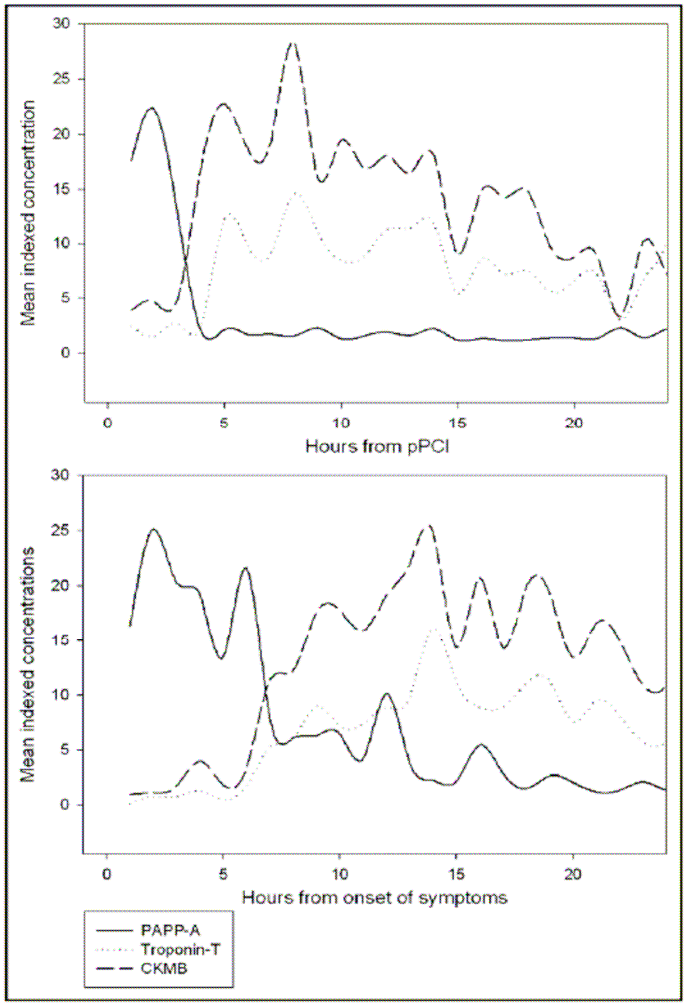
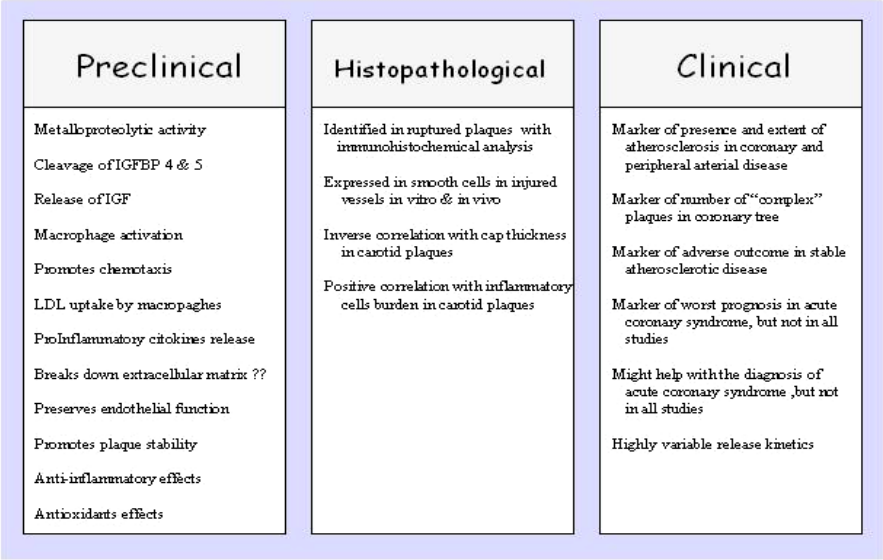


Figure 7. PAPP-A levels after PCI (above) and ST elevation ACS (below). Reproduced with permission from Iversen et al, Am J Cardiol. 2008;101:1389-94.



ACS = Acute coronary syndrome; CKMB = Creatinkinase isoenzyme MB; PCI = Percutaneous coronary intervention.

Figure 8. Summary of preclinical, histopathological and clinical evidences of PAPP-A in cardiovascular medicine. Reproduced with permission from Consuegra-Sánchez L et al, Drug News Perspect. 2009 (in press, 98).



Please see text for references.

Figure 9. Pregnancy associated plasma protein (PAPP-A) is commonly analysed using immunoassays employing an antigen "sandwich" format. An antibody is normally attached to a solid phase base of a plastic tube or well of a microtitreplate. A detection antibody, which is labelled with an enzyme or a fluorochromic molecule, then binds to the captured antigen. The most widely used assays detects and quantifies total PAPP-A (A) i.e. free PAPP-A plus the PAPP-A/proMBP complex. (B) An assay has been developed to specifically measure the PAPP-A/proMBP complex, and when used in conjunction with the total assay may provide a derived value for free PAPP-A. (C) A direct assay for free PAPP-A would be the ideal approach to investigate PAPP-A in cardiovascular disease. Reproduced with permission from Consuegra-Sanchez L et al. Atherosclerosis 2009;203:346-52 (ref. 97).

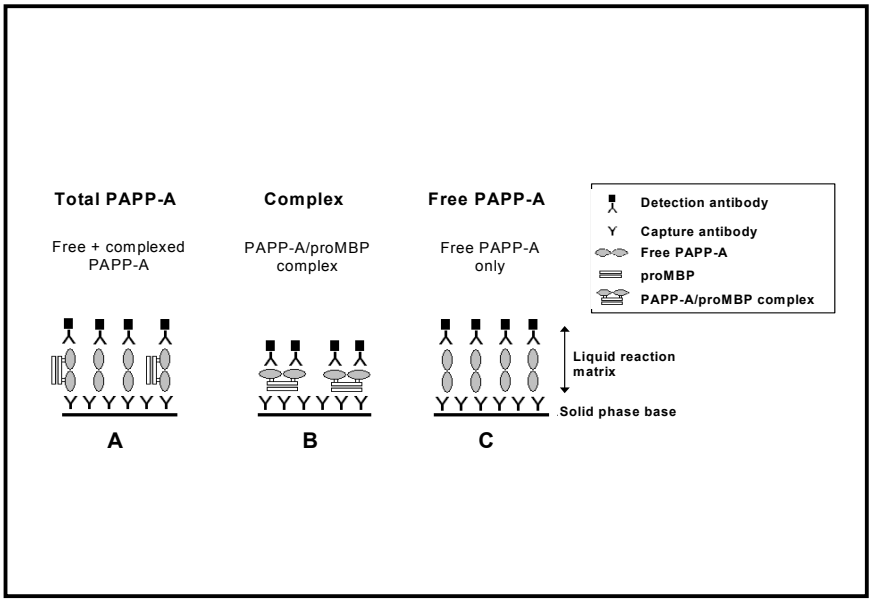
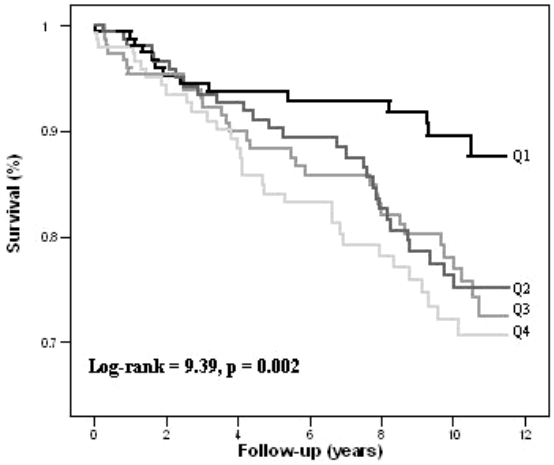
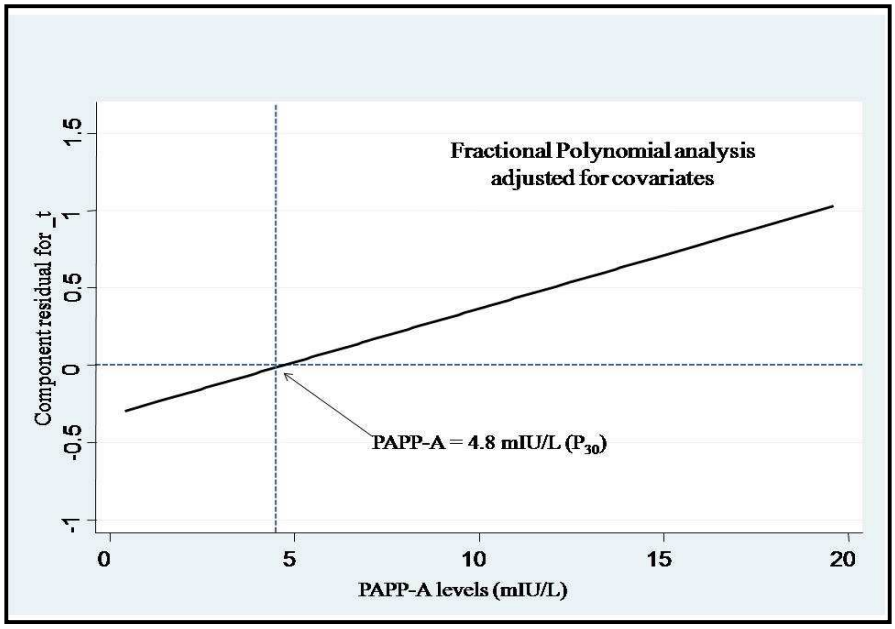


Figure 10. The Kaplan Meier curves of survival according to PAPP-A quartiles in mIU/L (Q1 = 1st quartile ≤ 4.6; Q2 = 2nd quartile 4.6-5.6; Q3 = 3rd quartile 5.6-6.8; Q4 = 4th quartile ≥ 6.8). Reproduced with permission from Consuegra-Sanchez L et al. Clin Chim Acta 2008;391:18-23 (ref. 153).



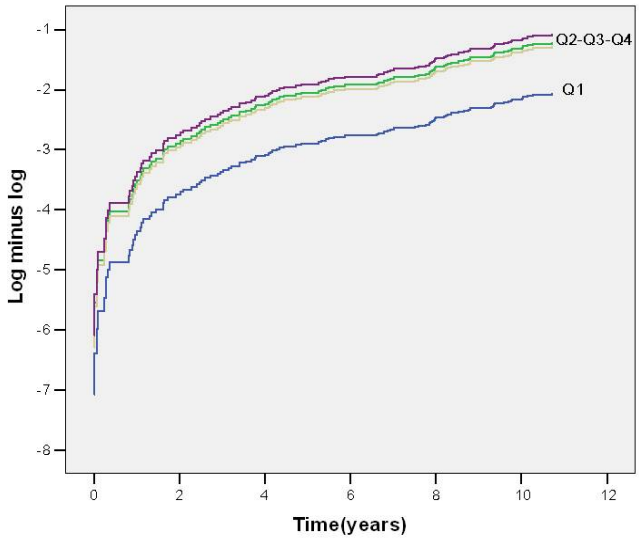
	Evaluation point (years)	2	4	6	8	10	12
Q1	Remaining cases	129	118	104	91	65	0
	Cumulative events	7	9	10	10	13	14
Q2	Remaining cases	128	112	99	88	69	0
	Cumulative events	7	14	19	23	28	31
Q3	Remaining cases	124	115	100	82	62	0
	Cumulative events	6	10	14	21	28	29
Q4	Remaining cases	115	102	92	71	49	0
	Cumulative events	9	16	21	26	31	32

Figure 11. Fractional Polynomial analysis supporting a "threshold effect" (4.8mUI/L, P_{30}) of PAPP-A levels in relation to adverse outcome, in patients with CSA.



Covariates were age, gender, hypertension, hyperlipidaemia, creatinine, vessel score, Sullivan extension score, LVEF, type 2 diabetes mellitus, revascularization at index hospitalization and CRP.

Figure 12. Log-minus-log curves to test the proportional hazard assumption.



Q1 to Q4 = Quartiles of PAPP-A levels at study entry.

Figure 13A & B. The Receiver Operating Characteristic (ROC) curves of PAPP-A levels (left) and the multivariable model with and without the incorporation of PAPP-A (right, p = non-significant).

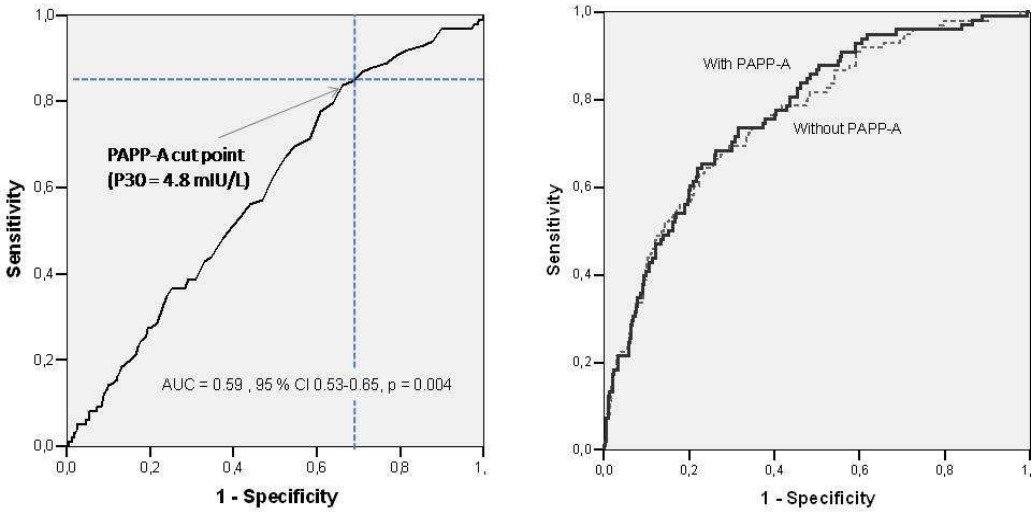
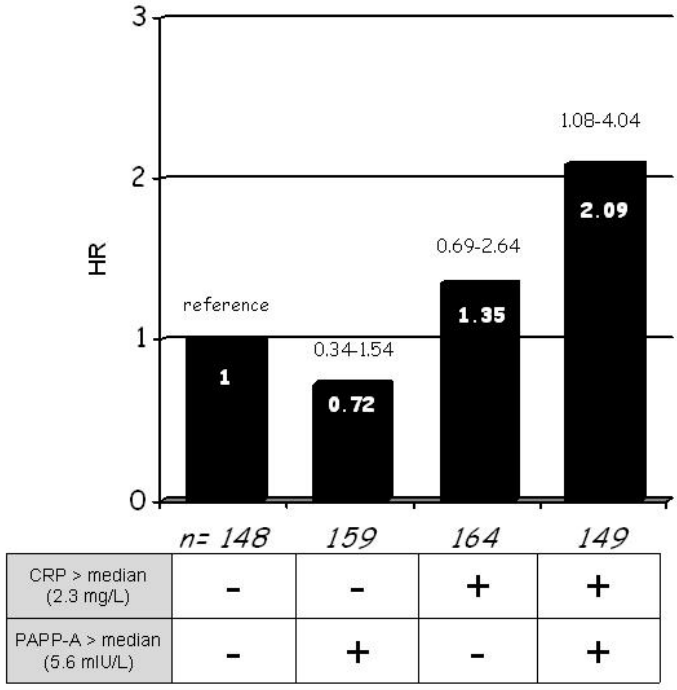


Figure 14. Hazard ratios (HR) for all-cause death according to baseline levels of CRP and PAPP-A in combined analysis. The relative risk and 95% CI for the patients is provided.



Note: The HR is adjusted by age, gender, hypertension, hyperlipidaemia, creatinine, vessel score, Sullivan extension score, LVEF, type 2 diabetes mellitus and revascularization at index hospitalization.

Figure 15 A & B. Kaplan-Meier curves of survival according to pro-MBP (left) & PAPP-A/pro-MBP ratio (right) quartiles at study entry.