Identificación de nuevos biomarcadores en los síndromes coronarios agudos

Judit Cubedo Ràfols

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Identificación de nuevos biomarcadores en los síndromes coronarios agudos

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Centro de Investigación Cardiovascular (CSIC-ICCC)

Doctoranda
Judit Cubedo

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Dra. Lina Badimon

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A mis padres

A David

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ÍNDICE

ABREVIATURAS

2-DE: electroforesis bidimensional (*bidimensional electrophoresis*)

ACEI: inhibidor de la enzima convertidora de angiotensina (*angiotensin-converting-enzyme inhibitor*)

ACS: síndrome coronario agudo (*acute coronary syndrome*)

AGE: productos finales de glicosilación avanzada (*advanced glycation end products)*

AhR: receptor de hidrocarburo de arilo (*aryl hydrocarbon receptor*)

AIIRA: antagonistas de los receptores de angiotensina II (*angiotensin II receptor antagonists*)

AMI: infarto agudo de miocardio (*acute myocardial infarction*)

ANP: péptido natriurético atrial (*atrial natriuretic peptide*)

Apo: Apolipoproteína (*apolipoprotein*)

ARNT: translocador nuclear del receptor de hidrocarburo de arilo (*aryl hydrocarbon receptor nuclear translocator*)

ASTEROID: *A Study to Evaluate the Effect of Rosuvastatin on Intravascular Ultrasound-Derived Coronary Atheroma Burden*

BMI: índice de masa corporal (*body mass index*)

BNP: péptido natriurético tipo B (*type-B natriuretic peptide*)

CK-MB: creatina quinasa-isoforma B muscular (*creatine kinase muscular isoform B*)

COX2: ciclooxigenasa-2 (*cyclooxygenase-2*)

CRP: proteína C reativa (*C-reactive protein*)

CVA: accidente cerebrovascular (*cerebrovascular accident*)

DiGE: electroforesis diferencial en gel (*Difference Gel Electrophoresis*)

DNA: ácido desoxirribonucleico (*deoxyribonucleic acid*)

dTTR: dímero de transtiretina (*transthyretin dimer*)

ECG: electrocardiograma (*electrocardiogram*)

ECM: matriz extracelular (*extracellular matrix*)

ELISA: ensayo por inmunoabsorción ligado a enzimas (*enzyme-linked immunosorbent assay*)

eNOS: óxido nítrico sintasa endotelial (*endothelial nitric oxide synthase*)

ESI: ionización mediante electroespray (*elecrospray ionization*)

FFAu: ácidos grasos libres no unidos a albumina (*unbound free fatty acid*)

FH: hipercolesterolemia familiar (*familial hypercholesterolemia*)

FRS: *Framingham Risk Score*

HDL: lipoproteínas de alta densidad (*high density lipoproteins*)

HIV: virus de la inmunodeficiencia adquirida (*human immunodeficiency virus*)

HPLC: cromatografía líquida de alta resolución (*high-performance liquid chromatography*)

Hsp: proteína de estrés térmico (*heat shock protein*)

HUVEC: células endoteliales de vena umbilical humana (h*uman umbilical vein endothelial cells*)

I/R: isquemia/reperfusión (*ischemia/reperfusion*)

ICAM-1: molécula de adhesión celular tipo-1 (*intercellular adhesion molecule 1*)

IDEAL: *Incremental Decrease in End Points through Aggressive Lipid Lowering*

IDL: lipoproteínas de densidad intermedia (*intermediate density lipoproteins*)

IEF: isoelectroenfoque (*isoelectrofocusing*)

IgA: inmunoglobulinas tipo A (*immunoglobulin A*)

IgG: inmunoglobulinas tipo G (*immunoglobulin G*)

IL-6: interleucina-6 (*Interleukin-6*)

IL-8: interleucina-8 (*Interleukin-8*)

IMA: albúmina modificada por isquemia (*ischemia modified albumin*)

IPA: *Ingenuity System Pathway Analysis*

IPG: gradiente de pH inmovilizado (*immobilized pH gradient*)

IPost-Co: post-condicionamiento isquémico (*ischemic post-conditioning*)

IT: trampa iónica (*ion tramp*)

LC: cromatografía líquida (*liquid cromatography*)

LCAT: lecitin colesterol aciltransferasa *(lecitin-cholesterol acyl transferase*)

LDL: lipoproteínas de baja densidad (*low density lipoproteins*)

LVEF: fracción de eyección del ventrículo izquierdo (*left ventricular ejection fraction*)

m/z: relación masa/carga (*mass/charge relation*)

MALDI: deserción/ionización mediante láser asistida por matriz (*matrix – assited laser desorption/ionization time-of-flight*)

MCP-1: proteína quimioatrayente de monócitos tipo 1 (*monocyte chemotactic protein-1*)

MMP: metaloproteasas (*metaloproteases*)

MP-EX: microparticulas-exosomas (*microparticles-exosomes*)

MPO: mielo-peroxidasa (*myeloperoxidase*)

mRNA: ácido ribonucleico mensajero (*messenger ribonucleic acid*)

MS: espectrometría de masas (*mass spectrometry*)

mTTR: monómero de transtiretina (*transthyretin monomer*)

NO: óxido nítrico (*nitric oxide*)

NT-proBNP: propéptido natriurético tipo B N-terminal (*N-terminal natriuretic propeptide type B*)

PAI-1: inhibidor-1 del activador del plasminógeno (*plasminogen activator inhibitor-1*)

PAPP-A: proteína plasmática A asociada al embarazo (*pregnancy-associated plasma protein A*)

PGI_2: prostaglandina I_2 (*I_2 prostaglandin*)

pI: punto isoeléctrico (*isoelectric point*)

PLTP: proteína de transporte de fosfolípidos (*phospholipid transfer protein*)

PNGasaF: péptido-N-glicosidasa tipo F (*Peptide-N-glycosidase F*)

PON-1: paraoxonasa-1 (*paraoxonase-1*)

PVD: enfermedad vascular periférica (*peripheral vascular disease*)

PVDF: membranas de polivinilo (*polyvinylidene difluoride membranes*)

RA: artritis reumatoide (*rheumatoid arthritis*)

RBP4: proteína plasmática de unión del retinol (*retinol binding protein-4*)

RNA: ácido ribonucleico (*ribonucleic acid*)

r-LDL: receptor de las lipoproteínas de baja densidad (*low density lipoproteína receptor*)

ROS: especies reactivas del oxígeno (*reactive oxigen species*)

sCD40L: ligando soluble del CD40 (*soluble CD40 ligand*)

SCORE: *Systematic Coronary Risk Evaluation*

SDS-PAGE: electroforesis en geles de poliacrilamida en presencia de SDS (*sodium dodecyl sulfate polyacrylamide gel electrophoresis*)

SELDI: deserción/ionización mediante láser optimizada en superficie (*surface enhanced laser desorption ionization*)

SLE: lupus eritomatoso sistémico (*systemic lupus erythematosus*)

SMC: células musculares lisas (*smooth muscle cells*)

TF: factor tisular (*tissue factor*)

TNF-α: factor alfa de necrosis tumoral (*tumor necrosis factor-alpha*)

TNT: *Treating to New Targets*

TOF: tiempo de vuelo (*time of flight*)

TTR: transtiretina (*transthyretin*)

tTTR: trímero de transtiretina (*transthyretin trimer*)

ttTTR: tetrámero de transtiretina (*transthyretin tetramer*)

VCAM-1: proteína de adhesión celular vascular tipo 1 (*vascular cell adhesion protein-1*)

VLDL: lipoproteínas de muy baja densidad (*very low density lipoproteins*)

WB: western blot

WBC: contaje de células blancas (*white blood cells count*)

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INTRODUCCIÓN

1. ANTECEDENTES

Las enfermedades cardiovasculares son actualmente la mayor causa de muerte en el mundo. De hecho datos del Framingham Heart Study advierten que 1 de cada 2 hombres y 1 de cada 3 mujeres desarrollarán enfermedad cardíaca sintomática a lo largo de su vida (D'Agostino et al. 2000) siendo la aterosclerosis su causa subyacente más frecuente. La aterosclerosis, engrosamiento de la pared arterial en zonas discretas en relación al flujo sanguíneo, suele progresar gradualmente. Cuando las placas ateroscleróticas se complican con un trombo ocasionan una obstrucción brusca de una arteria con obliteración del flujo en el órgano irrigado por dicha arteria. Según su localización, esta oclusión puede dar lugar a un accidente cerebrovascular (CVA), una obstrucción arterial periférica o a un síndrome coronario agudo (ACS), que pueden ocasionar muerte súbita o dejar graves secuelas a quienes los sufren (Badimon et al. 2002). Aunque se han producido grandes avances en el tratamiento de esta enfermedad, la medicina actual no es capaz de predecir de una forma adecuada el riesgo de sufrir patología cardiovascular y por tanto prevenir en individuos susceptibles el avance de la enfermedad. Por ello, uno de los mayores retos de la medicina cardiovascular es encontrar la manera de predecir el riesgo de un individuo de sufrir un evento trombótico agudo**.**

2. EVOLUCIÓN DE LA ATEROTROMBOSIS Y SUS COMPLICACIONES

La aterotrombosis es la conjunción de la aterosclerosis, que se caracteriza por un engrosamiento de la luz del vaso, con el proceso trombótico asociado que se dispara por la rotura o erosión de placas ateroscleróticas de alto riesgo (Badimon et al. 2002).

2.1. PRIMERAS FASES DE LA LESIÓN ATEROSCLERÓTICA

En las primeras fases la enfermedad progresa lenta y silenciosamente, sin manifestación clínica (Moreno 2001).

La presencia de factores de riesgo aterogénicos inducen la disfunción del endotelio caracterizada por una disminución en la biodisponibilidad del óxido nítrico (NO), un incremento en la permeabilidad de la capa endotelial, así como un aumento en la

secreción de compuestos quimiotácticos solubles (MCP-1, IL-8) (Libby et al. 2002) y la expresión de moléculas de adhesión como integrinas y selectinas (ICAM-1, VCAM-1, E-selectina) (Kinlay et al. 2001; Horstman et al. 2004). Dichas moléculas quedan expuestas en la superficie del endotelio vascular favoreciendo la adhesión de monocitos y linfocitos T. La presencia de zonas con disfunción endotelial favorece el flujo de las LDL hacia el subendotelio donde sufren modificaciones, tales como la agregación, fusión, oxidación y demás modificaciones enzimáticas, incrementando su aterogeneicidad y formando la estría grasa (Napoli et al. 1997; Llorente-Cortes et al. 2005; Badimon et al. 2006).

En las zonas donde el subendotelio está enriquecido en LDL modificadas es donde se da preferentemente la transmigración de los monocitos (Sima et al. 2009). De hecho hay evidencias que demuestran que niveles elevados de colesterol-LDL inducen la entrada selectiva de monocitos y células T en las lesiones ateroscleróticas (Swirski et al. 2007; Badimon et al. 2011). Cuando los monocitos alcanzan la íntima se diferencian en macrófagos y expresan receptores *scavenger,* que incorporan colesterol de las LDL modificadas convirtiéndose en células espumosas (Tabas 2009). Estas células espumosas secretan citoquinas, factores de crecimiento, metaloproteasas (MMP), especies reactivas del oxigeno (ROS) y factor tisular (TF) perpetuando la repuesta inflamatoria, induciendo el remodelado vascular y aumentando la susceptibilidad de la placa a la rotura y la consiguiente formación del trombo. Los diferentes estímulos aterogénicos inducen un cambio fenotípico en las células musculares lisas (SMC) que pasan de un fenotipo contráctil y no-proliferante a uno proliferante con capacidad de migrar y sintetizar matriz extracelular (ECM). Una vez dentro de la íntima, las SMC expresan receptores de colesterol participando en el proceso de internalización celular de lípidos y acumulación lipídica de la placa aterosclerótica. De hecho, la cantidad de SMC es un factor determinante de la susceptibilidad de la placa. Mientras que en las lesiones iniciales las SMC representan el 90-95% del componente celular, esta proporción va disminuyendo a un 50% en las lesiones ateroscleróticas avanzadas (Llorente-Cortes et al. 2004).

2.2. LESIÓN AVANZADA Y PRESENTACIÓN CLÍNICA

Las lesiones progresan a medida que se van incorporando lípidos extracelulares a los depósitos de células cargadas de colesterol y lípidos residuales de células espumosas que entran en apoptosis. Éstos, junto con las células espumosas, forman el núcleo lipídico de la lesión ateromatosa (Fuster et al. 1992; Falk et al. 1995). Las lesiones avanzadas se acaban estructurando como placas fibrocelulares que contienen núcleos lipídicos de tamaño variable (Fuster et al. 1992). Estas placas se mantienen estables mientras sean ricas en células musculares, predomine el componente esclerótico, colágeno y elastina **(Figura 1A).** Estudios anatomopatológicos en humanos han revelado que las características que definen una placa vulnerable son: (a) un gran núcleo lipídico con necrosis; (b) una capa fibrosa delgada; (c) un aumento de células inflamatorias en la capa fibrosa; (d) una reducción de la cantidad de colágeno y SMC; y (e) presencia de neovascularización (Fuster et al. 2005).

Esta situación, a medida que se va comprometiendo el lúmen vascular de forma gradual, puede continuar siendo clínicamente asintomática o acabar manifestándose como síntomas isquémicos crónicos a expensas del incremento en la demanda de oxígeno (angina crónica estable, claudicación intermitente). Por el contrario la placa se convertirá en inestable si predomina el componente ateromatoso: colesterol, fosfolípidos, agua y un reducido número de SMC **(Figura 1B)** (Davies et al. 1985).

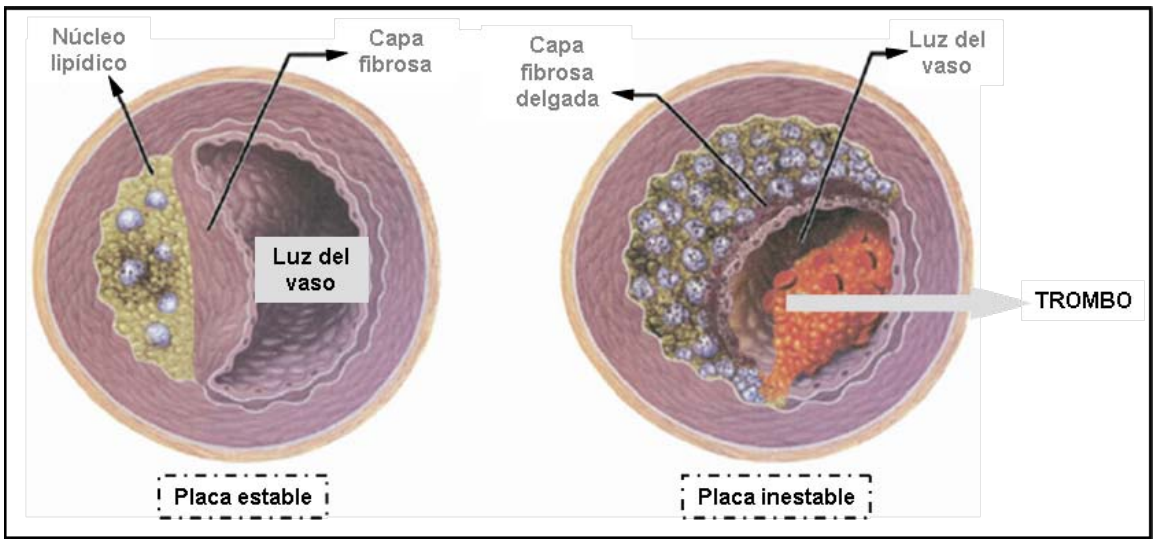

Figura 1. A: placa estable; **B:** placa inestable.

El riesgo de sufrir una complicación trombótica depende más de la composición bioquímica y celular de la placa que del grado de estenosis. A medida que la capa fibrosa disminuye, la placa se va haciendo más susceptible a la ruptura con la consiguiente exposición del núcleo lipídico, altamente trombogénico, hacia el lúmen del vaso (Fuster et al. 1992). Estudios llevados a cabo para evaluar la trombogeneicidad de diferentes componentes de la placa han demostrado que el núcleo lipídico es seis veces más trombogénico que el resto de componentes (Fernandez-Ortiz et al. 1994). Cuando se da la rotura de la placa, la trombosis asociada puede dar lugar a síntomas de isquemia aguda y derivar en un infarto si el trombo ocluye el vaso, o bien, incorporarse en la placa favoreciendo el crecimiento de la misma y comprometiendo cada vez más la luz del vaso **(Figura 2)** (Fuster et al. 1992; Falk et al. 1995).

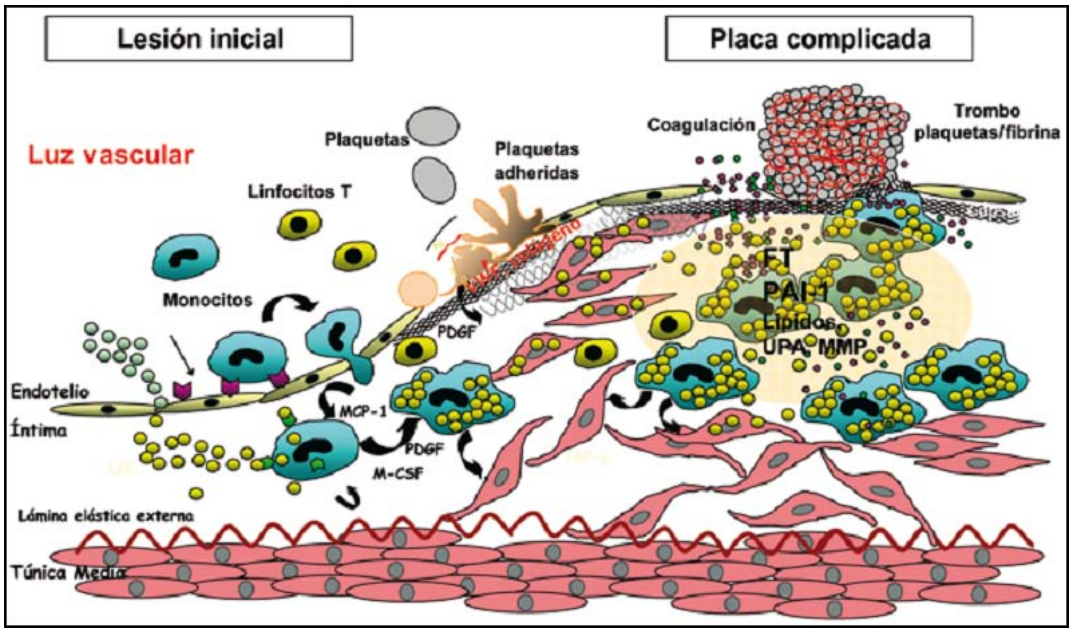

Figura 2. Evolución lesión aterosclerótica hasta la formación del trombo (Badimon et al. 2009).

La trombosis luminal se da tanto por la rotura como por la erosión de la placa. La rotura de la placa se da a partir de lesiones con un núcleo necrótico y un capa fibrótica muy fina que acaba derivando en una trombosis luminal como consecuencia del contacto de las plaquetas con el núcleo altamente trombogénico (Thim et al. 2008). La activación de las plaquetas juega un papel crucial en la progresión de la patología y la precipitación de

la manifestación clínica. La disfunción del endotelio induce el rodado de las plaquetas (mediado por P-selectina) y su posterior adhesión (mediada por integrinas) (Collins et al. 2000; Manka et al. 2001; Massberg et al. 2002; Burger et al. 2003). Adicionalmente, las plaquetas activadas liberan el contenido de sus gránulos promoviendo la adhesión celular, supervivencia y proliferación, coagulación y proteólisis, que aceleran y magnifican el proceso inflamatorio (Jennings 2009).

Las placas susceptibles de rotura tienen una elevada infiltración de macrófagos y en cambio no tienen células musculares lisas o tienen pocas. Las placas erosionadas son ricas en células musculares lisas y proteoglicanos en su parte luminal y presentan una menor calcificación comparadas con las rotas. La mayoría de placas erosionadas no contienen un núcleo necrótico pero en caso de tenerlo no se comunica con el lúmen por tener una capa fibrótica gruesa. La capa media está intacta e incluso es más gruesa que en los sitios de rotura de la placa. En estos casos hay una trombosis aguda en contacto directo con la íntima en un área ausente de endotelio. La erosión de placas está presente en un 20% de las muertes súbitas y en un 40% de los trombos coronarios de pacientes con aterosclerosis coronaria que mueren repentinamente (Virmani et al. 2000). Los factores de riesgo involucrados en la erosión de las placas son diferentes de los implicados en la rotura de las mismas. Estas placas erosionadas tienen una mayor frecuencia de trombos no oclusivos y presentan una menor estenosis. Se ha visto una mayor frecuencia de placas erosionadas en mujeres e individuos jóvenes (Farb et al. 1996; Virmani et al. 2006).

3. MANIFESTACIONES CLÍNICAS DE LA PATOLOGÍA ATEROTROMBÓTICA

3.1. TIPOS DE MANIFESTACIONES

Los síndromes aterotrombóticos derivan en diferentes tipos de manifestaciones clínicas en función de la zona del cuerpo que afectan **(Figura 3).**

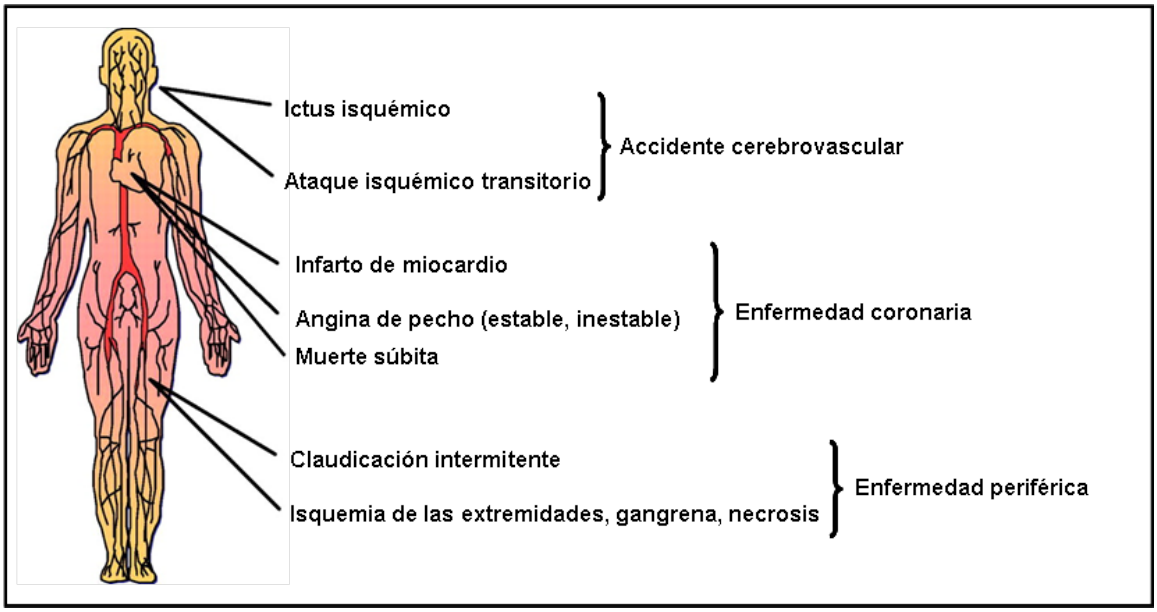

Figura 3. Manifestaciones clínicas de la enfermedad aterotrombótica (adaptado de Viles-Gonzalez et al. (Viles-Gonzalez et al. 2004)).

Entre las diferentes manifestaciones clínicas de la patología cardiovascular la más frecuente es la enfermedad coronaria que representa el 48.8 y el 40.7%, en hombres y mujeres respectivamente (datos de la OMS 2007).

3.2. FACTORES DE RIESGO

La patología cardiovascular es una enfermedad de tipo multifactorial a la que contribuyen mayoritariamente dos grupos de factores de riesgo: no modificables y modificables.

Entre los factores de riesgo no modificables encontramos la edad, el sexo y la predisposición genética. Los factores de riesgo modificables son patologías como la hiperlipemia, la diabetes, la hipertensión arterial y la obesidad, y hábitos adquiridos como el tabaquismo **(Figura 4)**. En base a estos factores existen diferentes escalas de estratificación del riesgo (tablas estudio Framingham, el proyecto SCORE) que sirven para calcular el riesgo de sufrir un evento cardiovascular en un período de tiempo concreto. La presencia de unos factores de riesgo determinados influye en el área

arterial afectada, de tal forma que el tabaquismo está asociado a la arteriosclerosis en arterias de extremidades inferiores, mientras que la hipertensión está más relacionada con la arteriosclerosis en arterias cerebrales. La hipercolesterolemia en cambio juega un papel importante en la aterogénesis a nivel coronario (Padró et al. 2008).

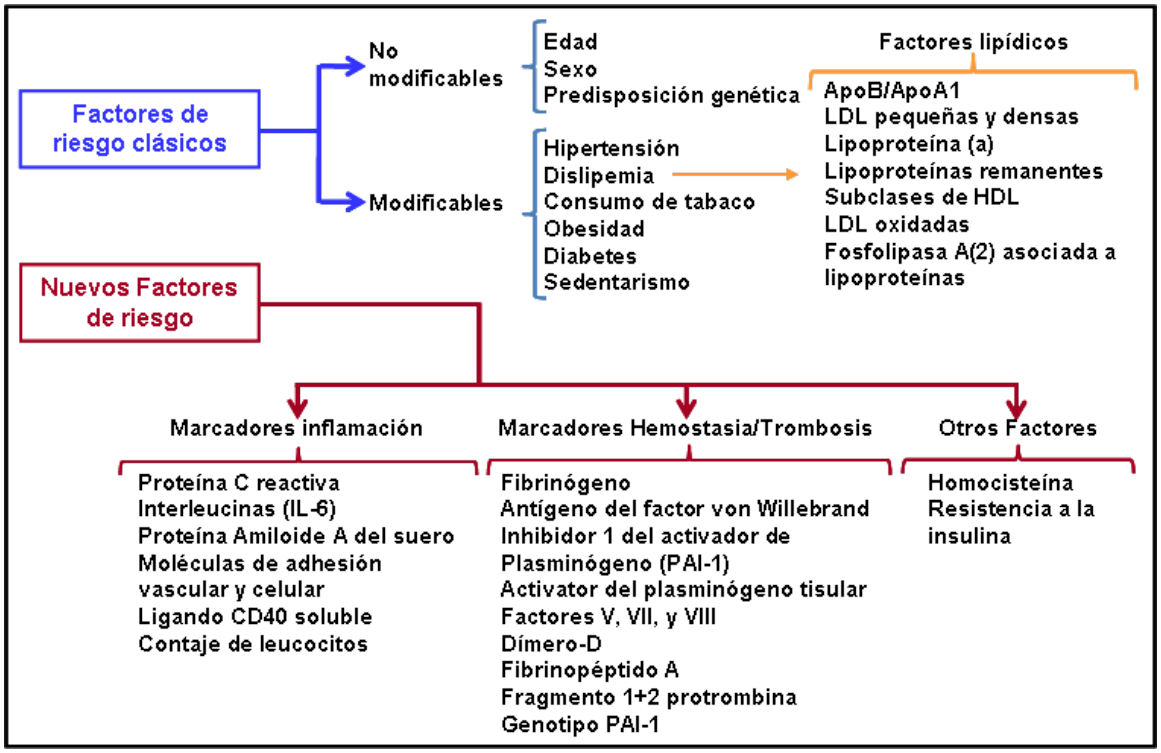

Figura 4. Clasificación factores de riesgo cardiovascular.

3.2.1. EDAD, SEXO Y PREDISPOSICIÓN GENÉTICA

La enfermedad cardiovascular tiene una mayor incidencia después de los 45 años en hombres y de los 55 años en las mujeres. El grado de incidencia de enfermedad cardiovascular es inferior en mujeres entre 30 y 50 años que en hombres de su misma edad (Sytkowski et al. 1996). Es más, las mujeres presentan una menor incidencia y extensión de placas ateroscleróticas a nivel de arterias coronarias y una menor prevalencia de enfermedad coronaria obstructiva que el hombre (Nicholls et al. 2007; Han et al. 2008). Por el contrario, la mujer tiende a presentar mayor número de resultados adversos y peor pronóstico tras sufrir un infarto agudo de miocardio y

someterse a cirugía de derivación arterial coronaria (Vaccarino et al. 1999; Vaccarino et al. 2002). Estudios experimentales y clínicos sugieren que la diferencia entre hombres y mujeres pre-menopáusicas viene dada por un efecto beneficioso de los estrógenos sobre el endotelio vascular, tanto a nivel celular como molecular (Stampfer et al. 1991; Rosano et al. 1993; Hsia et al. 2006). De este modo, los estrógenos inducen un aumento en la biodisponibilidad del NO vascular y una respuesta genómica que conlleva cambios en la transcripción de diferentes genes con elementos de respuesta a estrógenos (Haynes et al. 2000; Joy et al. 2006; Pedram et al. 2007; Traupe et al. 2007).
Los antecedentes familiares de enfermedad cardiovascular precoz (antes de los 55 años en hombres y de los 65 años en mujeres) también aumentan significativamente el riesgo (Perk 2009).

3.2.2. DISLIPEMIAS

Las lipoproteínas son estructuras compuestas de proteínas y fosfolípidos que facilitan el transporte de lípidos en el torrente circulatorio y se clasifican de forma creciente en función de su densidad: quilomicrones, VLDL (lipoproteínas de muy baja densidad), IDL (lipoproteínas de densidad intermedia), LDL (lipoproteínas de baja densidad) y HDL (lipoproteínas de alta densidad). Además también difieren en su composición lipidico-proteica. Las LDL tienen una mayor proporción de colesterol y los quilomicrones de triglicéridos. Las HDL son las más densas por contener un mayor número de proteínas. Específicamente, las LDL son una clase heterogénea de lipoproteínas con una densidad de 1.019-1.063 g/mL formadas por un núcleo hidrofóbico que contiene triglicéridos y ésteres de colesterol, y una cubierta hidrofílica formada por fosfolípidos, colesterol libre y apolipoproteínas, predominantemente Apo B-100. La presencia de niveles elevados de LDL ha demostrado tener un papel clave en el inicio y la progresión del desarrollo de la aterosclerosis (Badimon et al. 2006; Badimon et al. 2009; Badimon et al. 2011). Está demostrado además que los pacientes con hipercolesterolemia presentan hipercoagulabilidad y una mayor reactividad plaquetar en zonas de daño vascular (Badimon et al. 1991). Teniendo en cuenta el importante efecto deletéreo del colesterol-LDL, las estrategias farmacológicas hipolipemiantes se han convertido en la diana por excelencia en la prevención y el tratamiento de la patología cardiovascular. Hay varios estudios en los que se ha demostrado una reducción del 20-30% del riesgo cardiovascular gracias al tratamiento

con una clase de fármacos hipolipemiantes, los inhibidores de la 3-hidroxi-3-metilglutaril coenzima A reductasa (estatinas) (Scandinavian Simvastatin Survival Study 1994; Shepherd et al. 1995; Sacks et al. 1996; Downs et al. 1998; Heart Protection Study Collaborative Group 2002; Cannon et al. 2004). Es más, los resultados obtenidos en el ensayo clínico ASTEROID (*A Study to Evaluate the Effect of Rosuvastatin on Intravascular Ultrasound-Derived Coronary Atheroma Burden*) han revelado que un tratamiento hipolipemiante agresivo es también capaz de regresar la placa aterosclerótica coronaria ya establecida (Nissen et al. 2006). Varios estudios han demostrado que las estatinas poseen efectos beneficiosos más allá de la reducción del colesterol LDL (WOSCOPS 1998). De hecho, las estatinas muestran propiedades antiinflamatorias, antioxidantes, y antitrombóticas (Badimon et al. 1991; Kinlay et al. 2006; Libby et al. 2006; Tsimikas 2006). A pesar de la reducción del riesgo por el tratamiento intensivo con estatinas aún hay un alto riesgo residual (~75%) de sufrir un evento cardiovascular. Por este motivo hay una importante necesidad de encontrar estrategias alternativas y complementarias a las estatinas para la reducción del riesgo residual, y en este contexto cada vez hay más estudios dirigidos a las HDL como diana terapéutica (Brown et al. 2010; Badimon et al. 2012). Las HDL, son lipoproteínas pequeñas de alta densidad, compuestas tanto por lípidos (fosfolípidos, colesterol, ésteres de colesterol y triglicéridos) como por proteínas (siendo la Apo A-I la proteína mayoritaria). Se ha visto que una reducción en los niveles de colesterol asociado a lipoproteínas de alta densidad (HDL) está claramente asociada con el desarrollo de aterosclerosis, así como con un peor pronóstico en pacientes con patología cardiovascular establecida (Abbott et al. 1988; Assmann et al. 1998). De hecho, las HDL se considera que juegan un papel fundamental en el futuro del tratamiento de las enfermedades aterotrombóticas por su papel en el transporte reverso del colesterol (Choi et al. 2006). El posible papel terapéutico de las HDL se mostró por primera vez mediante la administración de HDL3 homologas en un modelo experimental donde se vio una inhibición del desarrollo de placas ateroscleróticas (Badimon et al. 1989) así como una regresión de las mismas (Badimon et al. 1990). Junto al papel en el transporte reverso del colesterol, las HDL poseen diferentes propiedades anti-inflamatorias, inmunomoduladoras y antioxidantes que hacen aún más importante su papel en la patología cardiovascular (Barter et al. 2004). Cada vez más estudios apoyan que el papel ateroprotector de las HDL reside más en su calidad que en la cantidad. De hecho, varios estudios apoyan que para la terapia basada en HDL se tendría que tener en cuenta

la función de las HDL más que los niveles de colesterol asociados a las mismas (Barter et al. 2007; Barter 2009; Blasi et al. 2009). Es más, dentro de las HDL existen varios tipos de subpoblaciones que varían en tamaño, densidad y composición tanto de lípidos como de proteínas. Se ha visto que las HDL3, más densas y pequeñas, poseen más propiedades antiaterogénicas que las HDL2, de menor densidad y más grandes (Camont et al. 2011). El estudio detallado de la composición (lipídica y protéica) de las diferentes clases de HDL aportará nueva información sobre sus diferencias en la protección frente a la patología cardiovascular.

A los trastornos del metabolismo lipídico se les conoce como dislipemias. Existen varios tipos de dislipemias en función de la lipoproteína alterada, según la causa (primaria si es de origen genético o secundaria si es por un factor externo que altera el metabolismo lipídico) y según el fenotipo en sangre periférica. Esta última clasificación es la más utilizada en la práctica clínica y se basa en el aumento de colesterol y/o triglicéridos (hipercolesterolemia aislada, hipertrigliceridemia aislada o dislipemia mixta). A su vez, también existen las hipolipidemias que se caracterizan por la presencia de concentraciones muy bajas de colesterol.

- HIPERCOLESTEROLEMIAS PRIMARIAS
  A. HIPERCOLESTEROLEMIA FAMILIAR (FH): Es un trastorno del metabolismo lipídico caracterizado por un aumento en la concentración de colesterol total y colesterol-LDL. Es una patología de herencia autosómica dominante por mutaciones en el gen que codifica el receptor de las lipoproteínas de baja densidad (r-LDL). Junto con los niveles elevados de LDL, se caracteriza por un fenotipo con presencia de xantomas cutáneos y tendinosos, arcos corneales y una elevada prevalencia de enfermedad coronaria en edades tempranas debida a una aterosclerosis prematura. De hecho, la mortalidad por enfermedad cardiovascular en la población con FH heterocigótica es 11 veces mayor que en la población general, por lo que la prevención de esta patología es el gran reto terapéutico en estos pacientes (Mabuchi et al. 1986). Se han descrito más de 200 mutaciones puntuales responsables de la FH (Heath et al. 2001). Se estima que uno de cada 400 a 500 personas en la población general padece la variante heterocigótica y uno de cada millón la forma homocigótica (Goldstein et al. 2001).

B. DEFECTO FAMILIAR DE APO B-100: Es una enfermedad autosómica dominante debida a una mutación en el gen de la Apo B-100 que impide la unión al receptor de las LDL por lo que su concentración en sangre aumenta. Las manifestaciones clínicas y bioquímicas son idénticas a la FH y sólo se pueden diferenciar mediante diagnóstico genético (Innerarity et al. 1987).

C. HIPERCOLESTEROLEMIA POLIGÉNICA: Como su nombre indica, no es monogénica, es el resultado de la interacción entre factores genéticos y ambientales. Es la más frecuente ya que representa más del 85% de las mismas, afectando alrededor de un 5% de la población general. Sus mecanismos patogénicos no se conocen muy bien. Se manifiesta a partir de la edad adulta con altos niveles de colesterol. Tienen un riesgo elevado de cardiopatía isquémica a partir de los 50 años, pero no tanto como los individuos con hipercolesterolemia familiar heterocigótica (Joven et al. 1991).

D. HIPERLIPEMIA FAMILIAR COMBINADA: Los pacientes con esta patología pueden presentar los tres fenotipos, hipercolesterolemia aislada por un aumento de LDL, hipertrigliceridemia aislada por aumento de VLDL o dislipemia mixta por incremento de ambos. Tiene una prevalencia del 1%. Presenten un aumento de la síntesis hepática de Apo B y VLDL con un aclaramiento defectuoso de los triglicéridos plasmáticos. Los pacientes pueden presentar tanto niveles elevados de colesterol como de triglicéridos. Pueden presentar xantelasmas. Se suele diagnosticar a partir de los 30 años. Se asocia con un riesgo elevado de cardiopatía isquémica (Civeira et al. 2008).

- HIPERTRIGLICERIDEMIAS (Ferns et al. 2008)

A. HIPERQUILOMICRONEMIA FAMILIAR: Trastorno hereditario del metabolismo muy poco frecuente con niveles de triglicéridos en sangre transportados por quilomicrones superiores a 1000 mg/dL. La concentración de colesterol LDL y HDL suele ser normal o baja. Tienen un elevado riesgo de pancreatitis aguda. Los casos severos pueden presentar xantomas. No presentan un mayor riesgo de enfermedad cardiovascular (Pedro-Botet et al. 1997).

B. DÉFICIT DE LIPOPROTEINLIPASA: Es una enfermedad autosómica recesiva que suele diagnosticarse en la infancia. Se han descrito numerosas mutaciones en el gen de la lipoproteinlipasa responsables de la falta de actividad enzimática. Los homocigóticos presentan una acumulación masiva de quilomicrones en

sangre. Tienen las mismas manifestaciones clínicas que en la hiperquilomicronemia familiar. No existe un tratamiento farmacológico eficaz (Paglialunga et al. 2009).

C. DÉFICIT DE APO C-II: Es una rara enfermedad autosómica recesiva con una clínica similar al déficit de lipoproteinlipasa. Su diagnóstico se basa en la ausencia de Apo C-II en una electroforesis. El tratamiento consiste en la restricción de grasas de por vida.

D. HIPERTRIGLICERIDEMIA FAMILIAR: Es una enfermedad de herencia autosómica dominante pero aún no se ha identificado el gen responsable. Tiene una penetrancia variable en función de otros factores externos. Es la hipertrigliceridemia primaria más frecuente. Hay un aumento de producción hepática de VLDL y una disminución de su catabolismo lo que lleva a un aumento de los triglicéridos en sangre. Presentan un riesgo cardiovascular ligeramente mayor que los pacientes sin dislipemia (Brunzell 2007).

- DISLIPEMIAS MIXTAS PRIMARIAS

A. HIPERLIPOPROTEINEMIA TIPO III: Es un trastorno de origen genético que cursa con elevación de colesterol y triglicéridos plasmáticos debida a un aumento de IDL. En esta patología se da una alteración de la Apo E, apolipoproteína imprescindible para la unión de las lipoproteínas ricas en triglicéridos a los receptores hepáticos. Los pacientes con esta patología suelen presentar depósitos lipídicos en las palmas de las manos. Tienen una elevada frecuencia de cardiopatía isquémica, siendo la principal causa de muerte en estos pacientes, y enfermedad periférica, mientras que los accidentes cerebrovasculares sólo son algo más frecuentes que en la población control (Mahley et al. 1999).

- HIPOLIPIDEMIAS

A. HIPOALFALIPOPROTEINEMIA: Es una enfermedad caracterizada por una disminución en los niveles de colesterol HDL. La concentración final de HDL es el resultado de complejas interacciones entre factores genéticos y ambientales. Entre las principales hipoalfalipoproteinemias primarias podemos encontrar: la enfermedad de Tangier, la hipoalfalipoproteinemia familiar, la enfermedad de Gaucher, déficit de Apo A-I y el déficit de LCAT (Garg et al. 2007).

B. HIPOBETALIPOPROTEINEMIA FAMILIAR: Trastorno genético caracterizado por un defecto en la síntesis de Apo B. En la forma homocigótica prácticamente no se detectan quilimicrones, VLDL, LDL e IDL; en la forma heterocigótica, la más frecuente, la síntesis de estas lipoproteínas está disminuida. Puede cursar de forma asintomática. El riesgo de enfermedad vascular está disminuido con respecto a la población general (Tarugi et al. 2011).

- DISLIPEMIAS SECUNDARIAS

  Están provocadas por factores externos como la presencia de ciertas patologías, toma de medicamentos o consumo de alcohol (Garg et al. 2007).

3.2.3. DIABETES

La diabetes tipo 2 es la forma más prevalente y aparece en edades medias habitualmente en asociación a otros factores de riesgo. Este tipo de diabetes es un trastorno metabólico caracterizado por la presencia de hiperglucemia crónica que se debe a una secreción deficiente de insulina por parte de las células beta del páncreas y a una disminución de la biodisponibilidad de la glucosa por parte de los tejidos (resistencia a la insulina) (Chisholm et al. 1997; Gerich 1998). La resistencia a la insulina típicamente precede a la diabetes tipo 2 y suele estar acompañada de otros factores de riesgo: dislipemia, hipertensión y factores protrombóticos (Hopkins et al. 1996; Gray et al. 1998). La co-existencia de estos factores de riesgo da lugar al llamado síndrome metabólico. La presencia del síndrome metabólico comúnmente precede al desarrollo de la diabetes tipo dos por unos años (Haffner et al. 1990).

La otra forma de diabetes, la tipo 1, ocurre en edades tempranas de la vida y es consecuencia de la destrucción inmunológica de las células beta del páncreas (Unger et al. 1998).

La diabetes es un factor de riego independiente de enfermedad cardiovascular en ambos sexos (Wilson 1998; Wilson et al. 1998). Las mujeres diabéticas pierden su protección frente al desarrollo de enfermedad cardiovascular (Brezinka et al. 1994; Wilson et al. 1998). Las patologías cardiovasculares son las causantes de la muerte del 65% de las personas con diabetes (Geiss et al. 1995). Los diabéticos presentan un riesgo coronario unas 2 a 3 veces mayor (Kannel et al. 1979; Pyorala et al. 1987; Stamler et al. 1993).

Más concretamente, se ha sugerido que los pacientes diabéticos sin un infarto de miocardio previo tienen tanto riesgo de sufrir un infarto como los pacientes no diabéticos que ya han sufrido uno (Haffner et al. 1998). Cuando los pacientes diabéticos sufren patología cardiovascular tienen un peor pronóstico que los no diabéticos (Smith et al. 1984; Stone et al. 1989). Además, presentan 3 veces más riesgo de morir tras un ictus que los pacientes no diabéticos (Stamler et al. 1993). Aproximadamente el 13% de los pacientes con diabetes mayores de 65 años sufren un ictus (Kuller et al. 1995).

Los pacientes diabéticos presentan un aumento de su actividad plaquetar, posiblemente a través de una elevación en los niveles circulantes de factor von Willebrand. En consecuencia, las microembolias plaquetarias son frecuentes en los vasos pequeños de ciertos órganos y están asociadas al desarrollo de microangiopatías, como lo son la retinopatía, nefropatía y neuropatía diabéticas (Royo 2006).

Una de las manifestaciones de enfermedad vascular más comunes en los pacientes diabéticos es la enfermedad vascular periférica (PVD). Datos del Framingham Heart Study revelaron que el 20% de los pacientes con PVD sintomática son diabéticos (Hiatt 2001). Además los diabéticos tienen un peor pronóstico de PVD dado que en muchos casos la percepción del dolor está alterada por la presencia de neuropatía periférica. Por este motivo, los pacientes diabéticos son más propensos a sufrir úlceras isquémicas o gangrena que pacientes con PVD sin diabetes (Hoyt 2003).

Se sabe que los pacientes diabéticos presentan una predisposición genética al daño oxidativo, probablemente asociado a una marcada disfunción de las HDL (Orchard et al. 2010). Una de las consecuencias más importantes de un mal control glicémico tanto en la diabetes tipo 1 como en la tipo 2, es la glicosilación no enzimática de proteínas y la formación de productos finales de glicosilación avanzada (*AGEs*), especialmente la unión de estos *AGEs* a la apolipoproteína A-I (Brownlee et al. 1988). Esta unión de *AGEs* a la Apo A-I disminuye su capacidad para activar la lecitin-colesterol aciltransferasa (LCAT), enzima responsable de la conversión de las HDL nacientes a HDL maduras (Nobecourt et al. 2007) disminuyendo las propiedades anti-aterogénicas de las HDL. En consecuencia, la presencia de estos *AGEs* acelera el desarrollo de enfermedad cardiovascular en los pacientes hiperglicémicos (Che et al. 1997).

3.2.4. HIPERTENSIÓN ARTERIAL

La hipertensión se define por la presencia mantenida de cifras de presión arterial sistólica iguales o superiores a 140mmHg, presión arterial diastólica de 90mmHg o superior, o ambas. Diversos estudios epidemiológicos demuestran que tanto la presión sistólica como la diastólica están positivamente asociadas con la presentación de enfermedad cardiovascular (Stamler et al. 1993). Normalmente el aumento del riesgo se da en la población entre 40 y 89 años, rango de edad en el que por cada incremento de 20 mmHg en la presión sistólica o 10 mmHg en la diastólica se duplica el riesgo de mortalidad por enfermedad cardiovascular (Perk 2009). Entre las complicaciones vasculares asociadas a la hipertensión podemos encontrar la enfermedad coronaria, el ictus, la enfermedad arterial periférica, la insuficiencia cardíaca y la enfermedad renal crónica, estando todas relacionadas tanto con la presión sistólica como con la diastólica (Lewington et al. 2002). No obstante, a partir de los 55 años la relación es más estrecha con la presión arterial sistólica (Mancia et al. 2009). Los pacientes hipertensos tienen 1.6-2.5 veces más probabilidades de sufrir un evento cardiovascular que de morir por causa no cardiovascular. Específicamente, en los hombres hipertensos de menos de 60 años las probabilidades de sufrir un evento cardiovascular son 6 veces más altas que de morir por causa no cardiovascular. El tipo de evento difiere en función del sexo y la edad de aparición de la hipertensión. El primer evento más común en hombres jóvenes es la enfermedad coronaria (infarto y angina), mientras que en hombres de edad avanzada y en mujeres de todas las edades el ictus es el primer evento más común (Lloyd-Jones et al. 2005). De hecho, el factor de riesgo modificable más importante para el ictus es la presión arterial elevada (Kannel et al. 1996). Hay estudios que demuestran que los antecedentes de presión arterial están relacionados con el riesgo de sufrir un ictus, incluso tras la corrección por los niveles de presión arterial en el momento del evento (Seshadri et al. 2001). Con el envejecimiento general de la población la prevalencia de la hipertensión aumentará y su impacto como factor de riesgo crecerá por lo que el establecimiento de terapias óptimas para su reducción es de vital importancia.

La hipertensión se puede reducir tanto con cambios de hábitos como con tratamiento farmacológico. Los cambios de hábitos incluyen la reducción en la ingestión de sal, la pérdida de peso y la moderación del consumo de alcohol. Diversos estudios clínicos de terapias anti-hipertensivas han documentado reducciones drásticas en la presentación de

eventos cardiovasculares (Psaty et al. 2003). Entre los fármacos antihipertensivos más comúnmente utilizados encontramos: inhibidores de la enzima convertidora de angiotensina (ACEI), antagonistas del calcio y antagonistas de los receptores de angiotensina II (AIIRA). Junto con terapias anti-hipertensivas se ha visto que la aplicación de tratamientos hipolipemiantes y aspirina tiene efectos muy importantes en la prevención de enfermedad coronaria (Hansson et al. 1998; Sever et al. 2003).

3.2.5. TABACO

El tabaco es responsable del 50% de todas las muertes evitables y la mitad son debidas a enfermedades cardiovasculares (Bartecchi et al. 1994; MacKenzie et al. 1994). De hecho, los fumadores tienen más riesgo de sufrir un infarto agudo de miocardio (3 veces más), muerte súbita (2 veces más), ictus (1.5 veces más), aneurisma aórtico abdominal (de 2 a 9 veces más) y enfermedad vascular periférica (7 veces más) (Burns 2003). Muchos de los componentes del tabaco son protrombóticos y/o aterogénicos. El consumo de tabaco aumenta la coagulación de la sangre, la agregación plaquetar y la formación de trombos, disminuye el aporte de oxígeno, induce disfunción endotelial, aumenta el estrés oxidativo y la inflamación vascular, promueve la vasoconstricción coronaria, aumenta el trabajo cardíaco, y altera el metabolismo lipídico (Beckman et al. 2004; Chelland Campbell et al. 2008; Benowitz 2009). Se ha demostrado que el tabaco induce daño en las proteínas activando la autofagia y provocando la muerte por necrosis de las células endoteliales, y se ha propuesto este mecanismo como uno de los responsables del deterioro de la función del endotelio vascular y la iniciación de la aterosclerosis (Csordas et al. 2011). Se sabe que las catecolaminas circulantes pueden promover la agregación plaquetar y la generación de trombina (Ip et al. 1991). Los fumadores presentan un incremento en la reactividad plaquetar (Fuster et al. 1981), que también se ha asociado a la liberación de catecolaminas (Winniford et al. 1986). El estudio INTERHEART demostró que el consumo de tabaco es el factor de riesgo modificable más importante al que se le atribuye el 36% del riesgo de sufrir un infarto agudo de miocardio (Yusuf et al. 2004). A este respecto, varios estudios han demostrado la importante reducción en el riesgo cardiovascular tras la interrupción del consumo de tabaco (Ford et al. 2007; Hardoon et al. 2008). Es más, se ha demostrado que es la intervención que induce, por sí sola, una reducción más importante en la morbilidad y mortalidad de los fumadores con

enfermedad coronaria (Pipe et al. 2011). Dichos pacientes presentan una reducción del 36% en la mortalidad y un 32% en el riesgo de sufrir un AMI no fatal dentro de los dos primeros años sin fumar. Esta reducción es mayor que la que aportan otras terapias de prevención secundaria como el uso de estatinas (reducción del 29%), beta-bloqueantes (23%), aspirina (23%) o los inhibidores del enzima convertidor de la angiotensina (23%) (Critchley et al. 2003). Estudios recientes, como el TNT (*Treating to New Targets*) y el IDEAL (*Incremental Decrease in End Points through Aggressive Lipid Lowering*), indican que el cese en el consumo del tabaco tiene un mayor efecto en la reducción del riesgo en fumadores con enfermedad coronaria que el aumento en las dosis de estatinas**,** demostrando que el consumo de tabaco es un determinante importante de la salud cardiovascular (Frey et al. 2011).

3.2.6. OBESIDAD

En individuos adultos, el sobrepeso se define como un índice de masa corporal (BMI=peso/altura^2) entre 25 y 30kg/m^2 y la obesidad como un BMI $\geq$ 30kg/m^2 (Klein et al. 2004). También se usan otros índices que han demostrado tener más valor predictivo como la grasa corporal, la circunferencia de la cintura, el ratio cintura-cadera y el ratio peso-altura (Litwin 2008). La obesidad tiene un importante efecto adverso sobre la salud en general y de forma muy importante sobre la patología cardiovascular (Lavie et al. 2003). Además de ser un factor de riesgo independiente para la aterosclerosis y la enfermedad coronaria, es uno de los componentes más importantes del síndrome metabólico, y tiene una influencia fundamental sobre otros factores de riesgo como la hipertensión arterial, la dislipemia y la diabetes (Hubert et al. 1983; Lavie et al. 2005; Lavie et al. 2007). Este gran impacto de la obesidad se basa en el hecho de que el tejido adiposo es un órgano endocrino capaz de sintetizar y liberar una gran cantidad de moléculas a la sangre que pueden influir sobre la homeostasis cardiovascular como la interleucina-6 (IL-6), la adiponectina, la resistina, el inhibidor-1 del activador del plasminógeno (PAI-1), y el factor alfa de necrosis tumoral (TNF-α) (Hotamisligil et al. 1995; Lundgren et al. 1996; Wajchenberg 2000; Steppan et al. 2001). Concretamente, el 30% de IL-6 circulante se origina en el tejido adiposo (Mohamed-Ali et al. 1997; Yudkin et al. 1999). La IL-6 modula la producción de CRP en el hígado y la CRP es un marcador de inflamación crónica que es uno de los desencadenantes de los síndromes coronarios agudos (Ridker 2000). Hay estudios que demuestran que un incremento de

10kg en el peso corporal se asocia a un incremento de 3.0mmHg en la presión sistólica y de 2.3mmHg en la diastólica, lo que se traduce en un incremento del 12% en el riesgo de sufrir enfermedad coronaria y del 24% en el caso de ictus (Cinical Guidelines Overweight and Obesity in Adults 1998). Es más, un aumento de una unidad en el BMI se asocia a un incremento del 4% en el riesgo de sufrir un ictus isquémico y un 6% en el caso de ictus hemorrágico (Kurth et al. 2002; Poirier et al. 2006). Este incremento en el riesgo de ictus viene dado por el estado pro-trombótico/pro-inflamatorio que acompaña la acumulación excesiva de tejido adiposo (Rost et al. 2001).

3.2.7. OTROS

Niveles de ciertas proteínas hemostáticas, como el fibrinógeno, han sido considerados como factores de riesgo independientes para la enfermedad arterial coronaria (Meade et al. 1980; Kannel et al. 1987). Niveles elevados de fibrinógeno se han relacionado con la edad, obesidad, hiperlipemia, diabetes, tabaquismo y estrés, lo que explica, en parte, la asociación de estos factores de riesgo a la patología cardiovascular (Rosengren et al. 1990).

Diversos estudios han demostrado que los pacientes con patologías inflamatorias crónicas presentan un mayor riesgo de sufrir aterosclerosis prematura como por ejemplo enfermedades autoinmunes como el lupus eritematoso sistémico (SLE) o la artritis reumatoide (RA). En el caso del SLE se ha visto que un 40% de los pacientes presentan aterosclerosis subclínica (Schattner et al. 2003). El incremento de apoptosis de células endoteliales se ha relacionado con la progresión de la aterosclerosis en los pacientes SLE (Rajagopalan et al. 2004). En el caso de los pacientes con RA también se ha visto una mayor prevalencia de aterosclerosis preclínica independiente de los factores de riesgo tradicionales, lo que sugiere que la inflamación crónica y su severidad son aterogénicas en esta población (Roman et al. 2006). Es más, se ha relacionado la aterosclerosis pre-clínica en los pacientes RA con una disminución de las células endoteliales progenitoras circulantes y un aumento de los linfocitos-T pro-inflamatorios (Gerli et al. 2004; Grisar et al. 2005). Además de los mecanismos específicos inherentes a cada patología, la inflamación sistémica conlleva otros factores generales que contribuyen a la patogénesis de las enfermedades vasculares aterotrombóticas como por ejemplo, disfunción endotelial y reducción de la expresión de la oxido nítrico sintasa endotelial (eNOS), dislipemia secundaria caracterizada por niveles reducidos de HDL y

aumento de triglicéridos y activación de la cascada de coagulación (van Leuven et al. 2008).

De la misma manera, también hay evidencias que apoyan que las infecciones crónicas por bacterias y virus contribuyen a la iniciación y/o progresión de la aterosclerosis, ya sea por una infección directa de las células vasculares o por efectos indirectos de citoquinas o proteínas de fase aguda inducidas por infecciones no vasculares. Entre las infecciones crónicas que han sido relacionadas con la patología cardiovascular podemos encontrar Chlamydia pneumoniae, Porphyromonas gingivalis, Helicobacter pylori, influenza A virus, hepatitis C virus, cytomegalovirus, y el virus de la inmunodeficiencia adquirida (HIV) (Rosenfeld et al. 2011).

3.2.8. RELEVANCIA DE LOS FACTORES DE RIESGO

La presencia de factores de riesgo condiciona la presentación clínica de la patología cardiovascular. Por ejemplo, la hipercolesterolemia y la diabetes están asociadas con la rotura de placas. Por su parte el tabaquismo está asociado a la erosión de las mismas, de forma más frecuente en mujeres con muerte súbita cuando tienen menos de 50 años (Moreno 2001). La presencia de factores de riesgo en el momento de la disrupción de la placa influye en el tamaño de los trombos, su potencial patológico y la resistencia de los mismos a la lisis, y por lo tanto, en las diferentes manifestaciones clínicas (Badimon et al. 2010).

Pese a la gran influencia de los factores de riesgo clásicos en la patología cardiovascular una proporción importante de los eventos ocurren en individuos que no presentan ninguno de estos factores (Hackam et al. 2003). De ello deriva la actual necesidad de identificar nuevos biomarcadores que mejoren la predicción global de riesgo de enfermedad cardiovascular.

4. SÍNDROMES CORONARIOS AGUDOS

El término de síndrome coronario agudo (ACS) engloba un amplio rango de síntomas clínicos que resultan de una isquemia aguda del miocardio. Dentro de los ACS podemos encontrar la angina inestable, el infarto agudo de miocardio con y sin elevación de ST y la muerte súbita. Estas manifestaciones agudas de la enfermedad

cardíaca comparten un mismo fenómeno patofisiológico: la aterotrombosis coronaria **(Figura 5)**.

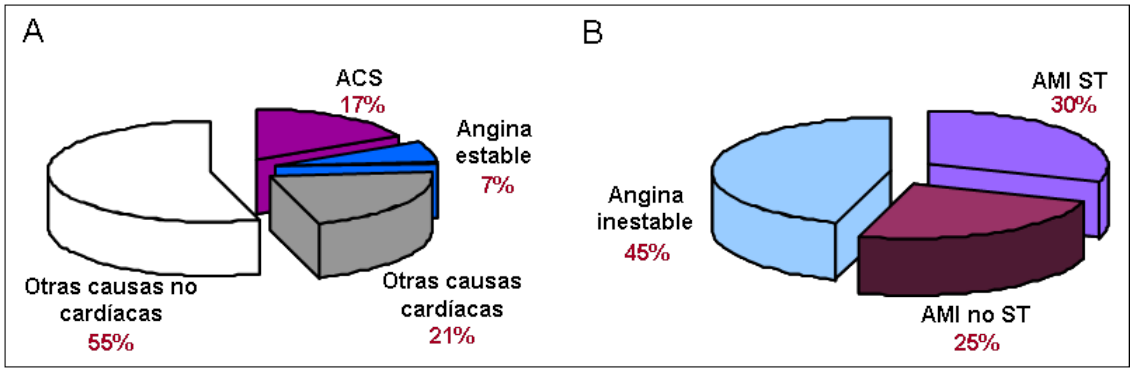

Figura 5A: Porcentaje de los ACS dentro de los pacientes que ingresan en el hospital con dolor torácico. **B:** Porcentaje de las diferentes manifestaciones dentro de los ACS (Datos obtenidos a partir de estudios Pope et al. (Pope et al. 2000), Marrugat et al. (Marrugat et al. 2002) y Hasdai et al. (Hasdai et al. 2002)).

En pacientes con enfermedad coronaria estable, la presentación de angina es el resultado de un aumento en la demanda de oxígeno por parte del miocardio que sobrepasa la capacidad de suministro de las arterias coronarias estenosadas. Por su parte, la angina inestable es una continuidad del proceso patológico y se caracteriza por una repentina disminución del flujo coronario (Fuster et al. 1992).

Dentro del infarto agudo de miocardio (AMI) podemos encontrar dos tipos: el infarto sin elevación ST (no-onda-Q) y el infarto con elevación ST (onda-Q). Un trombo no oclusivo o transitoriamente oclusivo frecuentemente deriva en angina inestable o en un infarto no ST, mientras un trombo oclusivo y más estable lleva a la presentación de un infarto con elevación ST (Fuster 1994). El infarto agudo de miocardio ocurre en pacientes que tienen aterosclerosis coronaria, con más de un 90% asociado a un trombo superpuesto en el lumen, mayoritariamente por la ruptura de la placa y menos comúnmente por la erosión de la misma (Burke et al. 2007). Se han encontrado trombos coronarios en un 98% de los pacientes que mueren por AMI, con un 75% de ellos causados por la ruptura de la placa y un 25% debidos a la erosión de la placa (Arbustini et al. 1999).

La muerte súbita frecuentemente es consecuencia de una progresión rápida de la lesión coronaria, en la que la rotura de la placa y la trombosis resultante llevan a una isquemia repentina y a arritmias ventriculares fatales. La ausencia colateral de flujo sanguíneo al miocardio distal a la oclusión y la presencia de microémbolos plaquetares pueden contribuir a la muerte súbita por isquemia (Falk 1985; Davies et al. 1989).

5. MARCADORES DE LOS ACS

Uno de los mayores retos de la medicina cardiovascular es encontrar herramientas que permitan no sólo un diagnóstico precoz sino también una predicción del riesgo de sufrir un evento trombótico agudo. En las últimas décadas, hay un gran interés en la búsqueda de biomarcadores que puedan ser identificados en sangre. Según su utilidad, los biomarcadores se pueden clasificar en diferentes tipos: **marcadores de riesgo** (*screening*), para identificar a pacientes sin manifestación cardiovascular previa, **marcadores para el diagnóstico**, para identificar a pacientes con sospecha de la patología, y **marcadores para el pronóstico**, para estudiar la evolución de pacientes que ya han presentado la patología **(Figura 6)** (Gerszten et al. 2011).

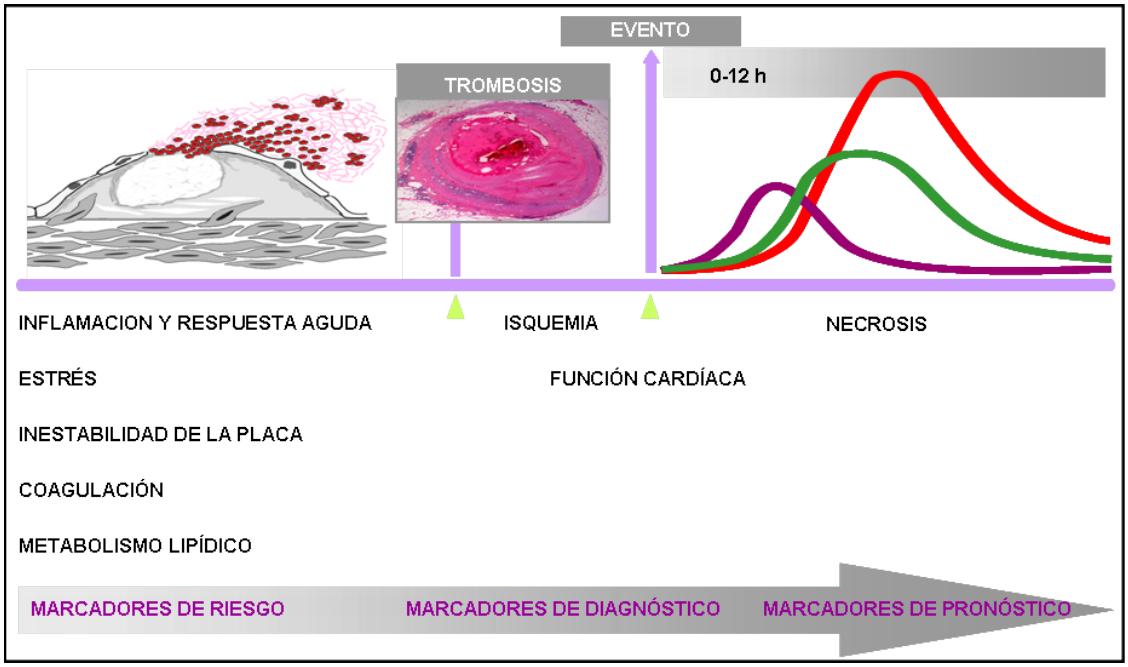

Figura 6. Tipos de marcadores de los ACS.

Tanto los marcadores de riesgo como los factores de riesgo se definen por la correlación entre la presencia del factor y el consiguiente desarrollo de la enfermedad. Pero el marcador, a diferencia del factor de riesgo, no tiene porqué estar ligado de forma causal a la patología sino que puede ser una medida del proceso patológico en sí (Vasan 2006). Un marcador de riesgo puede ser considerado factor de riesgo si la intervención para modular dicho factor resulta en una reducción en paralelo del riesgo (Borer 2008). Los marcadores cardíacos bioquímicos son macromoléculas intracelulares liberadas y detectables en la circulación periférica cuando hay un daño del miocardio (Kou et al. 2006). Aunque la mayoría de los biomarcadores estudiados hasta la actualidad se han basado en la posibilidad de que sean útiles para el diagnóstico y/o pronóstico, lo ideal sería que también constituyeran una diana terapéutica. Algunos, pese a no tener valor diagnóstico ni terapéutico, pueden proporcionar información adicional por su implicación en diferentes vías asociadas a la formación y el desarrollo de la aterosclerosis (Martin-Ventura et al. 2009).

El valor clínico de un biomarcador dependerá de su precisión y reproducibilidad en un método estandarizado de medición. Es muy importante que sea aceptado por el paciente y que sea de fácil interpretación por el especialista, aportando una gran sensibilidad y especificidad de forma independiente a la medición de otros marcadores (Vasan 2006). La información aportada por un biomarcador tiene que representar un cambio en el manejo del paciente de tal forma que su coste sea justificado (Vivanco et al. 2008).

Actualmente hay una falta de consenso sobre los marcadores disponibles **(Tabla 1)** para la detección del infarto de miocardio ya que no está claro si su uso facilitaría el diagnóstico y el pronóstico de los síndromes isquémicos aterotrombóticos (Tunon et al. 2010).

Tabla 1. Resumen marcadores ACS descritos.

Grupo	Marcador	Ventaja/Utilidad	Inconveniente
Necrosis	Troponinas (T, I)	Alta sensibilidad y especificidad	Detección daño irreversible
	CK-MB	Medición estandarizada	Menor sensibilidad y especificidad
	Mioglobina	Alta sensibilidad	Muy inespecífica
Isquemia	FFAu	Detección precoz y alta sensibilidad	Resultados preliminares
	IMA	Fácil detección	Muy inespecífica
Función cardíaca	BNP, Nt-proBNP	Valor pronóstico y diagnóstico	Falta datos aplicación clínica
Inflamación / Respuesta aguda	CRP	Valor pronóstico y diagnóstico	Falta umbral óptimo
	WBC	Simple y accesible	Falta datos aplicación terapias
	sCD40L	Valor pronóstico y diagnóstico	Falta especificidad
	MPO	Predicción de riesgo tras evento	Solapamiento pacientes y sanos
Estrés	Hsp 70	Posible papel citoprotector	Falta validación aplicabilidad
	Hsp 27	Posible marcador evolución patología	Falta validación aplicabilidad
Coagulación	Dímero-D	Papel fisiopatológico importante	Resultados contradictorios
Inestabilidad de la placa	MCP-1	Papel crítico inestabilidad placa	Solapamiento pacientes y sanos
	Colina	Alta sensibilidad y especificidad	Falta datos aplicación clínica
	PAPP-A	Detección previa a marcadores necrosis	Falta datos aplicación clínica
Metabolismo lipídico	Apo A-I	Predicción de riesgo	Riesgo residual
	PON-1	Predicción de riesgo	Falta validación aplicabilidad

CK-MB: creatina quinasa-isoforma B muscular; FFAu: ácidos grasos libres no unidos a albúmina; IMA: albúmina modificada por isquemia; BNP: péptido natriurético tipo B; Nt-pro-BNP: péptido natriurético tipo B N-terminal; CRP: proteína C reactiva; WBC: contaje de células blancas; sCD40L: ligando soluble de CD40; MPO: mielo-peroxidasa; Hsp: proteína de estrés térmico; MCP-1: proteína quimioatrayente de monocitos; PAPP-

A: proteína plasmática asociada al embarazo; Apo A-I: apolipoproteína A-I; PON-1: paraoxonasa-1.

5.1. MARCADORES DE NECROSIS

Las troponinas cardíacas son proteínas estructurales del músculo cardíaco generalmente consideradas como los biomarcadores por excelencia (Babuin et al. 2005). Actualmente hay métodos altamente sensibles para medir las troponinas I y T con una gran habilidad para detectar incluso áreas microscópicas de necrosis y tienen prácticamente absoluta especificidad del tejido miocárdico.

La CK-MB (creatina quinasa-isoforma B muscular), enzima involucrada en la transducción de energía, es la siguiente mejor alternativa disponible aunque tiene menos sensibilidad y especificidad que el análisis de troponinas (Babuin et al. 2005).

La mioglobina, proteína encargada del transporte de oxígeno, en cambio es altamente sensible pero muy inespecífica así que debe ser usada junto con las troponinas o la CK-MB (Yamamoto et al. 2004).

Tradicionalmente el inconveniente de las troponinas era que se detectaban de forma tardía y por lo tanto no eran capaces de detectar un infarto en sus fases iniciales. Por lo tanto, para pacientes que ingresaban dentro de las primeras seis horas y que inicialmente daban valores negativos de estos marcadores cardíacos, era imprescindible realizar una segunda medición entre las 8 y 12 horas del inicio de los síntomas. Actualmente existen las troponinas de detección precoz que permiten identificar un evento dentro de las 3 primeras horas, aunque son más inespecíficas (Melanson et al. 2007; Keller et al. 2009; Reichlin et al. 2009). La mioglobina puede detectar la necrosis temprana del miocardio a las 4-6 horas tras el ingreso hospitalario (Sallach et al. 2004).

A pesar de que estos marcadores son los más aceptados y usados en la práctica clínica sigue habiendo una necesidad de desarrollar marcadores tempranos que puedan detectar la isquemia del miocardio de una forma precoz con y sin la presencia de daño irreversible del tejido (Jesse 2003).

5.2. MARCADORES DE ISQUEMIA

Uno de los puntos clave en la detección precoz del infarto de miocardio, es la posibilidad de disponer de biomarcadores sensibles y específicos capaces de detectar el proceso isquémico antes de que se produzca una necrosis del miocardio (Morrow et al. 2003).

Se ha visto que tras la isquemia hay un aumento de ácidos grasos libres no unidos a la albúmina (FFAu) que se ha asociado a un aumento en la lipólisis del tejido adiposo y una disminución en la utilización de estos ácidos grasos por parte del organismo tras la isquemia. Se ha propuesto el uso del incremento de FFAu en pacientes con infarto de miocardio para la detección temprana del daño cardíaco. Estudios preliminares en pacientes han demostrado que el incremento de FFAu es anterior a la detección de los típicos marcadores de necrosis con una sensibilidad mayor al 90% (Kleinfeld et al. 1996; Panteghini 2004).

Bar-Or y sus colaboradores describieron que la albúmina presente en el suero de pacientes con isquemia miocárdica presentaba una menor capacidad de unión a metales que la albúmina de individuos sanos (Bar-Or et al. 2000; Bar-Or et al. 2001). A partir de estos resultados se desarrolló un método de análisis en el que el cobalto no unido al extremo N-terminal de la albúmina es detectado por un indicador colorimétrico (Christenson et al. 2001). Los pacientes con isquemia de miocardio presentan menos cobalto unido y por lo tanto un incremento de la albúmina modificada por isquemia (IMA). Sin embargo, también se han descrito incrementos de IMA en relación a procesos isquémicos asociados a daño en órganos diferentes del miocardio (Apple et al. 2002), sugiriendo una falta de especificidad de este marcador. Además se han detectado delecciones del extremo N-terminal de la albúmina en individuos sin isquemia, lo que daría lugar a falsos positivos (Bhagavan et al. 2003).

5.3. MARCADORES DE FUNCIÓN CARDÍACA

En este grupo de marcadores encontramos los péptidos natriuréticos cardíacos como el péptido natriurético atrial (ANP) y el péptido natriurético tipo B (BNP), que son hormonas que juegan un papel importante en la homeostasis cardiovascular regulando la natriuresis, diuresis y vascularización. Como el resto de hormonas natriuréticas, el ANP y el BNP, derivan de precursores, las pre-pro-hormonas, que

contienen un péptido señal en el extremo N-terminal, cuando éste es eliminado, las pro-hormonas se cortan en fragmentos N-terminales inactivos y en péptidos activos (Clerico 2002). Específicamente, el BNP y el Nt-pro-BNP, han mostrado tener un valor pronóstico de mortalidad a largo plazo durante las primeras fases de un evento coronario agudo, en pacientes AMI con y sin elevación ST, pacientes con angina inestable; pacientes con y sin elevación de troponinas; y pacientes con y sin evidencia clínica de fallo cardíaco (de Lemos et al. 2002; White et al. 2003). Incluso se ha visto que las concentraciones del péptido natriurético plasmático están relacionadas con el riesgo de sufrir eventos cardiovasculares y muerte en personas aparentemente asintomáticas (Wang et al. 2004). A pesar de estos resultados aún quedan muchas dudas por resolver sobre el uso de los péptidos natriuréticos cardíacos para llevarlos a la práctica clínica (Packer 2003).

5.4. MARCADORES DE INFLAMACIÓN Y RESPUESTA AGUDA

El papel de la inflamación local y sistémica en la patogénesis de los síndromes coronarios agudos está adquiriendo cada vez más relevancia (Mulvihill et al. 2002). Diversos estudios evidencian la importancia de la inflamación en todas las fases del proceso aterosclerótico. Las vías de inflamación están implicadas en las primeras fases de la aterogénesis, en la progresión de la lesión y en las complicaciones trombóticas de la enfermedad (Tedgui et al. 2006) como el infarto de miocardio**.**

Durante la progresión de la enfermedad cardiovascular las moléculas anti-inflamatorias intentan modular la reacción inflamatoria sistémica, es más, éstos factores anti-inflamatorios se han propuesto como moléculas clave en el pronóstico de la enfermedad aterosclerótica (Tziakas et al. 2007). En los ACS la interacción entre diferentes factores hemostáticos e inflamatorios podría estar asociada a la recurrencia de trombosis e isquemia, y a la mortalidad cardiovascular. De hecho, varios factores inflamatorios presentes durante un AMI se han asociado a un mal pronóstico (Mariotti et al. 2001; Ziakas et al. 2006; Zairis et al. 2007). Por lo que se ha propuesto que los marcadores plasmáticos de inflamación pueden mejorar la estratificación del riesgo y ayudar a identificar grupos de pacientes que podrían beneficiarse de estrategias terapéuticas específicas (Blake et al. 2003).

La respuesta de fase aguda es una reacción inflamatoria inespecífica que se da después de cualquier daño tisular, como en un ACS, donde la situación de estrés incrementa los

niveles de proteínas de fase aguda que han demostrado tener efectos autocrinos y paracrinos en las células cardíacas como respuesta adaptativa al daño cardíaco (Padmasekar et al. 2007).

Entre estos marcadores de inflamación el más estudiado es la proteína C reactiva (CRP), que es una proteína de fase aguda del sistema de la inmunidad innata primitiva. Varios estudios han demostrado el valor predictivo de la CRP (Liuzzo et al. 1994; Morrow et al. 1998; Heeschen et al. 2000; Lindahl et al. 2000), siendo éste independiente y añadido al de las troponinas cardíacas. Es más, la CRP ha demostrado tener valor pronóstico incluso en pacientes con troponinas negativas y sin evidencia de necrosis (Morrow et al. 1998; Heeschen et al. 2000; Lindahl et al. 2000). A pesar de todo, el uso de la CRP presenta problemas metodológicos. No se ha determinado el umbral óptimo para la definición de elevación de CRP en pacientes con ACS (Pearson et al. 2003). Y lo que es más importante, no hay evidencias de que la CRP sea útil para que los pacientes de ACS se beneficien de algún tratamiento en particular (Heeschen et al. 2000).

El contaje de células blancas de la sangre (WBC) también se ha propuesto como marcador de respuesta inflamatoria por su papel en evolución de la patología cardiovascular. Incrementos en WBC se han asociado con un peor pronóstico y una mayor tasa de mortalidad en los pacientes de ACS (Barron et al. 2000; Cannon et al. 2001; Mueller et al. 2003). Podría ser un buen marcador por su simplicidad y accesibilidad, pero hacen falta más estudios para ver si se podría usar para hacer terapias dirigidas.

El CD40 ligando (CD40L) es una proteína trimérica transmembrana presente en las plaquetas y, junto con su receptor CD40, contribuye de forma significativa a los procesos inflamatorios que llevan a trombosis coronarias (Henn et al. 1998). Tras la estimulación plaquetar, el CD40L es rápidamente translocado a la superficie donde se corta generando el CD40 ligando soluble (sCD40L) el cual tiene actividad protrombótica (Andre et al. 2002). En varios estudios se ha demostrado la relevancia del sCD40L en los SCA. Niveles elevados de sCD40L indican un mayor riesgo de sufrir eventos cardíacos en un seguimiento de seis meses (Heeschen et al. 2003). Es más, en pacientes negativos para troponinas cardíacas, el sCD40L es capaz de identificar subgrupos de alto riesgo cardíaco, lo que sugiere que la medición de sCD40L puede tener beneficios añadidos a las técnicas bioquímicas estándar para la detección y pronóstico del AMI (Varo et al. 2003). Hay un amplio espectro de condiciones

inflamatorias que podrían elevar los niveles de sCD40L, por lo que el uso de este marcador podría no ser específico (Andre et al. 2002).

La mielo-peroxidasa (MPO) es una enzima secretada por varios tipos de células inflamatorias, incluyendo los neutrófilos activados y los monocitos/macrófagos, como los que se encuentran en las placas ateroscleróticas (Blake et al. 2003). Tiene propieadades pro-inflamatorias y puede contribuir directamente al daño tisular (Eiserich et al. 2002). Se ha evaluado el poder predictivo de MPO en el riesgo cardíaco. Estos estudios demuestran que las mediciones de MPO en el momento del ingreso predicen el riesgo de sufrir eventos cardíacos adversos en periodos de 30 días a seis meses, incluso en ausencia de necrosis y con niveles de sCD40L por debajo del umbral de diagnóstico (Baldus et al. 2003; Brennan et al. 2003). Estos resultados ponen de manifiesto que la activación de neutrófilos representa un evento patofisiológico que acompaña a los ACS y que es independiente de la activación plaquetar.

En general, el inconveniente de estos marcadores de inflamación es el solapamiento de sus concentraciones plasmáticas entre pacientes e individuos sanos, por lo que no serían capaces de diagnosticar ACS en casos individuales.

5.5. MARCADORES DE ESTRÉS CELULAR

Eventos como el infarto agudo de miocardio representan situaciones de estrés celular que provocan alteraciones en las proteínas, tanto a nivel funcional como estructural (Westfall et al. 1992). Las conocidas como proteínas de estrés térmico (heat shock proteins; Hsp) son proteínas encargadas de reparar las proteínas desnaturalizadas, corregir el plegamiento de las mismas gracias a su actividad chaperona, y/o promover la degradación de las mismas, con el fin de reestablecer la homeostasis celular (Lindquist et al. 1988; Ellis et al. 1989; Gething et al. 1992; Hartl 1996). Las Hsp presentan una dualidad en cuanto a su papel en la patología cardiovascular. Por un lado cambios en sus niveles en respuesta a situaciones de estrés las convierte en posibles marcadores de la patología, pero además, debido a su papel fisiológico, pueden suponer una herramienta terapéutica.

Diversos estudios han descrito un incremento de la Hsp70 en respuesta a la isquemia (Benjamin et al. 1990; Knowlton et al. 1991). De forma similar, situaciones de estrés oxidativo inducen modificación en el nivel de fosforilación de la Hsp27 (Huot et al. 1996). Es más, se ha visto que los niveles plasmáticos de Hsp27 secretada disminuyen

en pacientes con aterosclerosclerosis, proponiéndose esta proteína como un potencial índice de la patología (Martin-Ventura et al. 2004).
Por otro lado, diversos estudios han relacionado el beneficio de ciertas aproximaciones cardioprotectoras con un incremento de los niveles de Hsp (Donnelly et al. 1992; Marber et al. 1993; Marber et al. 1994). Por lo que actualmente se considera que la sobreexpresión de ciertas Hsp está asociada a citoprotección.

5.6. MARCADORES DE COAGULACIÓN

El dímero-D es el producto primario de degradación de la fibrina y sirve, por lo tanto, como marcador directo de la coagulación y la fibrinolisis (Hunt et al. 1985). La relación entre los niveles de dímero-D y los ACS no está clara, de hecho los resultados publicados hasta el momento son contradictorios. Hay estudios que han descrito valores normales de dímero-D en pacientes con infarto (Gurfinkel et al. 1995), en cambio otros estudios han demostrado niveles de dímero-D elevados en pacientes con AMI y angina inestable (Bayes-Genis et al. 2000; Orak et al. 2010). Otros estudios han propuesto que el dímero-D además de ser un marcador de diagnóstico también podría ser un factor de riesgo del desarrollo de complicaciones tras un AMI (Lee et al. 1997).

5.7. MARCADORES DE INESTABILIDAD DE LA PLACA

La importancia de la rotura de la placa aterosclerótica en la patogénesis de los ACS ha hecho que cada vez se dé más relevancia a los marcadores de inestabilidad de la placa como biomarcadores de riesgo de la presentación de un evento cardiovascular (Rabbani 2001).
La MCP-1 (proteína-1 quimioatrayente de monocitos) es una quimioquina responsable del reclutamiento de los monocitos a sitios de inflamación que juega un papel crítico en la inestabilidad de la placa (Szmitko et al. 2003). Algunos estudios han asociado las concentraciones plasmáticas de MCP-1 a la restenosis tras una angioplastia coronaria (Cipollone et al. 2001). En un estudio posterior se vio un solapamiento considerable de los valores de MCP-1 de pacientes con ACS e individuos sanos (de Lemos et al. 2003).
Estudios experimentales han demostrado que la activación de la fosfolipasa D y la liberación de colina a la sangre, proceso derivado de la rotura de los fosfolípidos de la membrana, están relacionadas con la mayoría de procesos coronarios de

desestabilización de la placa (Jesse 2003). De hecho la medición de colina ha demostrado ser capaz de detectar pacientes con angina inestable de alto riesgo negativos para troponina, con una sensibilidad y una especificidad del 86% (Danne et al. 2003).
La proteína plasmática A asociada al embarazo (PAPP-A) se mide típicamente durante el embarazo para la detección del síndrome de Down. Por su papel en la disrupción de la capa protectora del ateroma se ha propuesto como una molécula potencialmente pro-aterogénica (Lawrence et al. 1999). Se ha detectado PAPP-A en placas inestables de pacientes que murieron súbitamente por causas cardíacas y se han encontrado niveles aumentados en suero de pacientes con angina inestable e AMI (Bayes-Genis et al. 2001). La medición de PAPP-A es capaz de detectar pacientes sin elevación de marcadores de necrosis (Lund et al. 2003). Hay estudios que muestran que la PAPP-A circulante durante los ACS es diferente a la PAPP-A aislada del suero de embarazadas (Qin et al. 2002).

5.8. MARCADORES RELACIONADOS CON METABOLISMO LIPÍDICO – HDL

Los niveles lipídicos plasmáticos **(Tabla 2)** por su papel fundamental en el desarrollo de la patología cardiovascular son factores de riesgo con causalidad con una clara asociación con el riesgo de sufrir la patología.

Tabla 2. Niveles lipídicos.

	Nivel sérico (mg/dL)	Comentario
Colesterol total	< 200*	Deseable
	200-239	Límite alto
	≥ 240	Alto
Colesterol-LDL	< 100*	Óptimo
	100-129	Casi óptimo
	130-159	Límite alto
	160-189	Alto
	≥ 190	Muy alto
Colesterol-HDL	< 40	Bajo
	> 40	Deseable en hombres
	> 60	Deseable en mujeres
Triglicéridos	≤ 200	Deseable
	≤ 180	Óptimo

Clasificación de los rangos de niveles lipídicos (Royo 2006). *La versión de 2012 de las guías europeas de prevención de enfermedad cardiovascular en la práctica clínica (Perk et al. 2012) recomiendan valores de colesterol total < 190mg/dL y colesterol-LDL < 115mg/dL en la población general, y en cambio en pacientes con enfermedad cardiovascular y/o diabetes recomiendan valores de colesterol total < 175mg/dL y colesterol-LDL < 100mg/dL. Dichas guías no definen objetivos terapéuticos para el colesterol-HDL ni triglicéridos aunque utilizan dichos niveles como marcadores de riesgo aumentado (colesterol-HDL < 40mg/dL en hombres, < 46mg/dL en mujeres y triglicéridos > 150mg/dL representan un aumento del riesgo).

Dentro del nuevo grupo de marcadores de enfermedad vascular aterosclerótica los biomarcadores relacionados con el metabolismo lipídico (LDLs, HDLs, Apolipoproteína A-I y B, y paraoxonasa-1) han adquirido una gran relevancia (Seed et al. 2001; Walldius et al. 2001; Nordin Fredrikson et al. 2003; Kabaroglu et al. 2004; Schwartz 2009). Diversos ensayos clínicos han demostrado que niveles elevados de lipoproteínas de alta densidad (HDL) tienen una fuerte correlación con la reducción del riesgo cardiovascular (Miller et al. 1975; Miller et al. 1977; Castelli et al. 1986). Pero cada vez más estudios sugieren que la relevancia de las HDL en las patologías cardiovasculares viene dada por su composición proteica, estructura y función más que por sus niveles de colesterol (Navab et al. 2007). Es más, hay evidencias clínicas y experimentales que postulan que las HDL podrían perder proteínas con propiedades anti-inflamatorias y citoprotectoras durante los procesos de respuesta de fase aguda (Van Lenten et al. 2001). Por lo que variaciones en la composición de las HDL pueden influir sobre el riesgo cardiovascular.

La estructura y la capacidad de transporte de colesterol de las HDL vienen determinadas por las propiedades de sus componentes apolipoproteicos intercambiables (Lund-Katz et al. 2010). La Apolipoproteína A-I (Apo A-I) es la proteína mayoritaria de las HDL y representa el 70% de su componente proteico total. En referencia a la Apo A-I se descubrió una mutación, la Apo A-I Milano, cuyos portadores, pese a presentar niveles reducidos de colesterol HDL tenían un riesgo cardiovascular 4.3 veces más bajo que la población control (Nissen et al. 2003). Incluso cambios en los niveles de otras proteínas más minoritarias de la HDL pueden tener repercusión en la patología. Se ha visto que la disminución en los niveles séricos de paraoxonasa-1, una de las enzimas antioxidantes más importantes de las HDL (Mackness et al. 1995), tiene la capacidad de predecir

eventos coronarios agudos (Mackness et al. 2003). Estos estudios remarcan la importancia de los componentes de las HDL en la predicción del riesgo cardiovascular.

6. BÚSQUEDA DE NUEVOS BIOMARCADORES MEDIANTE TÉCNICAS PROTEÓMICAS

Tradicionalmente la aproximación "OMICA" por excelencia ha sido la genómica. Las técnicas genómicas permiten identificar los genes asociados a una patología en concreto y en consecuencia determinar posibles candidatos para la predicción del riesgo de sufrir esa patología. En el humano hay de 30000 a 40000 genes (Venter et al. 2001), pero un gen puede dar lugar a varias proteínas debido a procesamientos alternativos (*splicings*) y modificaciones post-traduccionales. El genoma es estable y da por lo tanto la información del potencial de un organismo, el proteoma, por el contrario, es dinámico y refleja los procesos biológicos que se dan en un momento concreto (Tunon et al. 2010). Así, mientras las alteraciones a nivel génico son marcadores de riesgo, las modificaciones a nivel proteico aportan información sobre los diferentes mecanismos que dan lugar a la aparición y/o desarrollo de un proceso patológico. La proteómica se define como la ciencia que pretende caracterizar y cuantificar los efectores celulares y moleculares que reflejan la expresión génica (Thomas et al. 2011). Las proteínas constituyen una diana excelente para el diagnóstico de una patología y son buenos candidatos como marcadores de pronóstico para el desarrollo de estrategias terapéuticas. En los últimos años la proteómica se ha convertido en una estrategia básica para el estudio del perfil proteico y de asociaciones proteicas complejas en una muestra biológica (Vlahou et al. 2005). Mediante el uso de técnicas proteómicas se pueden determinar modificaciones en la estructura de una proteína, así como sus niveles de expresión y la presencia de modificaciones post-traduccionales, que pueden estar asociadas a una patología determinada y tener, por lo tanto, valor diagnóstico, pronóstico y terapéutico. Los estudios proteómicos se basan en la integración de varias tecnologías que incluyen la preparación de la muestra, la separación de las proteínas y el análisis de perfiles proteicos diferenciales, identificación y caracterización de formas específicas por espectrometría de masas, y el análisis *in silico* para determinar las vías moleculares y las posibles dianas. Uno de los protocolos más aceptados y utilizados para el análisis proteómico es la electroforesis bidimensional (2-DE) para la separación de las proteínas y la visualización de las mismas con

diferentes tinciones o fluorocromos (Smith 2009). El análisis del patrón proteómico resultante puede ser cuantitativo mediante el uso de programas específicos para el análisis de imágenes de 2-DE que permiten cuantificar las proteínas y comparar los perfiles proteómicos de diferentes grupos (Patton 1999). La separación de proteínas también se puede llevar a cabo mediante técnicas de cromatografía líquida (LC). El método más utilizado para la identificación de proteínas es la espectrometría de masas (Haynes et al. 1998; Haynes et al. 1998).

6.1. ANÁLISIS DIFERENCIAL DE PATRONES PROTEICOS

La proteómica descriptiva se basa en el análisis del contenido proteico de una determinada muestra mediante la identificación de las proteínas y la obtención de mapas proteómicos de dicha muestra. Las técnicas proteómicas también permiten el análisis proteómico diferencial en el que se comparan los patrones de expresión proteicos mediante la identificación, cuantificación y comparación de los mapas obtenidos en diferentes estados fisiológicos y/o patológicos a partir de un mismo tipo de muestra en un momento específico (Patton 1999). Este tipo de análisis comparativo no sólo permite determinar las proteínas que aumentan o disminuyen en una muestra en concreto y frente a una situación patofisiológica determinada, sino que también permite el estudio de los cambios en las modificaciones post-traduccionales. Éste área de la proteómica diferencial está adquiriendo cada vez más importancia ya que las modificaciones post-traduccionales juegan un papel esencial en la presentación de una patología (Unwin et al. 2007). Entre las diferentes modificaciones que puede sufrir una proteína podemos encontrar glicosilaciones, fosforilaciones, oxidaciones y acetilaciones**.** De hecho, varios estudios evidencian la importancia del análisis del los fosfo (Zhou et al. 2009) y glicoproteomas (Carpentieri et al. 2010) del suero como fuente para la búsqueda de biomarcadores. La modificación post-traduccional más importante en el suero es la glicosilación y estudios recientes sugieren una relación entre estados diferenciales de glicosilación de las proteínas séricas y determinadas patologías (Abbott et al. 2010; Mann et al. 2010; Zeng et al. 2010).

6.2. LA COMPLEJIDAD DEL PROTEOMA SÉRICO

El suero/plasma tiene la ventaja de ser una de las muestras más accesibles como fuente de identificación de nuevos biomarcadores y además desde el punto de vista diagnóstico y pronóstico son de gran utilidad ya que reflejan el estado fisiológico del individuo. Sin embargo, desde el punto de vista del análisis proteómico, presenta la desventaja de ser el proteoma humano más complejo ya que contiene los sub-proteomas derivados de otros tejidos. Su gran complejidad reside, no sólo en el gran contenido proteico sino en el amplio rango de concentraciones que dichas proteínas abarcan y la heterogeneidad de las proteínas predominantes que están glicosiladas (Anderson et al. 2004; Hortin et al. 2008). Cerca del 85% del proteoma sérico está representado por un reducido número de proteínas que son conocidas como la fracción de mayor abundancia o proteínas mayoritarias **(Figura 7).** Estas proteínas están muy bien caracterizadas y habitualmente carecen de interés para el estudio de nuevos biomarcadores. Las candidatas a biomarcadores pueden ser proteínas intracelulares o de membrana derivadas de la señalización celular, la disrupción y/o remodelado del tejido, y los procesos de apoptosis o necrosis (Gerszten et al. 2011). Estas proteínas se encuentran en el suero a muy baja concentración por lo que se ven enmascaradas por las proteínas mayoritarias como por ejemplo la albúmina y las IgGs (Hortin et al. 2008).

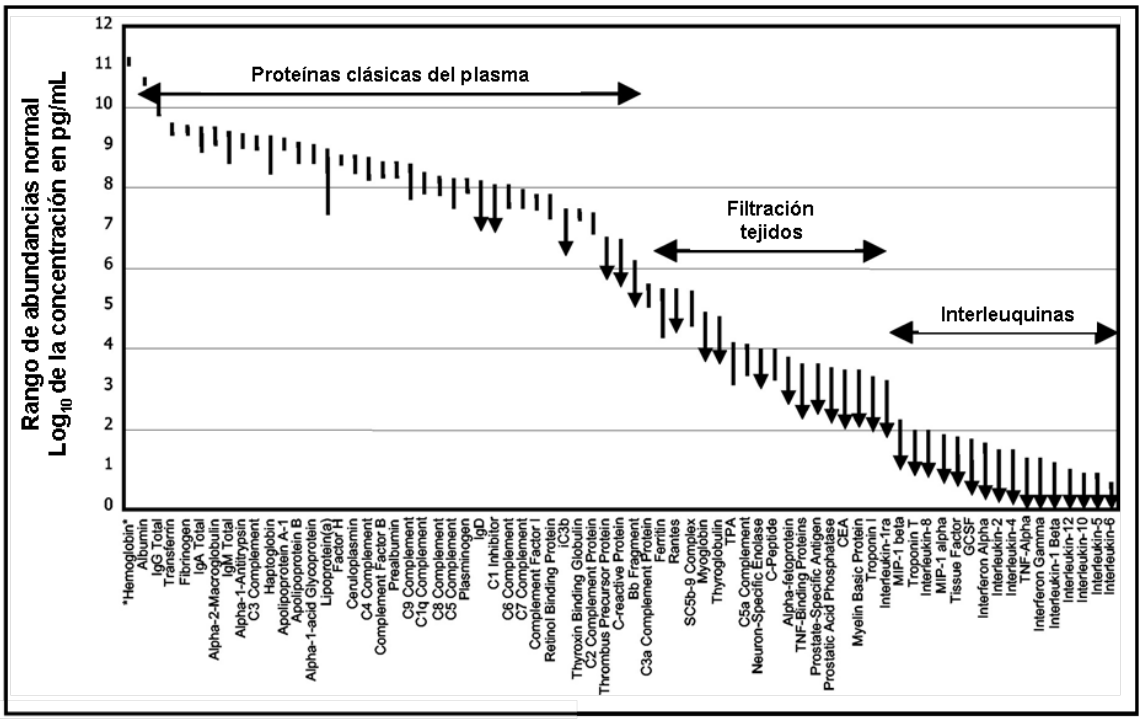

Figura 7. Rango de abundancia de las proteínas plasmáticas (Anderson et al. 2004).

Por este motivo, las muestras de suero deben ser sometidas a métodos de sub-fraccionamiento con tal de simplificar su contenido proteico previamente al análisis proteómico.

En un principio la albúmina se eliminaba del suero utilizando columnas de *Cibacron Blue* (Altintas et al. 2006). En el caso de las IgGs, comúnmente se han utilizado las resinas con proteína A y/o G inmovilizada (Fountoulakis et al. 2004). El problema de estos métodos es que carecen de especificidad y reproducibilidad. Hoy en día existen varios métodos basados en columnas con una mezcla de anticuerpos inmovilizados que permiten eliminar de forma específica varias proteínas mayoritarias simultáneamente (Pieper et al. 2003; Zolotarjova et al. 2005; Qian et al. 2008).

6.3. ESTRATEGIAS PARA EL ANÁLISIS PROTEÓMICO

La electroforesis bidimensional (2-DE) es una de las técnicas más utilizadas y aceptadas en proteómica (Dupont et al. 2005; Sung et al. 2006; Barderas et al. 2007; Lepedda et al. 2009; Urbonavicius et al. 2009). Ésta técnica se basa en la separación de mezclas complejas de proteínas dentro de una misma muestra mediante la aplicación de dos electroforesis sucesivas en diferentes dimensiones sobre una misma muestra, y la posterior visualización de las mismas con diferentes tinciones o fluorocromos (Smith 2009). Algunas estrategias desarrolladas recientemente consisten en la digestión previa de la muestra y la separación de los péptidos por cromatografía líquida de alta resolución (HPLC) y la generación de mapas proteicos basados en el análisis *in silico*. Este último método presenta una gran resolución y permite la identificación directa de un gran número de proteínas por muestra. Sin embargo, el mayor defecto de esta técnica es la carencia de información cuantitativa, aunque el desarrollo de técnicas de marcaje isotópico de proteínas está aportando una nueva perspectiva a esta metodología (DeSouza et al. 2005).

6.3.1. ELECTROFORESIS BIDIMENSIONAL (2-DE)

Esta técnica se basa en la separación de proteínas a partir de muestras complejas en dos dimensiones: en la primera dimensión o isoelectroenfoque (*Isoelectrofocusing*, IEF) las proteínas se separan en base a su punto isoeléctrico (carga) en un gradiente de pH en condiciones reductoras; en la segunda dimensión se lleva a cabo una electroforesis convencional en geles de poliacrilamida en presencia de SDS (SDS-PAGE), donde las proteínas se separan en función de su masa molecular (Smith 2009). El uso de estas dos propiedades fisicoquímicas de las proteínas (carga y masa) permite una gran resolución y la obtención de un amplio mapa proteico de forma simultánea (Schoenhoff et al. 2009). La técnica de la 2-DE fue desarrollada por Kenrick & Margolis (Kenrick et al. 1970) y Klose (Klose et al. 1984) entre otros, pero se extendió tras la aparición de los gradientes de pH inmovilizados (*immobilized pH gradients*, IPG) que mejoraron notablemente la reproducibilidad de la técnica (Gorg et al. 1999; Hoving et al. 2000; Wildgruber et al. 2000). El resultado de la técnica de 2-DE es un mapa de puntos proteicos en el que cada una representa una proteína o una isoforma de la proteína **(Figura 8)**. Tras la visualización de las proteínas mediante tinciones

específicas (*Coomassie Blue*, plata o tinciones fluorescentes) los geles obtenidos de diferentes condiciones, por ejemplo situación patológica y control, se digitalizan y se analizan mediante programas especializados. Posteriormente, los puntos proteicos de interés se recortan de los geles y se caracterizan mediante espectrometría de masas (Moxon et al. 2009).

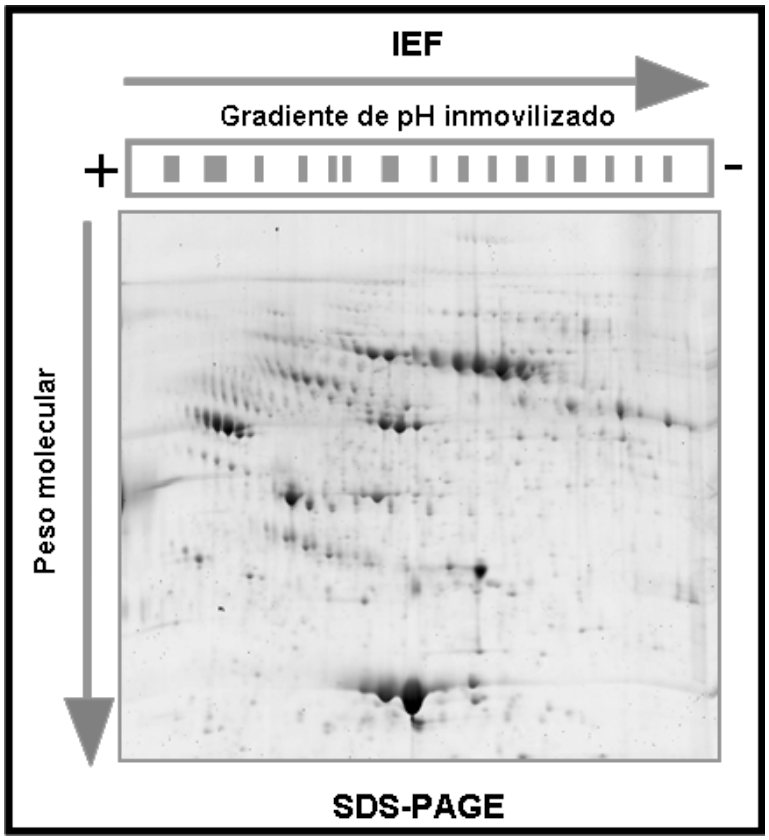

Figura 8. Esquema de las dos dimensiones de la electroforesis bidimensional.

En los últimos años se ha desarrollado la tecnología DiGE (*Difference Gel Electrophoresis*) que se basa en el uso de diferentes fluorocromos (Cy2, Cy3 y Cy5) para el marcaje simultáneo de varias muestras a la vez. En este caso al marcar las muestras previamente a la 2-DE y con fluorocromos que se visualizan a diferentes longitudes de onda, las muestras se juntan y se hace una única 2-DE en la que se obtendrán de forma simultánea los patrones proteómicos de las tres muestras (Lilley et al. 2004).

6.3.2. ESPECTROMETRÍA DE MASAS E IDENTIFICACIÓN DE PROTEÍNAS

Tras la separación y la detección, las proteínas de interés deben identificarse. La aparición de la espectrometría de masas (MS) para la identificación de proteínas marcó de forma importante la evolución de la proteómica (Jungblut et al. 1997; Yates 2000). Previo paso a MS, las proteínas de los geles deben ser extraídas y digeridas para obtener péptidos que serán convertidos en iones en fase gaseosa que se separarán en función de su relación masa/carga (m/z).

Los espectrómetros de masas suelen constar de tres partes: una fuente de ionización, un analizador de masas y un detector de iones. Los datos registrados por el detector son procesados por programas de ordenador que generarán los resultados en forma de gráficos que expresan la abundancia relativa de los iones frente a su relación m/z. Estos gráficos son los conocidos como espectros (Thomas et al. 2011).

Entre las fuentes de ionización más comunes están la de deserción/ionización mediante láser asistida por matriz (*matrix – assited laser desorption/ionization* - MALDI) sobre muestras en estado sólido, y la de ionización mediante electroespray (*elecrospray ionization* - ESI) directamente de muestras en fase líquida (Zhou et al. 2008). MALDI es el proceso de ionización más adecuado para el análisis de compuestos de alto peso molecular como las proteínas. En este sistema la muestra se mezcla con una matriz de componentes orgánicos que cristaliza. Las masas peptídicas obtenidas se comparan con las masas teóricas de las bases de datos (Jonsson 2001). ESI es un proceso utilizado tanto para lípidos como para proteínas que permite el análisis de un amplio rango de moléculas como son desde los péptidos hasta las proteínas (Griffiths et al. 2009). Este espectrómetro vaporiza la muestra directamente desde la fase líquida y un campo eléctrico la dispersa (Griffiths et al. 2001). En este caso el paso previo de separación de proteínas se suele llevar a cabo por cromatografía líquida (*liquid chromatography* - LC).

Tras la ionización, los iones precursores se filtran en el analizador según su m/z antes de la detección (Mann et al. 2001). Hay diferentes analizadores de masas, entre los que encontramos, la separación de iones por "tiempo de vuelo" (*time of flight* - TOF), separación por campos electromagnéticos generados por "cuadrupolos" (Q) y la separación por expulsión en un espacio tridimensional tipo trampa (*ion trap*-IT) (Thomas et al. 2011). En los detectores tipo TOF el tiempo de vuelo es proporcional a la relación masa/carga del péptido (m/z) ya que las moléculas más pequeñas vuelan más

rápido que las grandes. En estos detectores es posible mejorar la resolución trabajando en modo reflector de tal forma que los iones son reflejados hacia el detector aumentando la exactitud de la medición (**Figura 9).**

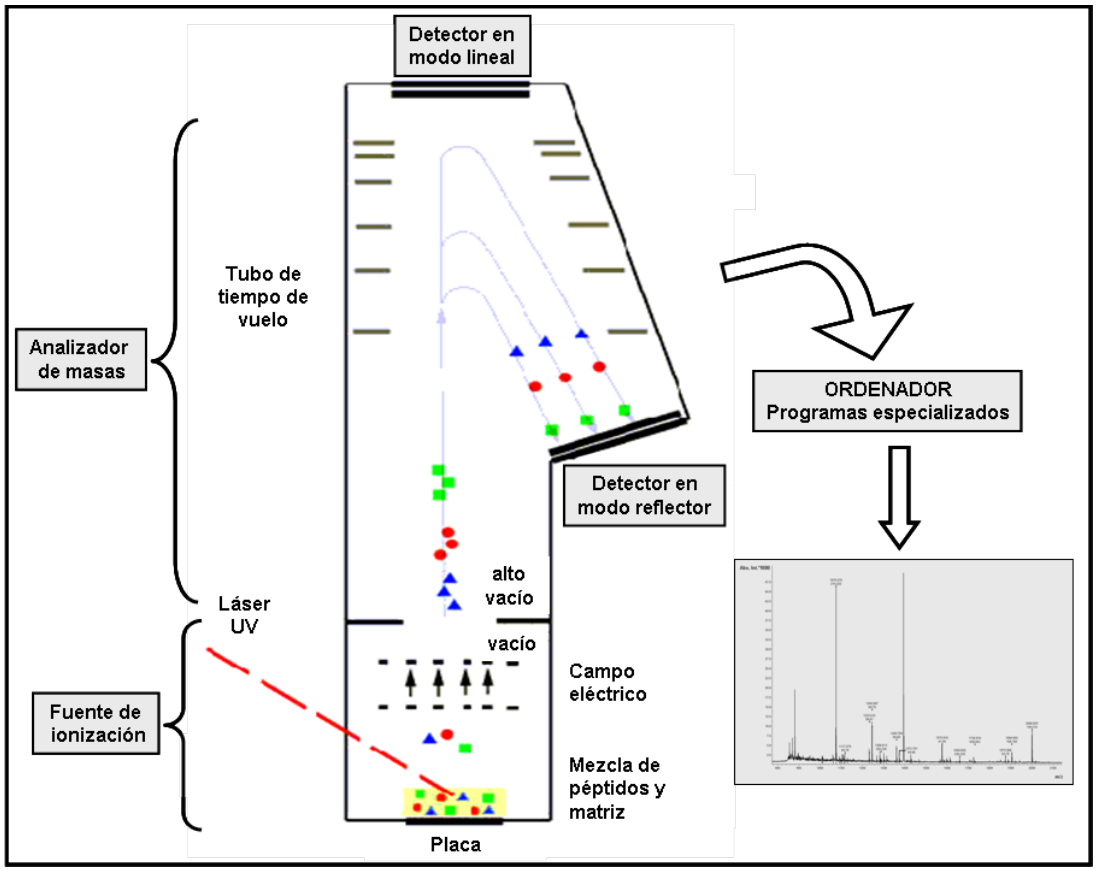

Figura 9. Esquema de un espectrómetro de masas, concretamente un MALDI-TOF.

Para identificar las proteínas de forma más fiable junto con los espectros m/z se ha desarrollado la estrategia de la huella peptídica (*mass fingerprint*) por la cual la proteína separada mediante 2-DE se extrae del gel y se somete a digestión enzimática para la obtención de los péptidos de dicha proteína. Hay varias enzimas (endo y exoproteasas) así como diferentes métodos para la optimización de la digestión y la obtención de péptidos (Courchesne et al. 1997; Staudenmann et al. 1998). La enzima más comúnmente utilizada para la digestión de las proteínas en péptidos es la tripsina. Esta proteasa corta de forma altamente fiable y reproducible en el extremo C-terminal de los

residuos de lisina (L) y arginina (K). Mediante esta técnica se contrasta la masa peptídica obtenida (real) con la masa peptídica teórica obtenida en la digestión virtual en las bases de datos (Mascot, ProFound) lo que permite la identificación de la proteína (Peng et al. 2001; Griffiths et al. 2009). Una mejora de esta tecnología para evitar las identificaciones ambiguas es la fragmentación de péptidos mediante el uso de espectrometría de masas en tándem (MS/MS). Esta técnica fragmenta los péptidos por la colisión con un gas y permite determinar la secuencia de aminoácidos de un péptido. Los espectros de fragmentación son una herramienta muy potente para la caracterización de modificaciones post-traduccionales y la secuenciación *de novo* de proteínas (Tunon et al. 2010).

La espectrometría de masas también se puede utilizar como método de identificación de proteínas sobre la muestra directamente sin pasar por la fase gel de la 2-DE. Estas técnicas son muy rápidas y permiten la identificación de un mayor número de proteínas y sin el problema de enmascaramiento de las técnicas basadas en geles. Pero la aplicación directa de la espectrometría de masas no permite detectar variantes de una misma proteína. La 2-DE, en cambio, por la gran sensibilidad a cambios de carga en la primera dimensión y por la elevada sensibilidad de la segunda dimensión a variaciones en el tamaño peptídico es muy eficiente revelando variantes genéticas (un tercio de las cuales difieren de la variante natural en su carga neta), formas proteolizadas y variaciones en el contenido de ácido siálico (Anderson et al. 2004). De hecho los eventos agudos, como el infarto de miocardio, inducen cambios rápidos en las modificaciones post-traduccionales de las proteínas (Arrell et al. 2001).

7. ANTECEDENTES EN PROTEÓMICA CARDIOVASCULAR

Durante la última década la proteómica ha sido mayoritariamente utilizada para el estudio del cáncer (Thomas et al. 2011). Los últimos avances en espectrometría de masas en cuanto a sensibilidad, selectividad y resolución han hecho que la proteómica sea una herramienta cada vez más aplicada al área cardiovascular (Domon et al. 2006). La proteómica tiene la capacidad de revolucionar el diagnóstico, la estratificación del riesgo, el pronóstico y las estrategias terapéuticas en la patología cardiovascular. De este modo, durante los últimos años se han descrito diferentes estudios que han utilizado técnicas proteómicas no sólo para la búsqueda de nuevos biomarcadores sino también para el análisis de la enfermedad cardiovascular y sus manifestaciones clínicas (Sung et

al. 2006; Barderas et al. 2007; Lepedda et al. 2009). De hecho, la integración de técnicas proteómicas con datos funcionales obtenidos a partir de métodos bioquímicos y fisiológicos puede llevar al conocimiento de la dinámica de diferentes proteomas en el desarrollo y evolución de las enfermedades cardiovasculares.

7.1. ESTUDIOS EN TEJIDO CARDÍACO

Se han hecho varios estudios en diferentes modelos de estrés cardíaco en los que se han observado alteraciones en proteínas de estrés (Hsp), tanto a nivel de expresión como a nivel de modificaciones post-traduccionales (Taylor et al. 2005; Cullingford et al. 2006; Dohke et al. 2006). Otros estudios proteómicos han revelado cambios en la función y/o señalización mitocondrial en diferentes fenotipos cardíacos (Brookes et al. 2002; Baines et al. 2003; White et al. 2005). Mediante el análisis proteómico específico de los complejos de la cadena respiratoria mitocondrial se pueden estudiar los sistemas de regulación de la función mitocondrial así como los de supervivencia/muerte celular (Schilling et al. 2006).

7.2. ESTUDIOS A NIVEL VASCULAR

La proteómica vascular es un área de la investigación proteómica que aún es muy reciente. El estudio del proteoma de vasos de diferentes tamaños es muy complejo debido mayoritariamente a que su composición celular es muy heterogénea (Mayr et al. 2006). Estudios realizados mediante 2-DE en placas ateroscleróticas obtenidas de endarterectomías carotídeas han demostrado que cuanto más complicada es la lesión más complejo es el proteoma que se obtiene (Duran et al. 2003). Más recientemente se ha aplicado la técnica DiGE para el análisis del proteoma de las lesiones ateroscleróticas de la capa íntima de la coronaria (de la Cuesta et al. 2011). Desafortunadamente este tipo de muestras no son fáciles de obtener y se tienen que buscar métodos alternativos. Como por ejemplo, la aparición de las líneas de ratón deficientes en Apo E y en el receptor de las LDL, modelo animal preferente para el estudio de las enfermedades cardiovasculares y los cambios a lo largo de las diferentes fases de la aterogénesis (Piedrahita et al. 1992; Plump et al. 1992; Zhang et al. 1992; Mayr et al. 2005). Una alternativa al uso de los vasos sanguíneos en sí es el uso de células en cultivo simulando los diferentes fenotipos de las lesiones ateroscleróticas, pero se tiene que tener en

consideración que las células vasculares están diseñadas para responder a las fuerzas mecánicas *in vivo*, mientras que las células en cultivo representan una situación artificial que permite estudiar la respuesta a estímulos aterogénicos más que conocer el proteoma exacto que expresan cuando forman parte de la pared vascular (Tunon et al. 2010). Actualmente el modelo *in vitro* más utilizado son las células endoteliales de venas umbilicales humanas (HUVEC), de las cuales hay mapas de 2-DE disponibles (Bruneel et al. 2003; Scheurer et al. 2004). También son muy comúnmente utilizadas las células musculares lisas (SMC), que en condiciones fisiológicas forman la capa media de las arterias y al migrar a la capa íntima desempeñan un papel clave en el desarrollo de la lesión aterosclerótica (Mahoney et al. 2005). Hay una gran variedad entre las diferentes sub-poblaciones de SMC (Frid et al. 1994; Frid et al. 1997), de hecho estas células pueden adquirir diferentes fenotipos en respuesta a estímulos aterogénicos (Schulick et al. 1998; Shanahan et al. 1998; Llorente-Cortes et al. 1999; Tyson et al. 2003; Davies et al. 2005). Además, debido a su gran plasticidad los estudios proteómicos en este tipo celular pueden ser muy útiles para entender los procesos de diferenciación celular e incluso llevar a una clasificación molecular basada en los patrones de expresión proteica de los diferentes tipos celulares vasculares (Mayr et al. 2006).

7.3. ESTUDIOS EN SUERO/PLASMA

La parte soluble de la sangre, tanto el plasma como el suero, representan la versión más amplia del proteoma humano ya que no sólo contiene las proteínas plasmáticas clásicas, si no que también se pueden encontrar las proteínas liberadas por los tejidos y varias clases de inmunoglobulinas (Anderson et al. 2004). La complejidad técnica de la muestra ha hecho que el número de estudios en plasma de pacientes con enfermedad cardiovascular sea limitado. Hace algunos años (Mateos-Caceres et al. 2004) se analizó mediante 2-DE el plasma de pacientes con angina inestable e AMI en comparación con controles sanos. En dicho estudio, al no sub-fraccionar la muestra antes de la 2-DE, sólo se describieron cambios en proteínas mayoritarias como la alfa-1-antitripsina, el fibrinógeno y la Apo A-I. También se han aplicado técnicas de LC-MS/MS para la búsqueda de marcadores de enfermedad coronaria mediante la comparación del perfil de mezclas de plasma de pacientes y de controles (Donahue et al. 2006). Más recientemente, el estudio mediante técnicas proteómicas del efluente coronario obtenido durante isquemia cardíaca de corazones de ratón, reveló la isoforma

cardíaca de la proteína C de unión a miosina como un posible candidato a biomarcador de isquemia (Jacquet et al. 2009). Recientemente el análisis mediante 2-DE y DIGE de la evolución del perfil proteómico en pacientes con enfermedad coronaria reveló que el mayor número de cambios se detectaba en los pacientes con enfermedad coronaria estable (Darde et al. 2010). Posteriormente se realizó un estudio similar en pacientes de AMI a diferentes tiempos desde el momento del ingreso mediante la tecnología del SELDI-TOF-MS (Silbiger et al. 2011) obteniendo, en este caso, un perfil de péptidos diferenciales asociados a la evolución del infarto.

7.4. ESTUDIOS EN HDL

En las últimas décadas se han desarrollado diversos métodos para el aislamiento de lipoproteínas: ultracentrifugación (separación por densidad), electroforesis (por carga), cromatografía de exclusión (por tamaño), y precipitación específica (Davidsson et al. 2010). Desde los primeros trabajos dónde se describió la ultracentrifugación secuencial de suero/plasma humano en soluciones de sales (KBr, NaI) para obtener las diferentes fracciones de lipoproteínas (VLDL, LDL y HDL) (Havel et al. 1955) esta técnica ha sido la más utilizada para llevar a cabo los estudios proteómicos. Sin embargo, tiene la limitación de someter a las lipoproteínas a fuerzas iónicas muy superiores a las que sufren en condiciones fisiológicas que pueden llevar a la pérdida de moléculas unidas por interacción de cargas (Skinner 1992). Posteriormente se han desarrollado técnicas de ultracentrifugación en tampones de D_2O y sucrosa que respetan las fuerzas iónicas y el pH fisiológicos (Hallberg et al. 1994; Davidsson et al. 2005; Stahlman et al. 2008). Pero la única ventaja de estos métodos refiere a la menor pérdida de las proteínas entre 2 y 10kDa de las LDL y HDL. Como contraposición a la ultracentrifugación se ha utilizado la cromatografía de afinidad con anticuerpos inmovilizados contra la Apo A-I y la Apo A-II para aislar las HDL (Rezaee et al. 2006). Esta técnica utiliza soluciones ácidas para disociar los complejos antígeno-anticuerpo pero no hay información sobre cómo estas soluciones pueden afectar a las lipoproteínas (Davidsson et al. 2010).

Mediante el uso de técnicas de proteómica se ha visto que las HDL transportan familias de proteínas implicadas en la activación del complemento, regulación de la proteólisis y procesos de respuesta de fase aguda (Vaisar et al. 2007) **(Figura 10).**

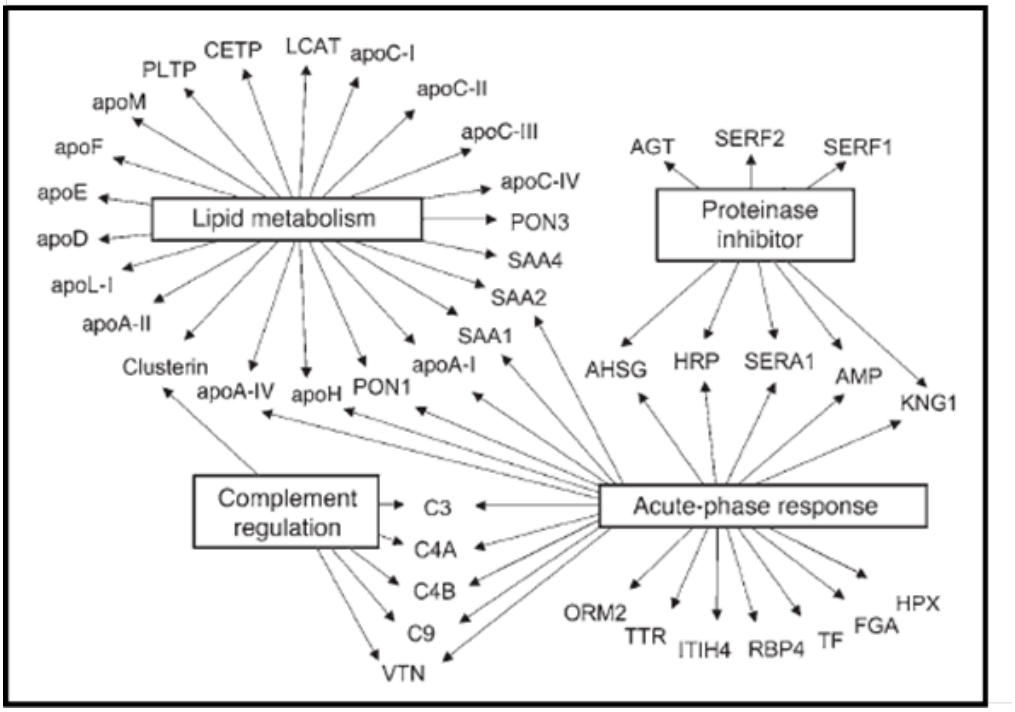

Figura 10. Proteínas relacionadas con las HDL. (Vaisar et al. 2007)

Estos resultados han sido corroborados por estudios posteriores de caracterización de las HDL (Gordon et al. 2010) y han puesto en evidencia la importancia de la calidad de las HDL más que de la cantidad de las mismas y el gran interés del estudio de la composición de estas lipoproteínas. De hecho se ha visto que hay una fuerte relación entre los niveles de los diferentes componentes de las subpoblaciones de HDL y sus propiedades anti-oxidantes. Concretamente las HDL3 contienen importantes niveles de paraoxonasa y apolipoproteína L-1 (Davidson et al. 2009). El análisis proteómico de HDL3 también se ha llevado a cabo para el estudio del efecto de la terapia con estatina/niacina en pacientes con enfermedad coronaria (Green et al. 2008). Este estudio ha revelado que las HDL3 de los pacientes antes del tratamiento estaban enriquecidas en Apo E pero tenían menos Apo J y proteína de transporte de fosfolípidos (PLTP) que los controles. El tratamiento con estatina/niacina aumenta los niveles de Apo J y PLTP sugiriendo una mejora en la participación de estas HDL3 en el transporte reverso del colesterol. Es más, un estudio reciente para comparar HDL de pacientes con ACS, enfermedad coronaria estable y controles, ha demostrado que tras un ACS las HDL

muestran un perfil proteómico enriquecido en proteínas inflamatorias (Alwaili et al. 2011).

8. FUTURAS PERSPECTIVAS

Pese al gran número de estudios que se han llevado a cabo para la búsqueda de nuevos biomarcadores de la patología cardiovascular sigue habiendo una falta de marcadores de diagnóstico de daño reversible del miocardio (Morrow et al. 2003), así como de marcadores de cribado que se puedan utilizar en la prevención primaria y permitan predecir el riesgo de desarrollar patología cardiovascular (Gerszten et al. 2011). Se han propuesto muchas moléculas como marcadores de la aterotrombosis, pero los resultados no son consistentes (Wang et al. 2006) y la mayoría de ellos no han llegado a utilizarse en la práctica clínica (Tunon et al. 2010). La obtención de marcadores de *screening* supondría un importante avance en la reducción del impacto socio-económico de estas patologías en la salud pública. Por eso cada vez hay una mayor necesidad de integrar las técnicas proteómicas en el descubrimiento de nuevos biomarcadores (Wilson 2004). La búsqueda de nuevos biomarcadores mediante técnicas proteómicas permitirá conocer nuevos mediadores y vías patofisiológicas sin una asociación previa a las patologías cardiovasculares. De hecho, el problema de la mayoría de los marcadores actualmente en uso es que son moléculas relacionadas con vías previamente asociadas a la aterosclerosis, como la inflamación, hemostasia y el metabolismo del colesterol, y la información que aportan puede reflejar, de hecho, la información que se basa en los factores de riesgo tradicionales ya conocidos. Un estudio reciente del Framingham Heart Study concluyó que el uso combinado de varios de los marcadores actualmente conocidos permitía sólo un modesto incremento en la predicción de riesgo frente a los factores de riesgo tradicionales ya utilizados (Wang et al. 2006). Las técnicas de proteómica nos permitirán identificar nuevos biomarcadores relacionados con nuevas vías, que junto con el uso de marcadores tradicionales a modo de multimarcador, nos permitirán obtener más información del grado de afección de un individuo, así como de su pronóstico y su respuesta al tratamiento, y convertirse por lo tanto en una clave de futuro para el desarrollo de abordajes de prevención de las enfermedades cardiovasculares.

HIPÓTESIS Y OBJETIVOS

El trabajo de esta tesis doctoral se basa en la hipótesis que en la fase aguda del infarto de miocardio se liberan proteínas marcadoras de isquemia miocárdica que pueden ser útiles para el diagnóstico temprano de lesión miocárdica y el pronóstico de aquellos pacientes susceptibles de llegar a un gran daño orgánico. El abordaje metodológico utilizado para probar esta hipótesis es el estudio del patrón proteico diferencial del suero de pacientes con infarto agudo de miocardio (AMI), y la identificación de nuevas dianas moleculares de interés en la patología cardiovascular y candidatas a estratificar el riesgo y a desarrollarse como potenciales biomarcadores del AMI.

De forma complementaria, el estudio en modelos animales de isquemia aguda de miocardio permite determinar cambios en el patrón proteómico a nivel del tejido cardíaco, en respuesta al daño producido por la isquemia y la posterior reperfusión, así como los posibles mecanismos moleculares implicados en estrategias de cardioprotección de uso clínico como el post-condicionamiento isquémico.

Los objetivos específicos de esta tesis son:

1. Establecer una metodología sensible y reproducible para el análisis del patrón proteómico sérico.
2. Estudiar, mediante electroforesis bidimensional, el perfil proteómico diferencial del suero en pacientes AMI comparado con individuos sanos.
3. Identificar, caracterizar y validar las proteínas diferenciales asociadas al AMI.
4. Analizar los candidatos proteicos seleccionados en base a los resultados de los puntos 2 y 3 en sub-poblaciones de alto riesgo cardiovascular como la diabetes y la hipercolesterolemia familiar heterocigótica.
5. Realizar estudios complementarios en modelos experimentales de AMI con relevancia preclínica para estudiar los cambios a nivel de miocardio.
6. Estudiar los cambios moleculares potencialmente involucrados en la cardioprotección frente a la isquemia mediante el uso de una técnica cardioprotectora como el post-condicionamiento isquémico.

MATERIALES Y MÉTODOS

1. ESTUDIO PROTEÓMICO DIFERENCIAL

Este estudio se centró fundamentalmente en la técnica de la electroforesis bidimensional (2-DE) como aproximación metodológica para la identificación y la caracterización de biomarcadores y dianas moleculares en los síndromes coronarios agudos. El desarrollo del plan de trabajo se realizó a partir de la aplicación de esta técnica tanto en muestras humanas de suero de pacientes AMI, como en muestras de tejido de miocardio obtenidas a partir del modelo porcino de isquemia y reperfusión, con y sin post-condicionamiento isquémico **(Figura 11).**

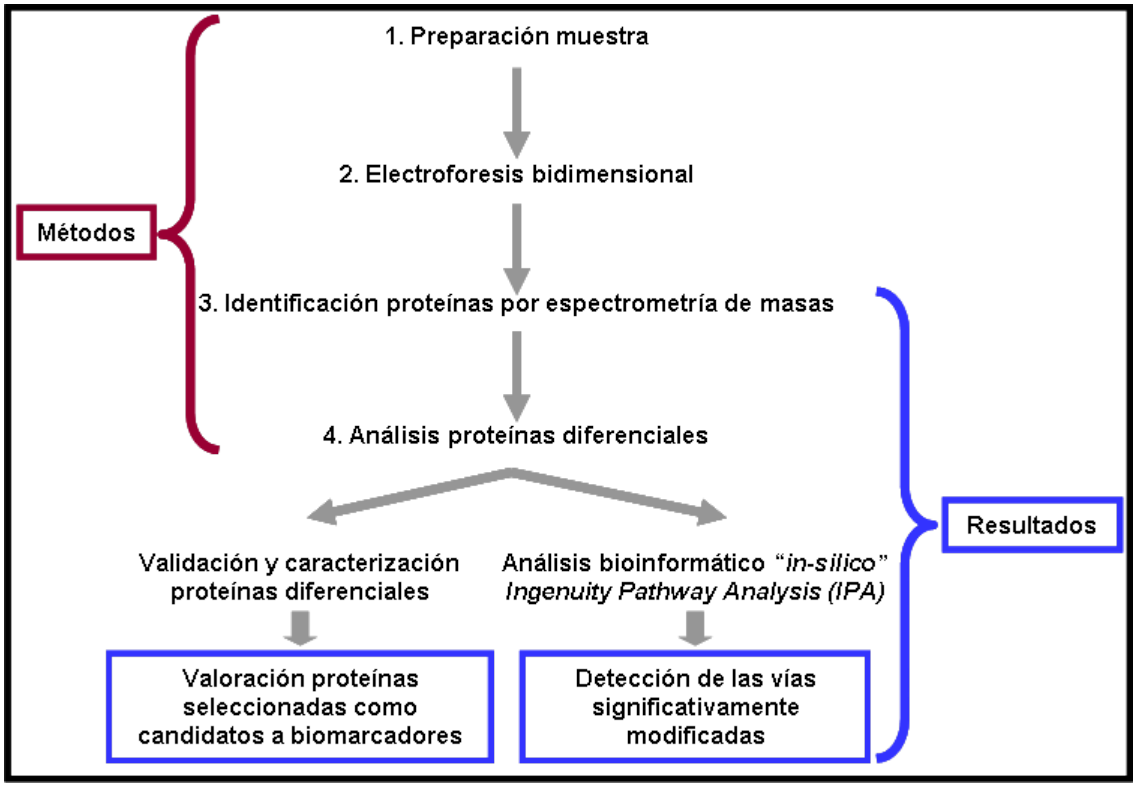

Figura 11. Esquema del plan de trabajo estudio diferenciales mediante 2-DE.

1.1. PREPARACIÓN MUESTRAS PARA ESTUDIOS PROTEÓMICOS

En este estudio se han puesto a punto diferentes protocolos en función del tipo de muestra que se quería analizar por 2-DE.

1.1.1. Subfraccionamiento del suero humano para la extracción de proteínas mayoritarias

En una fase preliminar del estudio se llevó a cabo la puesta a punto de la extracción de proteínas mayoritarias del suero.

Antes del sub-fraccionamiento las muestras de suero se sonicaron (en 6 ciclos de 15seg. sonicado + 30seg. descanso) en hielo (Quero et al. 2004) y se filtraron (0.22μm) mediante centrifugación para eliminar las impurezas.

Para la eliminación de las proteínas mayoritarias se compararon 2 métodos diferentes:

A. Método para eliminar albúmina e IgGs (Albumin and IgG renoval kit, GE Healthcare): Se basa en una resina que elimina de forma selectiva estas dos proteínas del suero humano. Mediante incubación la albúmina e IgGs son retenidas y las proteínas minoritarias se eluyen por centrifugación. Las proteínas unidas a la resina se descartan.
B. Método para eliminar seis proteínas mayoritarias, albúmina, transferrina, IgGs, IgAs, haptoglobina y α1-antitripsina (Multiple Affinity Removal Spin Cartridge, Agilent Technologies): Este método consiste en un único cartucho con una resina basada en anticuerpos que elimina las seis proteínas. Mediante una serie de centrifugaciones, las proteínas mayoritarias son retenidas en la resina y las minoritarias se eluyen. En contraste con el primer método, en este la fracción retenida de proteínas mayoritarias se puede recuperar eluyéndola con un tampón específico.

La comparación de los resultados obtenidos con ambos métodos se presenta en el Anexo I como puesta a punto de la metodología.

1.1.2. Concentración proteínas del suero

Las muestras se concentraron y se eliminaron las sales mediante centrifugación con filtros de 5kDa de corte. Durante esta fase el tampón de la muestra se cambió a un tampón urea (8M Urea, 2% Chaps), que es el tampón óptimo para solubilizar las proteínas del suero para la 2-DE.

1.1.3. Extracción proteínas HDL humanas

Las HDL se obtuvieron mediante ultracentrifugación en gradiente de KBr a partir de la fracción total del suero (Havel et al. 1955). La concentración de proteína de la fracción de HDL se cuantificó por el método del BCA y posteriormente las HDL se deslipidaron mediante la precipitación de proteína con 7 volúmenes de acetona pura fría durante 2 horas a -20ºC. Posteriormente las proteínas se solubilizaron con tampón urea/tiourea (7M Urea, 2M Thiourea, 2%Chaps).

1.1.4. Extracción proteínas LDL humanas

Las LDL también se obtuvieron mediante ultracentrifugación en gradiente de KBr a partir de la fracción total del suero y se deslipidaron siguiendo el protocolo descrito por Karlsson et al. (Karlsson et al. 2005) con algunas modificaciones. Se partió de 1 mg de LDL (1 g/l apoB) y se deslipidó incubando 90 min a -20ºC con 14ml de una mezcla de tributil fosfato:acetona:metanol (1:12:1), seguido de una centrifugación a 2800x g durante 15 min. Posteriormente, la proteína precipitada se lavó de forma secuencial con 1ml de tributil fosfato, acetona y metanol, y se dejó secar al aire. Los precipitados se hirvieron 3 min en una solución 0.325M DTT, 4% chaps, y 0.045M Tris, se dejaron enfriar a temperatura ambiente, se diluyeron (1:15) en la solución urea/tiourea (7M Urea, 2M Thiourea, 2%Chaps) y finalmente se incubaron a 35ºC durante 15 min.

1.1.5. Extracción proteínas tejido cardíaco de cerdo

El tejido cardíaco de la zona isquémica se pulverizó en N_2 líquido y la extracción de proteínas se llevó a cabo mediante homogeneización en tampón urea/tiourea (7M Urea, 2M Thiourea, 2%Chaps) con el sonicador. Posteriormente las muestras se incubaron 30 minutos en hielo y se centrifugaron 15 minutos a 4ºC a 16000x g.

1.1.6. Eliminación de sales

En el caso de las proteínas de HDL y tejido cardíaco, las muestras se limpiaron de sales y contaminantes utilizando un método comercial de precipitación de proteínas a base de acetona (2D Clean-up kit, Bio Rad). En el último paso las proteínas se resuspendieron en el tampón urea/tiourea (7M Urea, 2M Thiourea, 2%Chaps).

1.1.7. Cuantificación proteínas

En todos los casos, la concentración de proteína de las muestras preparadas para 2-DE se cuantificó utilizando un método comercial, el 2D Quant Kit (GE Healthcare). Este método es específico para muestras que contienen urea y funciona mediante la precipitación de las proteínas dejando las sustancias que pueden interferir en solución. Dichas proteínas precipitadas se cuantifican por su unión específica al cobre, de tal forma que el cobre no unido es el que reacciona por lo que la lectura de densidad está inversamente relacionada con la concentración de proteína. El umbral de sensibilidad de este ensayo es de 0,5µg.

1.2. ELECTROFORESIS BIDIMENSIONAL

1.2.1. Primera dimensión

En la primera dimensión se realizó un isoelectroenfoque (*isoelectrofocusing*, IEF) donde las proteínas se separan por su punto isoeléctrico en tiras de acrilamida con gradiente de pH inmovilizado. El rango de pH de la tira varía en función de la muestra analizada (ver **Tabla 3**).

Tabla 3. Condiciones de la 2-DE para los diferentes tipos de muestras.

Muestra	Tampón	Hidratación / Aplicación muestra	1ª Dimensión (rango pI)	2ª Dimensión (% gel SDS-PAGE)
Suero humano	8M urea, 2% chaps, 1.6%DTT, 0.2% anfolitos, 0.02% azul bromofenol	Cup-loading	4 – 7	10%
	8M urea, 2% chaps, 1.6%DTT, 0.2% anfolitos, 0.02% azul bromofenol	Cup-loading	4.7 – 5.9	15%
HDL humanas	7M urea, 2M tiourea, 2% chaps, 1.6%DTT, 0.2% anfolitos, 0.02% azul bromofenol	Pasiva	4 - 7	12%
LDL humanas	7M urea, 2M tiourea, 2% chaps, 1.6%DTT, 0.2% anfolitos, 0.02% azul bromofenol	Activa	4 - 7	10%
Miocardio cerdo	7M urea, 2M tiourea, 2% chaps, 1.6%DTT, 0.2% anfolitos, 0.02% azul bromofenol	Pasiva	3 - 10	10%

Estas tiras tienen la acrilamida seca para su conservación, por lo que antes de usarlas hay que hacer un paso previo hidratación de 12h.

Esta hidratación puede hacerse de diferentes maneras según la muestra:

- Hidratación pasiva: en este caso no se aplica corriente y las proteínas se van difundiendo hacia el gel de la tira. Este tipo de hidratación ha sido el utilizado para las muestras de HDL.
- Hidratación activa: se aplica una corriente de 50V de forma constante para que las proteínas entren en el gel. Este tipo de hidratación se ha usado para las muestras de LDL y tejido de miocardio.
- Hidratación pasiva + *cup-loading*: se realiza un tipo de hidratación pasiva con el tampón urea/chaps suplementado con un potente agente reductor (destreak) pero sin la muestra. Una vez hidratada la tira se aplica la muestra

por ambos extremos de a la vez **(Figura 12).** Este método ha sido el utilizado para las muestras de suero.

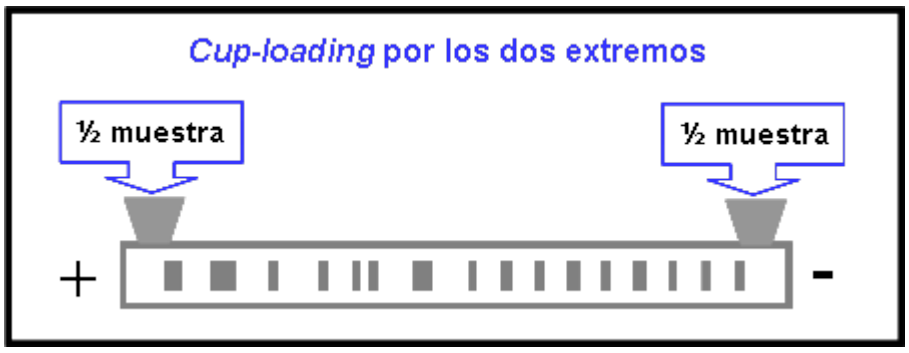

Figura 12. Esquema de la aplicación de la muestra mediante *Cup-loading* por ambos extremos de la tira.

Los programas de IEF constan de varias etapas en las que la conductividad eléctrica del gel va cambiando en función del movimiento de las proteínas. En esta tesis doctoral se han utilizado diferentes tipos de programa en función de la muestra **(Tabla 4).**

Tabla 4. Pasos programa IEF para los diferentes tipos de muestras.

	Suero humano	**HDL humanas**	**LDL humanas**	**Miocardio cerdo**
Hidratación	pasiva	pasiva	50V 12h	50V 12h
Pasos IEF: 1	250V 45min lineal	250V 15min lento	250V 15min lento	250V 15min lento
2	500V 1h lineal	10000V 3h lento	10000V 3h lento	10000V 3h lento
3	1000V 1h lineal	10000V hasta 55000V lento	10000V hasta 55000V lento	10000V hasta 55000V lento
4	4000V 1h lineal	-	-	-
5	10000V 1h lineal	-	-	-
6	10000V hasta 70000V lineal	-	-	-
Mantenimiento	100V 24h rápido	500V 24h rápido	500V 24h rápido	500V 24h rápido

1.2.2. Segunda dimensión

En la segunda dimensión se llevó a cabo una electroforesis convencional en geles de poliacrilamida en presencia de SDS (SDS-PAGE), donde las proteínas se separan en función de su masa molecular. Se utilizaron diferentes porcentajes en función del tipo de muestra (**Tabla 3**). Tras el IEF y previo paso a la segunda dimensión las tiras se equilibraron primero en una solución reductora (50mM Tris-HCl pH 8.8, 6M urea, 2% SDS, 30% glicerol y 2% DTT) y después en una solución alquilante (50mM Tris-HCl pH 8.8, 6M urea, 2% SDS, 30% glicerol y 2.5% iodoacetamida), cada paso durante 15 minutos, a fin de proteger las proteínas frente a procesos oxidativos y evitar la aparición de rayas verticales en los geles (*streaking*).

Para la visualización de las proteínas los geles se tiñeron con una tinción fluorescente (Flamingo fluorescent stain; Bio Rad).

Al final del proceso los geles se escanearon con un equipo Typhoon 9400 (GE Healthcare) con el fin de visualizar las proteínas y obtener el patrón proteómico de cada muestra analizada.

En el estudio proteómico se realizaron dos tipos de geles: los analíticos, en los que se cargaron 100-150μg de proteína y se usaron para comparar de forma sensible y reproducible el patrón proteómico de las diferentes muestras; y los geles preparativos, en los que se cargó una mayor cantidad de proteína (300-500 μg) para favorecer la identificación de proteínas por espectrometría de masas.

1.3. ANÁLISIS DIFERENCIAL

Para cada experimento independiente los extractos de los diferentes grupos se procesaron en paralelo para garantizar el máximo grado de comparación posible. Cada electroforesis se realizó un mínimo de dos veces. Los patrones proteómicos de las diferentes muestras se compararon mediante el programa de análisis PDQuest (versión 8.0; Bio Rad), que utiliza un gel patrón que incluye la imagen de todos los geles de cada experimento independiente. Este programa asigna a cada punto proteico un valor relativo que corresponde al volumen de cada punto comparado con la suma del volumen de todos los puntos del gel tras la sustracción de la señal de fondo. La normalización de las imágenes se llevó a cabo utilizando el modelo de regresión local (LOESS). El peso molecular de las proteínas se calcula en base a un marcador de peso molecular que se

aplica en los geles de 2-DE y los pI se calculan en base al rango lineal de pH de las tiras de IEF.

1.4. IDENTIFICACIÓN MEDIANTE ESPECTROMETRÍA DE MASAS (MS)

Tras el análisis diferencial, las proteínas de interés se identificaron.

En este proceso se realizaron los siguientes pasos:

- Los puntos proteicos de los geles se recortaron de forma automática utilizando un Ettan Spot Picker (GE Healthcare).
- Los pedazos de gel se lavaron con una solución 25mM Ambic.
- Se deshidrataron, primero en una solución 25mM Ambic / 50% ACN y después en 100% ACN.
- Se secaron (SeepVac).
- Se digirieron enzimáticamente con un volumen de gel de tripsina porcina modificada (Promega). Esta proteasa corta de forma altamente fiable y reproducible en el extremo C-terminal de los residuos de lisina (L) y arginina (K) de las proteínas.
- Los péptidos obtenidos en la digestión enzimática se limpiaron de restos de sales y se concentraron mediante el uso de filtros ZipTipU-C18 (Millipore).
- Los péptidos concentrados se mezclaron con matriz (-ciano-4-hidroxi-cinamínico 5mg/mL) en una proporción 1:1.
- La mezcla de la muestra y la matriz se aplicó sobre placas del tipo *Prespotted AnchorChip plates* (Bruker Daltonics) que contienen los calibradores en pocillos que rodean las posiciones donde se aplican las muestras.
- La mezcla de la muestra y la matriz se dejó secar para que cristalizasen.
- La placa se introdujo en el equipo para su análisis.

Para la identificación de las proteínas se utilizó un espectrómetro de masas de tipo MALDI-TOF (*matrix – assited laser desorption/ionization time-of-flight*; AutoFlex III Smartbeam MALDI-TOF/TOF de Bruker Daltonics) que está compuesto por una fuente de ionización del tipo deserción/ionización mediante láser asistida por matriz (MALDI) y un analizador de masas en el que la separación de iones se lleva a cabo por "tiempo de vuelo" (TOF). Como resultado se obtuvo un espectro para cada muestra en el que se representa la intensidad de cada ión/péptido detectado (eje "y") en función de su

relación masa/carga (m/z; eje "x"). Este equipo además permite trabajar en modo reflector de tal forma que los iones se reflejan hacia el detector aumentando la exactitud de la medición y la resolución. La relación de masas de los péptidos obtenidos a partir de la digestión enzimática de una proteína (masa real), lo que se conoce como huella peptídica (*mass fingerprint*), se contrastó con la masa peptídica teórica obtenida en la digestión virtual en las bases de datos utilizando la herramienta de búsqueda MASCOT en la base de datos Swiss-Prot 57.15, lo que permitió la identificación de la proteína. Adicionalmente y para evitar las identificaciones ambiguas se utilizó el equipo en modo MS/MS para fragmentar los picos de interés de las proteínas seleccionadas.

1.5. ESTUDIOS DE MODIFICACIONES POST-TRADUCCIONALES

Los cambios detectados mediante 2-DE a nivel de isoformas o de modificaciones post-traduccionales de una proteína se validaron mediante el uso de técnicas específicas.

1.5.1. GLICOSILACIONES

A. TINCIONES ESPECÍFICAS

Para la detección de las proteínas glicosiladas en geles de 2-DE se utilizó una tinción fluorescente que marca específicamente las glicoproteínas (ProQ Esmerald 300 glycoprotein staining, Molecular Probes). Este método se basa en el uso del ácido periódico que reacciona con las proteínas glicosiladas formando un grupo de carbohidrato peryodato oxidado, que reacciona con el fluorógeno dando una señal fluorescente-verde de las glicoproteínas. Este tipo de tinción tiene un umbral de detección de 0.5ng, aunque este valor varía en función de la naturaleza y el grado de glicosilación.

B. SUB-FRACCIONAMIENTO DE GLICOPROTEÍNAS

La fracción de proteínas glicosiladas del suero se purificaron con resinas de lectinas inmovilizadas en cartuchos para centrífuga (Qproteome Glycoprotein Kit from QIAGEN). Mediante centrifugaciones seriadas las glicoproteínas son retenidas en la

resina y posteriormente son eluídas con un tampón específico. Los cartuchos contienen diferentes lectinas en función del tipo de glicosilación que se quiere sub-fraccionar:

- Glicosilaciones totales: Se asilan con lectinas de concavalina A (*Concavalin A*, ConA) y aglutinina de germen de trigo (*Wheat germ agglutinin*, WGA).
- O-glicosilaciones: Se asilan con lectinas de jacalina (*Jacalin*, AIL) y aglutinina de cacahuete (*Peanut agglutinin*, PNA).

C. DESGLICOSILACIÓN ENZIMÁTICA

Para el estudio específico de proteínas N-glicosiladas se realizó una desglicosilación enzimática con la enzima PNGasa F (*Peptide-N-glycosidase F*, Sigma Aldrich). La PNGasa F desglicosila específicamente cortando los oligosacáridos unidos a asparagina (Asn) mediante la hidrólisis del extremo amida del residuo. La digestión con PNGasa F se realizó a partir de 1 µg/µl de proteína en solución de desnaturalización (20mM Ambic, 0.2% SDS, 100mM 2-Mercaptoetanol). El proceso de desglicosilación se llevó a cabo siguiendo los siguientes pasos:

- Las muestras se incubaron 10min a 100ºC.
- Se añadió 1µl de Tritón X-100 y 2µl de PNGasa F.
- Se incubó durante 1h a 37ºC.
- La reacción se paró incubando 5min a 100ºC.

2. TÉCNICAS COMPLEMENTARIAS DE VALIDACIÓN

La validación de las proteínas diferenciales se realizó mediante el uso de técnicas complementarias, entre las cuales podemos encontrar métodos cuantitativos y semicuantitativos.

2.1. MÉTODOS CUANTITATIVOS

2.1.1. ELISA

Este método se ha utilizado para determinar la concentración de las proteínas de interés en muestras de suero de los pacientes. Se han utilizado ELISAs comerciales de dos tipos siguiendo el protocolo del fabricante en cada caso:

- Tipo *sándwich*: En este tipo los pocillos de la placa están recubiertos de un anticuerpo primario al que se le une la proteína de interés. Luego se añade otro primario, que detectará la proteína unida, y un secundario marcado a partir del cual se desarrollará la reacción para la lectura de la absorbancia.
- Tipo *sándwich* competitivo: En este tipo los pocillos de la placa también están recubiertos de un anticuerpo primario, pero en este caso junto con la muestra se añade una cantidad determinada de la proteína de interés marcada con biotina, de tal forma que la proteína de la muestra y la externa compiten por unirse al anticuerpo. En este caso la absorbancia es inversamente proporcional a la concentración de la proteína de interés en la muestra.

2.2. MÉTODOS SEMICUANTITATIVOS

2.2.1. WESTERN BLOT

Las muestras de interés se sometieron a electroforesis en geles de poliacrilamida en presencia de SDS (SDS-PAGE), en condiciones reductoras o no reductoras (presencia o no de β-mercaptoetanol, respectivamente) en función de la proteína a analizar. Las proteínas del gel se transfirieron en condiciones semi-secas (Semi-dry transfer system, BioRad) a membranas de nitrocelulosa o PVDF (Polyvinylidene Difluoride), dependiendo de que se revelaran las bandas mediante quimioluminiscencia o fluorescencia:

- Método Quimioluminiscencia:

  Las membranas se bloquearon e incubaron con anticuerpos primarios y secundarios, según protocolos generales, y la señal se detectó mediante el sistema de la peroxidasa (SuperSignal chemiluminescence system, Pierce). La imagen se obtuvo con el ChemiDoc™ (BioRad) y se analizó la intensidad de las bandas mediante el programa ImageLab (BioRad).
- Método Fluorescencia:

  La detección de los anticuerpos primarios se llevó a cabo con un método de fluorescencia que permite combinar la tinción mediante fluorescencia de la proteína total y anticuerpos secundarios de fluorescencia para detectar las proteínas de interés (Dye Double Western Blot kit, Invitrogen). La fluorescencia se visualizó utilizando un escáner de fluorescencia (Typhoon

9400, GE Healthcare). La intensidad de las bandas se determinó mediante el programa ImageQuant (GE Healthcare).

Los anticuerpos utilizados se describen en la **Tabla 5.**

Tabla 5. Anticuerpos utilizados.

Antígeno	Casa comercial/ Referencia	Especie	Dilución
Apolipoproteína J	Clone CLI-9, Abcam (ab16077)	ratón	1:2000
Transferrina	Aviva Systems (AVAMM00004-4)	ratón	1:2500
Transtiretina	FL-147, Santa cruz (SC-13098)	conejo	1:1000
Apolipoproteína A-I	Calbiochem (178422)	conejo	1:1000
Aryl hydrocarbon receptor	Abcam (ab2770)	ratón	1:1000

2.2.2. INMUNOPRECIPITACIÓN

La inmunoprecipitación se llevó a cabo mediante el uso de anticuerpo primario contra la proteína de interés, y los complejos se aislaron de la muestra total con resina de proteína G (Protein G Sepharose 4 Fast Flow, GE Healthcare). Los complejos inmunoprecipitados se separaron de la resina de proteína G incubando la muestra 5 minutos a 95ºC en condiciones reductoras (Tris-HCl 2.5 M pH 6.8, 100% Glicerol, 20% SDS, azul de bromofenol).

3. POBLACIONES DE ESTUDIO

El estudio proteómico diferencial del suero se llevó a cabo en una población de 39 pacientes con infarto agudo de miocardio (AMI) de nueva presentación.
Criterios de inclusión:

1) Dolor torácico durante más de 30 minutos.
2) Elevación del segmento ST (>0.2mV) por lo menos en dos ciclos seguidos.
3) Admisión en el hospital dentro de las seis primeras horas desde el inicio del dolor.
4) Niveles séricos normales de CK y CKMB en el momento de la admisión.
5) Valores negativos de troponina T en el momento de la admisión (excluyendo infarto de miocardio sub-agudo).
6) Ritmo sinusal.

Los criterios de exclusión fueron:

1) Haber sufrido un AMI previo.
2) Haber recibido tratamiento antitrombótico por el AMI en el tiempo transcurrido entre el inicio del dolor y la recogida de muestra.

El estudio específico de las proteínas candidatas seleccionadas se llevó a cabo a partir de análisis complementarios en poblaciones de alto riesgo cardiovascular como pacientes diabéticos y pacientes con hipercolesterolemia familiar heterocigótica (ver **Tabla 6** para descripción de las poblaciones). Para cada uno de los estudios se ha utilizado subgrupos de pacientes y controles con un número similar de individuos, la misma edad y la misma proporción entre sexos. De la misma forma también se han tenido en cuenta los tratamientos farmacológicos así como los diferentes parámetros bioquímicos que podían influir en el análisis.

Tabla 6. Descripción población de estudio.

	Control	AMI	DMI	DMII	FH
N	99	39	19	36	124
Edad (media ± ES)	55 ± 1	61 ± 2	47 ± 1	59 ± 2	46 ± 1
Mujeres / Hombres	25 / 74	10 / 29	8 / 11	15 / 21	46 / 75
IMC	27.1 ± 0.4	28.2 ± 0.7	26.2 ± 0.5	25.1 ± 0.5	26.6 ± 0.5
Factores riesgo (%)					
Tabaco	24	51	74	36	44
Hipertensión	15	42	84	97	5
Dislipemia	30	49	74	94	100
Diabetes	1	27	100	100	2
Tratamiento (%)					
AAS	4	10	46	42	11
IECA	4	17	74	47	4
Estatinas	23	17	63	92	60
ARA2	3	5	16	86	3
B-bloqueantes	2	7	5	10	7
Antagonistas-Ca^{2+}	2	10	21	53	2
NTG	0	2	0	0	5
ADO	1	17	5	86	3
INS	0	0	84	78	0

4. MODELO EXPERIMENTAL PORCINO DE AMI

Cerdos de cruce comercial Landrace-Largewhite de 36-39kg fueron aclimatados durante una semana antes de realizar cualquier procedimiento experimental. Posteriormente, los animales fueron aleatoriamente divididos en cuatro grupos: I) 1.5h de oclusión de la arteria coronaria descendente anterior (DA) sin reperfusión (Grupo I; n=7); II) 1.5h oclusión DA seguidas de 2.5h de reperfusión (Grupo II; n=5); III) 1.5h oclusión DA seguidas de un periodo de post-condicionamiento isquémico (IPost-Co) y 0.5h de reperfusión (Grupo III; n=4); y IV) 1.5h oclusión DA seguidas de un periodo de post-condicionamiento isquémico (IPost-Co) y 2.5h de reperfusión (Grupo IV; n=5). El protocolo de IPost-Co se llevó a cabo aplicando 6 ciclos de 20 segundos de reperfusión y 20 segundos de re-oclusión. Se incluyó un grupo de control de intervención (n=4) en el que se llevó a cabo el mismo protocolo de operación pero sin isquemia **(Figura 13)**.

El protocolo fue aprobado por un comité institucional de investigación animal y fue aplicado según las Guías de Cuidado y Uso de Animales de Laboratorio publicada por el Instituto Nacional de Salud de los EU (NIH Publication No. 85-23, revised 1996).

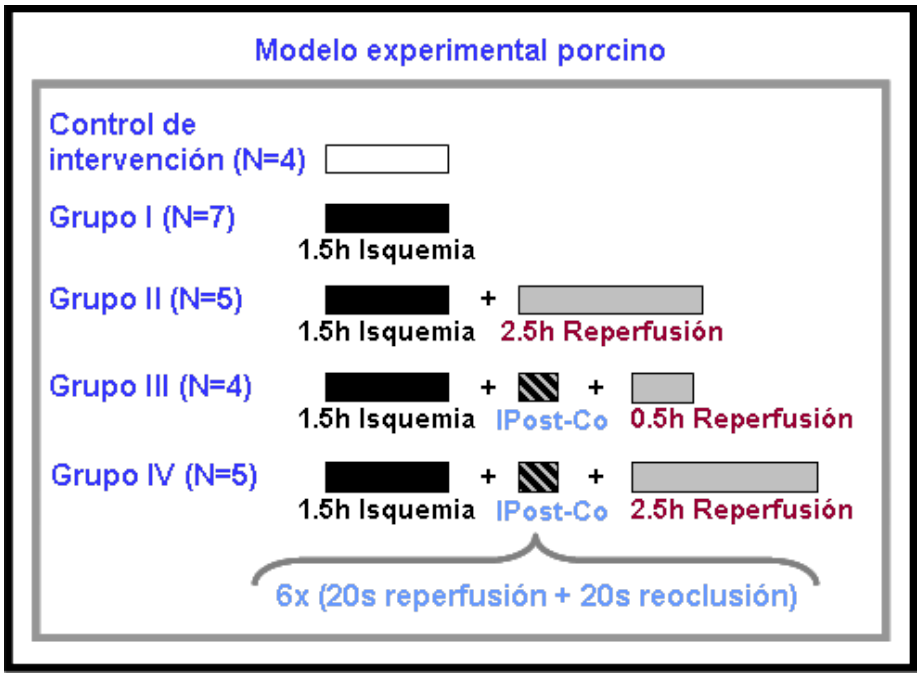

Figura 13. Grupos del estudio en el modelo experimental porcino de infarto de miocardio.

A todos los animales se les administró una dosis de carga de clopidogrel (150mg) 12h antes de la inducción experimental del AMI para evitar las complicaciones trombóticas derivadas de la manipulación de los catéteres.

Los animales se anestesiaron mediante una inyección intramuscular de zoletil® (7mg/kg), domtor® (7mg/kg) y atropina (0.03mg/kg). Posteriormente, se intubaron y la anestesia se mantuvo mediante inhalación de isoflurano (1.5-2%). El AMI se indujo de forma experimental mediante la oclusión completa con un balón de la arteria coronaria DA izquierda. Como medida profiláctica para evitar la aparición de arritmias malignas desde el inicio del procedimiento se administró una infusión continua de amiodarona (300mg, 75mg/h). Para la correcta disposición del balón (después de la primera diagonal) se utilizaron imágenes angiográficas y la oclusión se mantuvo durante 1.5h. Después del periodo de isquemia los animales se sacrificaron (Grupo I) o se desinfló el

balón y se dejó reperfundir el área isquémica (verificado mediante angiografía) durante 2.5h sin (Grupo II) o durante 0.5h y 2.5h con la aplicación previa del IPost-Co (Grupos III y IV). A lo largo de todo el procedimiento se monitorizaron la frecuencia cardíaca y el electrocardiograma (ECG). Se utilizaron ecocardiogramas 2D (Phillips iE33) para evaluar la función cardíaca global (fracción de eyección del ventrículo izquierdo – LVEF) en todos los animales antes del momento de la oclusión, después de la oclusión justo antes de reperfundir y al final del periodo de reperfusión (sacrificio).

En los estudios proteómicos primero se llevó a cabo el análisis de los cambios en el proteoma miocárdico inducidos por la isquemia y la reperfusión completa (2.5h) respecto al grupo de control de intervención, para posteriormente compararlos con el proteoma asociado al post-condicionamiento isquémico. Para tal fin se analizaron mediante 2-DE los extractos proteicos de la zona isquémica del miocardio de animales de control de intervención (n=4), animales sometidos a 1.5h isquemia (n=3), 1.5h isquemia+2.5h reperfusión (n=3) y 1.5h isquemia+IPost-Co+2.5h reperfusión (n=3).

5. TÉCNICAS ADICIONALES DESCRITAS CON MÁS DETALLE EN LOS ARTÍCULOS

- Evaluación por inmunohistoquímica del tejido de miocardio.
- Cultivos celulares: células musculares lisas de coronaria (VSMC) y células endoteliales de vena umbilical humanas (HUVEC).
- Análisis de expresión de mRNA por reacción en cadena de polimerasa (PCR) a tiempo real.

6. ANÁLISIS BIOINFORMÁTICO (IPA)

Con el fin de analizar el significado fisiopatológico de las proteínas diferenciales se ha utilizado un programa bioinformático (*Ingenuity System Pathway Analysis* - IPA) para el análisis de las vías en las que están involucradas de forma significativa las proteínas de interés. De esta forma se establecen las vías o funciones significativamente alteradas en cada condición.

7. ANÁLISIS ESTADÍSTICO

Los análisis estadísticos se han realizado mediante 2 herramientas estadísticas, un programa estadístico basado en el lenguaje "R" para la comparación de los perfiles proteicos obtenidos por electroforesis bidimensional y el programa estadístico Stat View 5.0.1. Para comparaciones de factores independientes se han utilizado test no paramétricos como Mann-Whitney y Kruskal-Wallis, y paramétricos como T-Student, ANOVA, y Fisher's PSLD como Post-hoc test. Para medidas repetidas se han utilizado test no paramétricos como Wilcoxon y Friedman, y paramétricos como ANOVA de dos factores. Las variables cuantitativas se han analizado mediante Chi-cuadrado. Los estudios de correlación se han realizado mediante test de Spearman (no paramétrico) y Pearson (paramétrico), así como análisis de regresión lineal y múltiple. Los test estadísticos se han realizado con un planteamiento bilateral y el nivel de significación estadística α establecido en todos los análisis fue de $P < 0.05$.

RESULTADOS

ARTÍCULO PRIMERO

"Proteomic signature of Apolipoprotein J in the early phase of new-onset myocardial infarction"

Judit Cubedo * † ¶, Teresa Padró * ¶, Xavier García-Moll ‡ ¶, Xavier Pintó §, Juan Cinca ‡ ¶, Lina Badimon * † || ¶

*Cardiovascular Research Center (CSIC-ICCC), †Ciber ObeNU, ‡Cardiology Service, Hospital Santa Creu i Sant Pau, §University Hospital Bellvitge, ||Autonomous University of Barcelona, and ¶ Biomedical Research Institute Sant Pau (IIB-Sant Pau). Barcelona. Spain

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Resumen resultados

Identificación en suero de la Apo J, una proteína asociada a las HDL

La apolipoproteína J, también llamada clusterina, es una proteína presente en las HDL que se detecta en la 2-DE de suero como un grupo de 12-13 puntos proteicos con un pI entre 4.5 y 5.0 y un peso molecular entre 37.1 y 47.3 kDa, que se corresponden con diferentes isoformas de la proteína.

La Apo J presenta un patrón de distribución diferencial en los pacientes AMI

Dentro de las seis primeras horas posteriores a un AMI de nueva presentación en el suero de los pacientes se detecta un cambio en la distribución de las isoformas de la Apo J. Concretamente hay un aumento en la intensidad de las formas de mayor pI y menor peso molecular.

Los pacientes AMI presentan cambios en las formas N-glicosiladas de la Apo J

El aislamiento específico de las proteínas glicosiladas del suero revela que la Apo J es una proteína glicosilada y que los pacientes AMI tienen una pérdida en la intensidad de las formas glicosiladas. La presencia de formas glicosiladas de la Apo J se corrobora mediante la 2-DE de la fracción de proteínas glicosiladas del suero. En este caso sólo se detectan 6 formas de Apo J con una disminución en su intensidad total en los pacientes AMI respecto a los controles.

La Apo J se encuentra en forma soluble y transportada por en las micropartículas

El análisis específico mediante WB de la fracción de micropartículas-exosomas del suero (MP-EX) y de la fracción de suero libre de MP-EX sugiere que los cambios observados en la Apo J se deben únicamente a la forma soluble.

Disminución en los niveles totales de Apo J en la fase temprana post-AMI

Los niveles totales en suero de Apo J medidos mediante ELISA a diferentes tiempos tras la presentación del AMI demuestra que dentro de las 6 primeras horas desde el inicio del dolor se observa una disminución significativa de los niveles de Apo J. Posteriormente, los niveles se van recuperando hasta alcanzar los valores de los controles a las 96 horas tras el ingreso.

Inmunodetección de Apo J en tejido cardíaco isquémico

La Apo J se detecta en cortes de tejido cardíaco isquémico pero no en tejido cardíaco sano. Concretamente la Apo J se detecta de forma mayoritaria alrededor de los micro-vasos.

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Proteomic Signature of Apolipoprotein J in the Early Phase of New-Onset Myocardial Infarction

Judit Cubedo,[#,†,¶] Teresa Padró,[#,¶] Xavier García-Moll,[‡,¶] Xavier Pintó,[§] Juan Cinca,[‡,¶] and Lina Badimon[*,#,†,||,¶]

Cardiovascular Research Center (CSIC-ICCC), CIBERobn, Cardiology Service, Hospital Santa Creu i Sant Pau, University Hospital Bellvitge, Autonomous University of Barcelona, and Biomedical Research Institute Sant Pau (IIB-Sant Pau), Barcelona, Spain

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Acute myocardial infarction (AMI) is one of the major causes of mortality and morbidity worldwide. Despite all the efforts, there is a lack of early markers for prevention, diagnosis, and treatment of ischemic syndromes. By applying a proteomic expression profiling approach to identify biomarkers of early stages of AMI, we have detected significant changes in Apolipoprotein J/clusterin (ApoJ) in patients with an acute new-onset myocardial infarction. ApoJ characterization by bidimensional electrophoresis (2-DE), followed by mass spectrometry (MALDI-TOF) depicted a cluster of 13 spots (p*I*, 4.5−5.0; M_w, 37.1−47.3 kDa) with a significantly different distribution between AMI-patients and controls. Specifically, spots 2, 3, 7, 10, and 13 showed a 2-fold increase in their intensity in AMI-patients ($P = 0.001$). Western-blot analysis (WB) for total serum ApoJ depicted two bands of 40−45 and 65−70 kDa. When only glycosylated forms were analyzed, the band of 65−70 kDa was the most predominant one. A 25% decrease ($P = 0.05$) of ApoJ glycosylated forms in AMI-patients was detected by 2-DE. Serum ApoJ levels, determined by a commercial ELISA, were significantly lower ($P < 0.001$) in AMI-patients ($n = 39$) immediately after the event than in controls ($n = 60$). In 60% of patients, the lowest ApoJ level was detected within 6 h after the onset of AMI. Between 72 and 96 h after admission, ApoJ values in AMI-patients had reached control levels. Our results demonstrate alterations in ApoJ proteomic profile, due to a differential glycosylation pattern, in AMI-patients within the first 6 h after the onset of the event. Therefore, the analysis of this isoform glycosylation shift in patients with AMI may be of better use to understand ApoJ function than the total serum levels of ApoJ and this isoform shift may become an early marker of AMI.

Keywords: myocardial-infarction • ApoJ • proteomics • HDL

Introduction

Ischemic atherothrombotic syndromes induce structural and functional modifications that are reflected in serum levels of proteins and other biomarkers. However, biomarkers characterization is nowadays incomplete and their prognostic value is not clear. Increased concentrations of inflammatory biomarkers such as C reactive protein (CRP), serum amyloid A, myeloperoxidase, and interleukin-6 (IL-6) are detectable in a substantial proportion of patients with acute coronary syndromes. Several biomarkers of myocardial ischemia are under investigation; ischemia-modified albumin (IMA) is among the most thoroughly studied of these markers.[1] There are also biomarkers for the detection of cardiac injury; from those ones, the protein of choice is troponin.[2]

The lack of consensus on some of those biomarkers stems from the fact that, up to now, it is not clear whether their measurement would be useful in the diagnosis and prognosis of ischemic atherothrombotic syndromes.[3]

Despite the success of cardiac troponins as markers of myocardial injury, there is still a need for the development of early markers in the first hours after the onset of the myocardial infarction.

Within the group of new surrogated markers of atherosclerotic vascular disease, the lipid metabolism related biomarkers (low-density lipoproteins (LDLs), high-density lipoproteins (HDLs), Apolipoprotein A-I and B, LDL oxidase, paraoxonase) have reached a significant relevance.[4−8] A recent study using a proteomic approach has reported that HDL carries protein families implicated in complement activation, regulation of proteolysis, and acute-phase response processes.[9] Some authors[10] suggest that the relevance of HDL in cardiovascular diseases is not through its cholesterol concentration but through its composition, structure, and function. Even more,

* To whom correspondence should be addressed. Cardiovascular Research Center, CSIC-ICCC, Hospital de la Santa Creu i Sant Pau, Av. S. Antoni M. Claret, 167 08025 Barcelona, Spain. Tel: +34-93-5565880. Fax: +34-93-5565559. E-mail: lbadimon@csic-iccc.org.
[#] Cardiovascular Research Center (CSIC-ICCC).
[†] Ciber ObeNU.
[¶] Biomedical Research Institute Sant Pau (IIB-Sant Pau).
[‡] Cardiology Service, Hospital Santa Creu i Sant Pau.
[§] University Hospital Bellvitge.
[||] Autonomous University of Barcelona.

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clinical and experimental evidence suggests that HDL might loose proteins with anti-inflammatory and cytoprotective properties during acute phase response processes.[11] Focusing our study in the serum proteome, we can analyze a subgroup of HDL-associated proteins and, therefore, we may reach a better understanding of their biomarker and functional role.

Apolipoprotein J or Clusterin (ApoJ) is a disulfide-linked heterodimeric protein that associates to ApoA-I in HDL.[12] ApoJ is represented by a series of isoforms that are differentially expressed in serum depending on the physiological status or in relation to disease.[13] ApoJ has been suggested to have anti-inflammatory,[14] cytoprotective,[15,16] and antiapoptotic properties.[17] In relation to atherothrombotic disease, ApoJ seems to be abundant in atherosclerotic lesions[18] and it has been proposed that ApoJ reduces fatty acid-mediated toxicity through its binding to enzymatically modified LDL.[19]

Up to now, contradictory ApoJ findings in acute coronary syndrome patients have been reported,[20,21] and despite the efforts, the relevance of this apolipoprotein in cardiovascular disease remains to be elucidated.

Increasing evidence relates the amount of circulating microparticles with atherothrombosis and vascular damage in immflamatory disorders.[22] However, the relevance of microparticles composition in this syndrome is not yet clear. Therefore, the analysis of ApoJ in the microparticles fraction was performed to elucidate if the observed changes were due to soluble ApoJ or if there was an important contribution of microparticles to ApoJ serum changes.

In this study, by analyzing serum obtained from patients with new-onset acute myocardial infarction (AMI), we have used a proteomic approach to characterize differential serum proteins in the early phase of AMI and found that ApoJ shows significant changes in patients with new-onset AMI. These ApoJ changes are not due to modifications in ApoJ content in circulating microparticles.

Materials and Methods

Study Population. The study population comprised 39 new-onset AMI-patients (29 men and 10 women; mean age: 61 ± 13 years) who were admitted with chest pain and suspected of acute coronary syndrome (ACS) at the Emergency Room of Santa Creu i Sant Pau Hospital.

At the emergency department, routine diagnostic procedures were applied to establish the onset of symptoms as accurately as possible (i.e., description of chest pain, pulmonary edema, severe dyspnea, and syncope). In addition to the general patient history, clinical examination, 12-lead electrocardiogram (ECG), and laboratory tests were also run to characterize AMI-patients. All AMI-patients showed (1) typical chest pain lasting more than 30 min; (2) ST segment elevation >0.2 mV in at least 2 contiguous leads; (3) admission to the hospital within the first 6 h after the onset of chest pain; (4) normal serum CK and CKMB levels at admission; (5) negative T-troponin at admission (excluding subacute myocardial infarction); and (6) sinus rhythm. Exclusion criteria were a previous documented or suspected myocardial infarction and antithrombotic treatment because of the AMI onset before the time of blood collection. Background description of AMI-patients is listed in Table 1.

The control group included 60 healthy individuals (45 men and 15 women; mean age: 62 ± 8 years) who attended to a routine health check.

The Ethics Committee of the Santa Creu i Sant Pau Hospital approved the project and the studies were conducted according to the principles of Helsinki's Declaration. All participants gave written informed consent to take part in the study.

Table 1. Background Description of AMI-Patients

	AMI patients (n = 39)
Risk Factors, n (%)	
Tobacco smoking	51
Hypertension	42
Dyslipemia	49
Diabetes Mellitus	27
Background Medication, n (%)[a]	
ASA	9.8
ACEI	17.1
Statins	17.1
A2RA	4.9
β-blockers	7.3
Ca-Antagonists	9.8
NTG	2.4
OAD	17.1

[a] ASA = Acetylsalicylic acid; ACEI = Angiotensin-converting enzyme inhibitors; A2RA = Angiotensin 2 receptor antagonists; NTG = Nitroglycerine; OAD = Oral antidiabetic drugs.

Cardiovascular Magnetic Resonance (CMR). Delayed contrast-enhanced (CE) CMR studies were performed within the first week after acute ST-segment elevation in all myocardial infarction patients (n = 39) with a Philips Intera 1.5-T scanner (Philips Medical Systems). After obtaining the usual scout planes, steady-state free-precision cine −R images were acquired in long-axis planes and in multiple 10-mm-thick short-axis slices from the atrioventricular ring to the apex of the left ventricle. Sixteen phases of the cardiac cycle were acquired for each slice and displayed as a loop. Intravenous gadobutrol (Gadovist, Schering AG) was injected at a dose of 0.1 mmol/kg. A 3-dimensional inversion−recovery, segmented, gradient echo sequence was acquired 10 min after contrast administration to assess delayed contrast hyperenhancement. Inversion times were adjusted to null the signal from normal myocardium (200−300 ms). This sequence was prescribed in multiple short-axis planes using the same orientation as the cine −R images and acquired during a breath-hold of 20 s. A MI location as detected by CE-CMR imaging was described according to the statement of North American Societies of Imaging, which divided the left ventricle into 2 zones (anteroseptal and inferolateral), 4 walls (anterior, septal, inferior, and lateral), and 17 segments. Delayed CE images were scored visually by 3 experienced observers who were blinded to other clinical or CE-CMR data, and each segment was evaluated according to established criteria.[23]

Blood Collection and Sample Preparation. 1. Blood Samples Procurement. Venous blood samples of AMI-patients, before starting any medication, and control individuals were collected to prepare serum that was aliquoted and stored at −80 °C.

2. Serum Subfractionation. For proteomic studies, serum samples were sonicated (six cycles of 15 s each) in ice[24] and filtrated (0.22 μm) by centrifugation to avoid the presence of impurities. The six most abundant serum proteins were depleted using a specific affinity cartridge with binding capacity for albumin, IgGs, IgAs, transferrin, α1 antitrypsin, and haptoglobin (Multiple Affinity Removal Spin Cartridge, Agilent Technologies) as reported by the providers. Serum depleted samples (called the total serum fraction) were concentrated and desalted by centrifugation with 5 kDa cutoff filter devices and

sample buffer was exchanged to a urea containing buffer (8 M urea, 2% CHAPS). The glycosilated serum fraction was purified using a commercial kit (Qproteome Total Glycoprotein Kit, Qiagen). Protein concentration in serum extracts was measured with 2D-Quant Kit (GE Healthcare). All processed samples were stored at −80 °C until used.

3. Microparticle and Exosome (MP-EX) Isolation. Serum was subjected to centrifugation at 100 000*g* for 45 min at 20 °C (OPTIMAX Ultracentrifuge, Beckman Coulter) to isolate the MP-EX fraction. After two washes, the MP-EX pellets were resuspended in a urea/thiourea containing buffer (7 mol/L urea, 2 mol/L thiourea, 2% CHAPS). Serum MP-EX were concentrated and desalted by centrifugation with 5 kDa cutoff filter devices and sample buffer was exchanged to a urea containing buffer (8 M urea, 2% CHAPS). Fractions of serum derived MP-EX and serum free of MP-EX were aliquoted and stored at −80 °C.

4. HDL Preparation. Human HDLs were obtained from normocholesterolemic total serum fraction as described previously.[25] HDLs were analyzed to determine lipoprotein and cholesterol concentrations. The protein fraction was obtained by precipitation of 2 mg of HDLs with pure ice-cold acetone for 2 h at −20 °C.

Biochemical Analysis. C-reactive protein (CRP) levels were measured with a commercial assay (Roche) with a detection interval between 0.1 and 20 mg/L. When CRP levels were above the detection limit, samples were further diluted. Urea levels were measured by the urease-kinetics method. Creatinine levels were analyzed using the alkaline picrate test. Bilirubin levels were measured by the 2,5-dichlorophenyldiazonium (DPD) method. GOT (glutamic-oxaloacetic transaminase) and GPT (glutamic-pyruvic transaminase) levels were tested in Tris buffer by the measurement of aspartate (>100 mM) and alanine (>225 mM) concentration, respectively. Urea, creatinine, bilirubin, GOT, and GPT measurements were performed using a modular analytics system D/P Hitachi (Roche). Lipids were measured by conventional enzymatic methods within 8 h after the onset of the event. The measurements within this time period are considered basal lipid levels. Total cholesterol levels were analyzed by the CHOD-PAP method and triglycerides by TOD-PAP (Boehringer-Mannheim). HDL- cholesterol levels were measured after manual lipoprotein precipitation with PEG 6000 method (Boehringer-Mannheim). If triglycerides were above 3.36 mmol/L, LDL-cholesterol levels were determined by centrifugation, and if triglycerides were bellow 3.36 mmol/L, LDL-cholesterol levels were estimated by the Friedewald formula. T-troponin levels were measured with a commercial kit from Roche Diagnostics IVth generation and were analyzed with a Roche Diagnostics Elecsys 2010.

Proteomic Analysis. 1. Two-Dimensional Gel Electrophoresis (2-DE). For analytical and preparative gels, respectively, a protein load of 120 and 300 μg protein of the urea/CHAPS soluble extracts was applied to 18-cm dry strips (pH 4−7 linear range, GE Healthcare) and the second dimension was resolved in 10% SDS-PAGE. Gels were developed by fluorescent staining (analytical gels) or Coomassie blue (preparative gels). For each independent experiment, 2-DE for protein extracts from controls and patients were processed in parallel to guarantee a maximum of comparability. Each 2-DE run was at least repeated twice. Analysis for differences in protein patterns was performed with the PD-Quest 8.0 (BioRad), using a single master that included all gels of each independent experiment. Each spot was assigned a relative value that corresponded to the single spot volume compared to the volume of all spots in the gel, following background extraction and normalization between gels.

2. Mass Spectrometry Analysis. Protein spots of interest were excised from 2-DE gels, washed (25 mM ammonium bicarbonate (Ambic)), dehydrated (25 mM Ambic/50% acetonitrile (ACN) followed by 100% ACN), dried, and enzymatically digested with one gel volume of sequence-grade modified porcine trypsin (Promega). Peptides from in-gel-trypsin digestion were desalted and concentrated by ZipTipU-C18 (Millipore), mixed 1:1 with 5 mg/mL α-cyano-4-hydroxy-cinnamic, and spotted on a stainless steel mass spectrometry slide. Protein identification was performed by peptide-mass fingerprinting using an Ettan MALDI-TOF Pro (matrix-assisted laser desorption/ionization time-of-flight mass spectrometer, GE-Healthcare) operating in delayed extraction/reflector mode. MALDI-generated mass spectra were internally calibrated using trypsin autolysis products, Ang III (angiotensin III), and ACTH (adrenocorticotropic hormone) peaks. The peptide masses were searched against the National Center for Biotechnology Information nonredundant mammalian database using ProFound and confirmed using a Mascot 2.3 search from Matrix Science, selecting the Swiss-Prot database. For the present study, protein identification was based on the measurement with a Mascot score higher than 55. Minimal expectation for valid identification was <0.005 and $P < 0.05$.

Protein Immunoprecipitation. ApoJ was specifically immunoprecipitated by adding 2 μg of the specific monoclonal antibody (Apolipoprotein J, clone CLI-9, Abcam) to previously precleared samples with 50% Protein G Sepharose 4 Fast Flow (GE Healthcare; 1 h at 4 °C). The antibody−antigen complexes were precipitated with 50% Protein G Sepharose 4 Fast Flow. Elution of the immunoprecipitated complexes from protein G agarose beads was achieved by boiling the sample for 5 min under reduction conditions (Tris-HCl 2.5 M, pH 6.8, 100% glycerol, 20% SDS, and bromephenol blue).

Western Blot Analysis. Sample extracts were resolved by SDS-PAGE under reducing conditions and electrotransferred to PVDF (polyvinylidene difluoride) membranes in semidry conditions (Semidry transfer system, BioRad). Detection was performed with a monoclonal antibody (mAb) against ApoJ (clone CLI-9, 1:2000 dilution, Abcam). The anti-mouse antibody was labeled with Cy3 fluorochrome (1:20 000 dilution, GE Healthcare). Band fluorescence was determined with Typhoon 9400 (GE Healthcare) and band quantification was performed using ImageQuant TL software (GE Healthcare).

Deglycosylation with Peptide-*N*-Glycosidase F (PNGase F). Deglycosylation of ApoJ was performed with the enzyme PNGase F (Sigma Aldrich). Briefly, 5 μg of protein was placed in 10 μL of denaturing solution (20 mM Ambic, 0.2% SDS, 100 mM 2-mercaptoethanol). Samples were boiled 10 min at 100 °C; 1 μL of Triton X-100 and 2 μL of PNGase F were added the solution was further incubated for 1 h at 37 °C. The reaction was stopped by boiling the mixture 5 min at 100 °C. Transferrin was used as a positive control of the enzyme activity.

Quantification of ApoJ Serum Levels. Serum clusterin concentration was determined by an enzyme-linked immunosorbent assay (Clusterin ELISA from Biovendor) that uses two mouse monoclonal antibodies in a sandwich arrangement, as described by the providers. The detection limit of the assay was 0.5 ng/mL. Samples from AMI-patients were analyzed at the moment of admission and at different time periods after the admission time (8, 16, 24, 48, 72, and 96 h).

The intraindividual variability of the assay assessed in 24 samples measured in three independent assays was of 6.4% ± 3.8%.

Immunohistochemistry. Human myocardium was obtained from ischemic and nonischemic hearts upon transplants performed at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). The specimens were immersed in fixative solution (4% paraformaldehyde), and after embedding in paraffin, they were cut into 5 mm thick serial sections and placed on poly-L-lysine coated slides. The primary antibody was a rabbit polyclonal to ApoJ 1:50 (ab69644, Abcam). Before incubation with primary antibody (2 h), sections were washed, suppressing endogenous peroxidase activity with H_2O_2 and blocking nonspecific bindings with goat serum. The primary antibodies were detected using the avidin−biotin immunoperoxidase technique. The sections were incubated with an appropriate biotinylated secondary antibody (1:200, VectorÒ). The chromogen used was 3,3′-diaminobenzidine. Haematoxylin was used for nuclear stain.

Statistical Analysis. For quantitative analysis (biochemical, lipid parameters and ELISA results), data are expressed as mean and standard deviation except when indicated. For semiquantitative analysis (2-DE and WB analysis) data are expressed as median and interquartile range [IQR]. *N* indicates the number of subjects tested. Statistical analysis was performed with Stat View 5.0.1 software. Student's *t* test and Mann−Whitney testing were used for comparison between control and AMI groups for variables with and without parametric distribution, respectively. The ANOVA test was used in analysis where the effect of variables acting as covariates was added to the model, and in multiple comparisons analysis using Fisher's PSLD as Posthoc Test. Correlations between variables were determined by single and multiple regression models. A *P*-value ≤ 0.05 was considered significant.

Results

Clinical Characteristics of the Study Population. The characteristics of AMI and control groups are outlined in Table 2. There was no significant difference in age, sex, and cholesterol levels (Total-, HDL-, and LDL-cholesterol) between AMI-patients and controls. Triglycerides levels were significantly increased in AMI-patients ($P = 0.02$). AMI-patients showed significant higher levels of urea ($P = 0.03$) and creatinine ($P < 0.001$), and also significant higher levels of hepatic parameters as bilirubin ($P < 0.001$), GOT ($P < 0.001$), and GPT ($P < 0.001$) when compared to controls. No T-troponin levels were detected at arrival to the hospital. The mean highest value for T-troponin was 7.3 ± 5.9 μg/L. AMI-patients were classified according to the Killip classification:[26] 87% of AMI-patients showed no clinical signs of heart failure (Killip class I), 10% of AMI-patients had crackles in the lungs and elevated jugular venous pressure (Killip class II), none of the patients showed acute pulmonary edema (Killip class III), and 3% of AMI-patients showed cardiogenic shock or hypotension (systemic blood pressure <90 mmHg) and evidence of peripheral vasoconstriction such as oliguria, cyanosis, or sweating (Killip class IV). As shown in Table 2, the percentage of necrotic mass of the myocardium (calculated as percentage of the total cardiac mass measured by CMR within the first week after the event) in AMI-patients was **13.5%** (±**9.9**). The left ventricular ejection fraction (LVEF) of AMI-patients was **55%** (±**11**).

Table 2. Biochemical and Lipid Parameters of the Subjects in the Present Study

	AMI patients	healthy donors	*P*-value
N	39	60	-
Age (mean ± SEM)	61 ± 13	62 ± 8	NS
Females/Males	10/29	15/45	-
Total Cholesterol (mg/dL)	204.6 ± 46.7	216.4 ± 42.2	NS
HDL-Cholesterol (mg/dL)	44.3 ± 11.9	48.8 ± 14.0	NS
LDL-Cholesterol (mg/dL)	130.5 ± 40.0	142.3 ± 37.2	NS
Triglycerides	169.6 ± 73.8	129.2 ± 83.8	0.02
Urea (mmol/L)	7.4 ± 3.2	6.4 ± 1.4	0.03
Creatinine (μmol/L)	107.0 ± 32	81.3 ± 19	<0.001
Bilirubin (μmol/L)	14.0 ± 6	7.6 ± 4	<0.001
GOT (U/L)[a]	69.0 ± 50	20.8 ± 5	<0.001
GPT (U/L)[a]	44.0 ± 27	22.2 ± 11	<0.001
CRP levels (mg/L)[b]	7.6 ± 9.9	-	-
TnTmax (μg/L)[c]	7.3 ± 5.9	-	-
Necrosis %[d]	13.5 ± 9.9	-	-
Killip class, *n*			
I	34 (87%)	-	-
II	4 (10%)	-	-
III	-	-	-
IV	1 (3%)	-	-
LVEF (%)[a]	55 ± 11	-	-

[a] GOT = Glutamic-oxaloacetic transaminase; GPT = Glutamic-pyruvic transaminase; LVEF = Left ventricular ejection fraction. [b] CRP levels: C-reactive protein values at admission. [c] TnTmax: Defined as the highest serum T-troponin levels after the event. [d] Necrosis (%): Percentage of necrotic mass of the myocardium (calculated as percentage of the total cardiac mass measured by cardiovascular magnetic resonance).

Proteomic Profile of HDL-Associated Proteins in Serum Samples. Serum samples (after removal of the six most abundant proteins) from 27 AMI-patients and healthy donors were analyzed individually in duplicates by 2-DE and proteins were identified by MALDI-TOF mass spectrometry. HDL-associated proteins, including ApoJ, PON1, and ApoA-I, were consistently identified in human serum samples (compare Figure 1A,B, and Table 3), both in controls and in AMI-patients. As shown in Figure 1, proteins were mainly detected as clusters of different spots. In the serum fraction, as occurred in the HDL fraction, ApoA-I was a major protein consisting of different spots with an apparent mass of approximately 25 kDa and p*I* within the 5.0−5.8 interval.

ApoJ Proteomic Profile from Serum of AMI-Patients Differed from Control Individuals. In serum, ApoJ was identified as a cluster of 12−13 different spots (MW, 37.1−47.3 kDa; p*I*, 4.5−5.0; Figure 2). Most of the spots significantly differed in relative volumes between AMI-patients (serum samples obtained at the moment of admission) and controls (Table 4). AMI-patients showed a shift toward protein isoforms with higher electrophoretic mobility. Thus, spots number 6 and 9 (MW 43.2−41.2 kDa) decreased more than 2-fold their relative volume ($P = 0.009$ and $P < 0.001$, respectively), whereas spots 7, 10, and 13 (MW 41.0−37.1 kDa) were more than 2-fold increased ($P \leq 0.001$) in AMI-patients compared with controls. The intensity of the spot 3 was also increased in AMI patients (1.8-fold increase; $P = 0.01$). ApoJ was not found in the serum fraction containing the six most abundant proteins (data not shown).

Immunodetection of Total and Glycosylated ApoJ. As shown in Figure 3A, WB analysis for total serum ApoJ revealed one band of approximately 40−45 kDa, representing the 65%

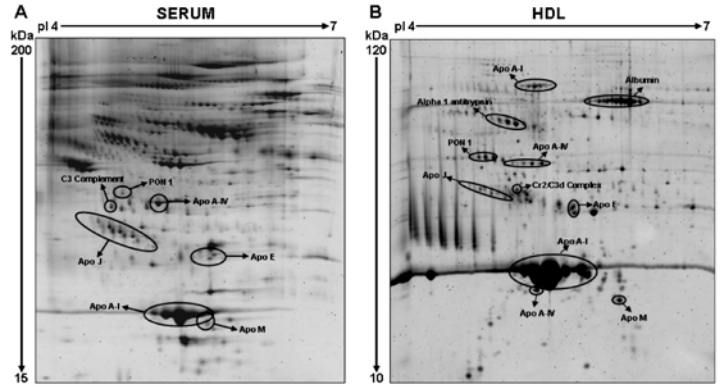

Figure 1. Representative 2-DE image of (A) human serum sample after immunodepletion of six high-abundance proteins (albumin, transferrin, haptoglobin, antitrypsin, IgGs, and IgAs) and (B) ultracentrifugation isolated human HDL. Spots marked with circles refer to proteins identified by MALDI-TOF analysis in both types of samples.

Table 3. Proteins Identified in Serum and in HDL Samples by 2-DE Analysis Followed by MALDI-TOF Identification[a]

protein	Swiss-Prot number	expectation	Mascot score	MW (kDa)	p*I*	serum	HDL
Albumin	P02768	0.000	91	69	5.8−6.3	Depleted	11 spots
Alpha 1 antitrypsin	P01009	0.000	96	60−67	4.9−5.2	Depleted	4 spots
Apo A-I	P02647	0.000	86	25	5−5.8	5 spots	5 spots
				75	5−5.6	UD	5 spots
Apo A-IV	P06727	0.000	110	55	5−5.4	1 spot	4 spots
				24	5.6	UD	1 spot
Apo E	P02649	0.000	85	36−37	5.8	3 spots	2 spots
Apo J	P10909	0.002	83	37.1−47.3	4.5−5	12−13 spots	7 spots
Apo M	O95445	0.002	59	23	5.7	1 spot	1 spot
C3 complement	P01024	0.000	75	52	4.8	1 spot	1 spot
PON 1	P27169	0.003	93	58	4.8−5.1	1 spot	3 spots

[a] Apo A-I = Apolipoprotein A-I; Apo A-IV = Apolipoprotein A-IV; Apo E = Apolipoprotein E; ApoJ = Apolipoprotein J; Apo M = Apolipoprotein M; UD = undetectable; PON 1 = Paraoxonase 1.

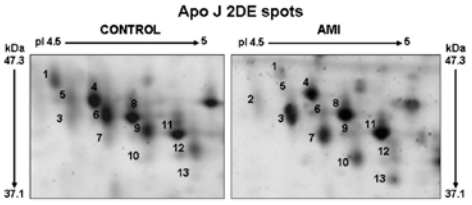

Figure 2. Representative pattern of ApoJ cluster in 2-DE gels from control and AMI serum samples. ApoJ in serum was detected as a cluster of 13 spots with a p*I* range between 4.5 and 5.0 and a molecular mass between 37.1 and 47.3 kDa. Spots identified as ApoJ were numbered from acidic to basic pH. Spot 2 was only apparent in AMI-patients. Note that spots marked as 1, 3, 7, 8, 10, 11, and 13 depicted enhanced detection levels in AMI, whereas spots 6 and 9 depicted a reduced intensity in AMI gels compared to the control group.

of the total detected ApoJ, and a second band with an apparent mass of 65−70 kDa, corresponding to the ApoJ glycosylated heterodimer. WB analysis for ApoJ in the serum-derived glycosylated fraction also revealed two bands with similar electrophoretic mobility as those detected in total serum although with a predominance of the high molecular band (83%) representing the higher glycosylated state of the detected ApoJ (compare Figure 3, panels A and B).

Glycosylation of the high molecular weight band was determined after immunoprecipitation of serum ApoJ followed by the incubation with and without PNGase-F, an enzyme that cleaves all types of asparagine-bound N-glycans. AMI-patients and controls revealed a band of 65−70 kDa. This band disappeared with PNGase F treatment and only one defined band, of approximately 40−45 kDa corresponding to the α/β polypeptidic chains of the protein, was detected in both groups (Figure 3C).

Proteomic Profile of ApoJ in the Serum-Derived Glycosylated Fraction. The proteomic profile of glycosylated serum ApoJ was studied after purification of glycosylated proteins through its binding to lectins. The glycoprotein-subproteome from AMI and control serums (pools of 10 individuals) were analyzed in triplicates (Figure 4). Patients having diabetes as a cardiovascular risk factor were excluded from this analysis. Only 6 spots (MW, 40−45 kDa; p*I*, 4.5−5.0) were identified as ApoJ in the AMI and control groups. These spots referred to those denoted as 1, 2, 4, 5, 8, and 11 in the 2-DE analysis of total serum fraction (compare Figure 4 with 2). AMI-patients showed a decrease ($P = 0.05$, Mann−Whitney) in the gycosilated ApoJ intensity when compared to the control population (AMI vs control: 11612 [10712−13620] vs 8944 [8907−9173], values

Table 4. ApoJ Proteomic Profile in Serum of AMI-Patients and Control Individuals by 2-DE[a]

spot number	expectation	Mascot score	pI	MW (kDa)	control (AU)	AMI (AU)	P-value[b]
1	0.003	72	4.6	47.3	446 [339−970]	821 [455−1099]	0.007
2	0.004	66	4.5	44.4	-	486 [428−546]	NS
3	0.002	93	4.6	44.8	685 [525−1490]	1218 [592−1798]	0.01
4	0.001	90	4.7	45.1	1990 [1923−2203]	1983 [1566−3012]	NS
5	0.002	73	4.7	43.5	116 [116−116]	460 [460−460]	NS
6	0.000	122	4.8	43.2	3113 [2138−4088]	1583 [769−2450]	0.009
7	0.002	69	4.8	41	447 [423−472]	972 [717−1587]	0.001
8	0.004	61	4.8	44	1929 [1577−4067]	3351 [2195−4014]	<0.001
9	0.002	58	4.9	41.2	2603 [1378−2915]	1282 [935−2026]	<0.001
10	0.001	94	4.9	39	262 [258−267]	739 [434−1018]	0.001
11	0.000	119	5	42.2	1287 [1108−2365]	2651 [2288−3131]	<0.001
12	0.001	78	5	41	1494 [1329−1660]	1447 [1172−1595]	NS
13	0.001	80	5	37.1	117 [94−141]	361 [279−500]	<0.001

[a] AU = arbitrary units; NS = no significant. [b] Acute myocardial infarction (AMI) patients vs Controls (Median [IQR]): Mann−Whitney.

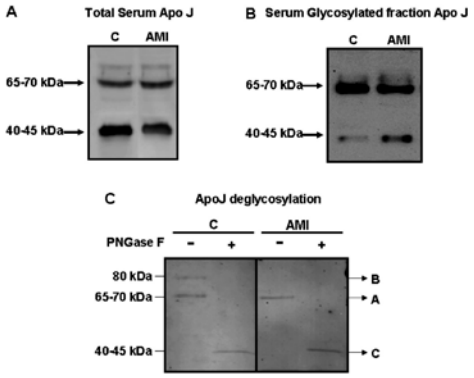

Figure 3. Representative Western blot for ApoJ in controls and AMI-patients in (A) total serum after immunodepletion of six high-abundance proteins and (B) the glycosylated fraction of serum. Two major bands were detected corresponding to the ApoJ heterodimer (65−70 kDa) and ApoJ α and β chains (40−45 kDa). The lowest MW band showed the highest intensity in total serum in controls and AMI-patients, whereas the major part of glycosylated ApoJ was represented by the band of 65−70 kDa. Differences in the band intensity of the glycosylated fraction were especially evident in the control group. (C) ApoJ deglycosylation profile after treatment with PNGase F in immunoprecipitated ApoJ from serum samples of controls and AMI-patients. ApoJ immunoprecipitation resulted in a band of approximately 65−70 kDa corresponding to glycosylated heterodimer (band A) in both samples (controls and AMI-patients). In controls, another band of 80 kDa was detected (band B) corresponding to a higher glycosylated state of ApoJ. After PNGase F treatment, bands A and B were not apparent and a band of 40 kDa was detected in both groups (band C) corresponding to a deglycosylated form of ApoJ.

indicate total intensity of the ApoJ isoforms normalized for intensity volume of all spots in the gel). When spots were individually compared, we found a significant underexpression of the spots 4 and 8 (two major glycosylated spots) in AMI-patients compared to controls (3271 [3239−3465] vs 2891 [2661−2975] and 4089 [3524−4890] vs 2662 [2599−2772], respectively; Mann−Whitney, $P < 0.05$ for both spots).

Analysis of ApoJ in Microparticles-Exosomes. In a subgroup of patients, ApoJ was analyzed in the microparticles-exosomes (MP-EX) fraction. Serum MP-EX fraction and MPs-EX-free serum fraction (soluble fraction) from healthy individuals ($n = 10$) and AMI-patients ($n = 10$) were analyzed by ELISA and WB. ApoJ was quantified in the soluble fraction and in a 10-fold concentrated fraction of serum MP-EX. The soluble fraction revealed an ApoJ concentration of 56.5 ± 6.3 μg/mL while ApoJ levels in MP-EX were under the detection limit of the assay (0.005 μg/mL). WB analysis of the serum soluble-fraction and serum-derived MP-EX fraction (10-fold concentrated) from controls and AMI-patients (Figure 5A) revealed the two ApoJ bands also depicted in the total serum and glycosylated fractions (compare Figure 5A with Figure 3A,B). In the soluble fraction, a significant difference in the band distribution was detected between AMI-patients and controls. The intensity of the band of 65−70 kDa (measured as the percentage of the total ApoJ intensity) was significantly lower in AMI-patients than in controls (Control, 62% [57−68] vs AMI, 43% [39−47]; $P = 0.03$), while the band of 40−45 kDa showed an increase in its intensity in AMI-patients compared to controls (Control, 39% [32−43] vs AMI, 57% [53−61]; $P = 0.03$). In the MP-EX fraction, the most representative was the low molecular weight band (92%) with no significant differences between AMI-patients and controls (Figure 5B).

ApoJ Serum Levels in AMI-Patients and Control Individuals. ApoJ serum levels were measured by a commercial ELISA in 39 AMI-patients at time zero (defined as the time of admission within 6 h after the onset of the event). The two monoclonal antibodies used in the ELISA kit are undisclosed by the providers, and therefore, the recognition sites in ApoJ are unknown. When compared to the control group, ApoJ levels were significantly lower in AMI-patients (Control, 50.8 ± 7.7 μg/mL vs AMI, 36.0 ± 18.3 μg/mL; $P < 0.001$; Figure 6A). Differences between AMI group and controls were still significant when men (Control, 48.7 ± 6.7 μg/mL vs AMI, 35.4 ± 19.0 μg/mL; $P < 0.001$) and women were analyzed separately (Control, 57.0 ± 7.2 μg/mL vs AMI, 37.9 ± 17.2 μg/mL; $P < 0.001$). Differences in ApoJ were also observed after adjustment for the effects of other variables as total-, LDL-, and HDL-cholesterol ($P < 0.001$). In 35 of the patients, ApoJ levels were determined at different time periods up to 96 h after the admission time. In 60% of these patients, the lowest ApoJ level was detected at admission (35.0 ± 19.0 μg/mL). At 8 and 24 h after the time of admission, ApoJ levels were significantly higher than those at time zero (8 h, 42.9 ± 11.1 μg/mL, $P = 0.01$; 24 h, 42.0 ± 8.8 μg/mL, $P = 0.02$) and serum ApoJ in AMI-patients had achieved control levels at 72 and 96 h after admission (72

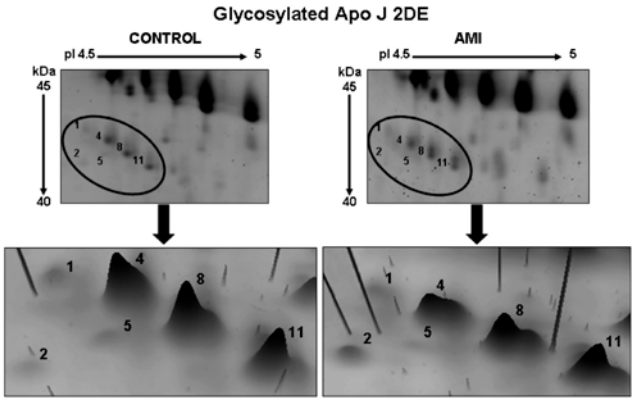

Figure 4. Representative 2-DE gel of the ApoJ cluster in the glycosylated serum fraction from (A) healthy donors and (B) AMI-patients. Six spots (1, 2, 4, 5, 8, and 11) corresponding in p*I* and MW with those of total serum were detected. As for Figure 2, spots were numbered from acidic to basic pH. Intensity of ApoJ spots was lower in AMI-patients than in controls. Decrease was more evident in spots 4 and 8.

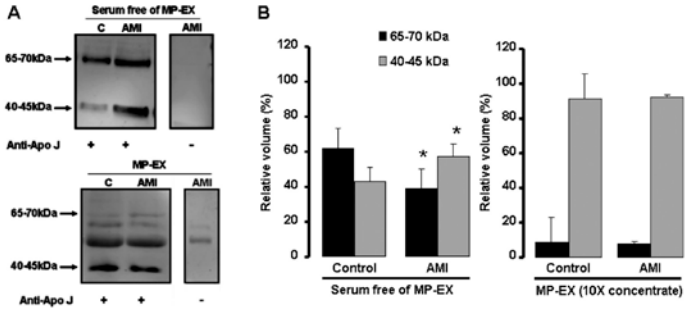

Figure 5. ApoJ in the serum derived microparticles-exosomes fraction (MP-EX) and MP-EX-free serum fraction (soluble fraction) of AMI-patients and control individuals. (A) Representative Western blot depicted two bands corresponding to the glycosylated ApoJ heterodimer (65−70 kDa) and the nonglycosylated α and β chains (40−45 kDa). (B) Bar diagram (median and interquartile range) for band relative volumes (calculated as % of the total ApoJ intensity). Note that intensity band percentages significantly varied between AMI and control groups in the MP-EX serum free fraction ($P = 0.03$).

h, 43.3 ± 11.5 μg/mL, $P = 0.02$; 96 h, 49.4 ± 12.8 μg/mL, $P = 0.001$; Figure 6B).

In the AMI group, serum ApoJ levels at admission did not correlate with serum T-troponin peak levels (defined as the highest T-troponin levels after the event), nor with the percentage of necrotic mass of the myocardium (calculated as percentage of the total cardiac mass measured by CMR). Contrarily, ApoJ levels showed a positive correlation with CRP levels at time zero ($r = 0.445$, $P = 0.02$; Table 5). No correlation was obtained if ApoJ levels were analyzed in relation to the peak values obtained for CRP up to 96 h after admission-time ($r = -0.081$, $P =$ NS; Table 5). Peak-CRP values positively correlated with T-troponin levels and the percentage of necrotic mass of the myocardium ($r = 0.383$, $P = 0.04$; $r = 0.454$, $P = 0.01$, respectively). ApoJ levels in the AMI group were not significantly modified in patients under statin treatment (Statins, 44.1 ± 20.9 μg/mL vs No statins, 34.0 ± 17.7 μg/mL; $P > 0.05$).

Immunohistochemistry. Positive ApoJ signals were consistently detected in ischemic heart tissue but not in normal healthy heart tissue (brown signals in Figure 7). ApoJ signal was mainly detected in the microvessels, but also ApoJ traces were seen in the rest of the myocardial tissue.

Discussion

Acute myocardial infarction (AMI) is one of the major causes of mortality and morbidity worldwide. However, the early systemic changes after the onset of an AMI have not been fully characterized, and therefore, other than CPK and troponins, there is a dearth of biomarkers that could indicate early ischemic damage. So there is a need to discover new serum biomarkers in the early phase after an AMI, in order to stratify the risk of patients with chest pain that arrive to the emergency room. In many instances, chest pain is not associated with myocardial infarction but with unstable angina or it has a noncardiac origin. In the present study, by applying proteomic technologies, we have identified in serum samples several proteins previously related to HDL, such as ApoA-I, A-IV, E, J,

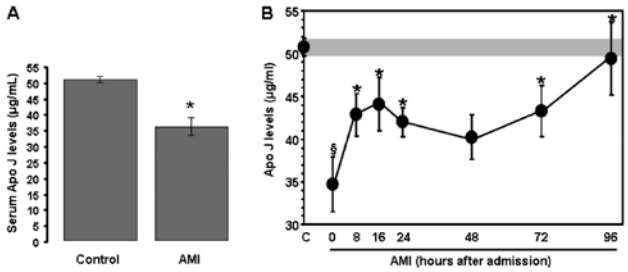

Figure 6. Serum ApoJ levels (μg/mL) in AMI-patients and healthy donors. Quantification of ApoJ levels was performed by a commercial ELISA. (A) Bar diagram of the mean (±SEM) serum ApoJ levels of the control group and AMI-patients at admission in the Emergency room (time 0) (control: $n = 60$; AMI $n = 39$; $^*P < 0.001$; Student's t test). (B) Time course diagram of mean (±SEM) serum ApoJ levels in AMI-patients between 0 and 96 h after the admission time. ($\S P < 0.001$ vs control; $^*P < 0.02$ vs time 0; ANOVA and Fisher's PSLD as Posthoc Test).

Table 5. ApoJ Correlations with Age and Serum Parameters in AMI and Control Populations

	control population		AMI population	
	r-value	*P*-value	*r*-value	*P*-value
Age	0.446	0.003	0.312	NS
Total-cho	0.230	NS	0.184	NS
HDL-cho	0.161	NS	0.032	NS
LDL-cho	0.103	NS	0.176	NS
Necrosis[a]	-	-	−0.126	NS
TnTmax[b]	-	-	−0.210	NS
CRP $t = 0$[c]	-	-	0.445	0.02
CRP max[d]	-	-	−0.081	NS

[a] Necrosis (%): Percentage of necrotic mass of the myocardium (calculated as percentage of the total cardiac mass measured by cardiovascular magnetic resonance). [b] TnTmax: Defined as the highest serum T-troponin levels after the event. [c] CRP $t = 0$: C-reactive protein values at admission. [d] CRP max.: Defined as the highest serum C-reactive protein levels after the event.

and PON1.[27] Among these HDL-related proteins, ApoJ has shown a significant change in its isoform distribution profile in serum of acute new-onset myocardial infarction patients. To our knowledge, our study has demonstrated for the first time a predominance of ApoJ isoforms of lower molecular weight and more basic p*I* in AMI-patients when compared to controls. The observed molecular mass differences for ApoJ isoforms in the proteomic study are due to the N-glycosylations of the ApoJ molecule, consisting of associated complex sugars contributing 20−30% to its molecular weight.[28−30] Serum ApoJ is represented by a series of different isoforms that are present in serum depending on the physiological status or in relation to disease.[13] Indeed, the specific analysis of the purified serum derived glycoprotein fraction revealed a decrease in the intensity of glycosylated ApoJ isoforms in AMI-patients. Further studies are needed in order to identify and characterize the most important ApoJ glycosylated isoforms in relation to AMI and henceforth to investigate their pathophysiological relevance. In fact, clinical implications of different ApoJ glycosylated isoforms have already been postulated for cancer[31] and in Alzheimer's disease.[32]

Beside the changes in the serum ApoJ proteome profile, we have observed a significant decrease in serum ApoJ levels in AMI, being the minimum peak detected within the first 6 h after the onset of the event. Because of the lack of information on the probes of the ELISA kit, we are unable to identify whether the measurement reflects an isoform or a global decrease of total serum ApoJ mass. ApoJ levels have been related to cardiovascular disease risk factors such as Type 2 diabetes[21] and aging.[33] Up to now, there are controversial reports relating ApoJ to coronary heart disease.[20,21] Additionally, in our study, after the early drop, serum ApoJ levels start to recover at 24 h after admission, reaching normal levels at 72−96 h. Therefore, the contradictory results previously reported in CAD[20,21] could be related to time of blood collection after presentation of AMI. The serum ApoJ levels we observed in healthy subjects were in the same range of values than those previously reported.[21] From our results, we cannot exclude the possibility that those patients having an AMI could have, before the clinical event, lower levels of ApoJ than the control population and therefore a higher susceptibility of suffering an AMI. However, in the Caerphilly prospective study,[34] patients having an acute coronary syndrome did not show any significant variation in ApoJ levels previous to the event. Even more, the fact that we detected a decrease in ApoJ levels after the onset of the event and a normalization of those levels 96 h afterward seems to reflect a transient situation induced by the AMI. Altogether these data suggest that ApoJ decrease is an immediate consequence of an acute situation of ischemia. Differing from our results in acute myocardial infarction, previous studies in chronic heart disease (CHD) have found increased ApoJ levels in patients with more than 50% of stenosis and have suggested ApoJ as a marker for CHD.[35] The same authors performed studies in patients with stable angina pectoris and related statin treatment with a decrease in ApoJ serum levels.[36] In contrast, in our study, AMI patients under statin treatment did not show any significant modification in ApoJ serum levels. The lack of effect of statin treatment in our study may indicate that the acute ischemic effect is unrelated to the chronic treatment. Indeed, the differences between these studies and ours may highlight a different implication of ApoJ in coronary heart diseases whether patients are in an acute or in a chronic situation.

The positive correlation between CRP and ApoJ levels at the moment of admission of AMI-patients may indicate the pro-inflammatory systemic balance during ischemia. It has been already described that ApoJ exhibits anti-inflammatory properties;[14] indeed, there are some inflammatory diseases, such as systemic lupus erythematosus[37] where a decrease in ApoJ levels has been described.

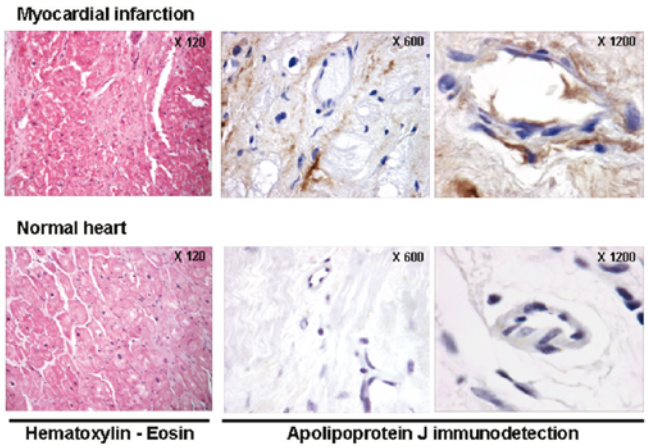

Figure 7. Representative photomicrographs of immunostaining for ApoJ in ischemic and normal myocardial tissue. Note the strong brown signals around capillaries in myocardial sections from a recent ischemic heart, whereas no signals were detected in capillaries of a non-ischemic myocardial tissue (normal heart).

Blood circulating microparticles have been recently associated with atherothrombotic diseases.[38] Previous proteomic studies have shown the presence of ApoJ in platelet derived microparticles.[39] In our study, we evidenced a very low presence of ApoJ in serum microparticles from both controls and AMI-patients with no significant differences between them. ApoJ is mainly present in the soluble fraction (microparticles-free serum fraction).

Anti-inflammatory effects of ApoJ have been reported to be mediated through NF-κB.[40] Interestingly, here we have shown that ApoJ is found in myocardium of ischemic human hearts but not in normal hearts, supporting previous data reporting deposition of ApoJ in infarcted myocardia.[41] Whether this deposition contributes to the healing process remains to be investigated.

As a limitation of this study, one may consider the low sample size. However, this is a proteomic proof of concept study that looks for specific characteristics of protein processing associated to new-onset MI and not yet a biomarker qualification analysis. Even more, controls and patients are age and sex matched and the observed differences are highly significant despite being a small population.

In summary, ApoJ characterization in patients with acute myocardial infarction has revealed significant changes in its proteomic profile and in serum levels in early stages after AMI. This data supports the hypothesis that during the early ischemic phase of AMI there are systemic responses that might try to compensate the inflammatory outburst. Indeed, in an experimental study of closed chest balloon induced ischemia/reperfusion, we showed that already after 30 min of myocardial ischemia significant pro-inflammatory systemic alterations could be measured.[42] Intriguingly, in the early phase of AMI, serum ApoJ isoforms are less glycosylated than in control individuals. The analysis of this isoform glycosylation shift in patients with AMI may provide a better understanding of ApoJ function than the total serum levels of ApoJ, and ultimately, the isoform shift may become a more reliable biomarker for detecting the early phase of AMI.

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Supporting Information Available: Representative *m/z* spectrum of human ApoJ. This material is available free of charge via the Internet at http://pubs.acs.org.

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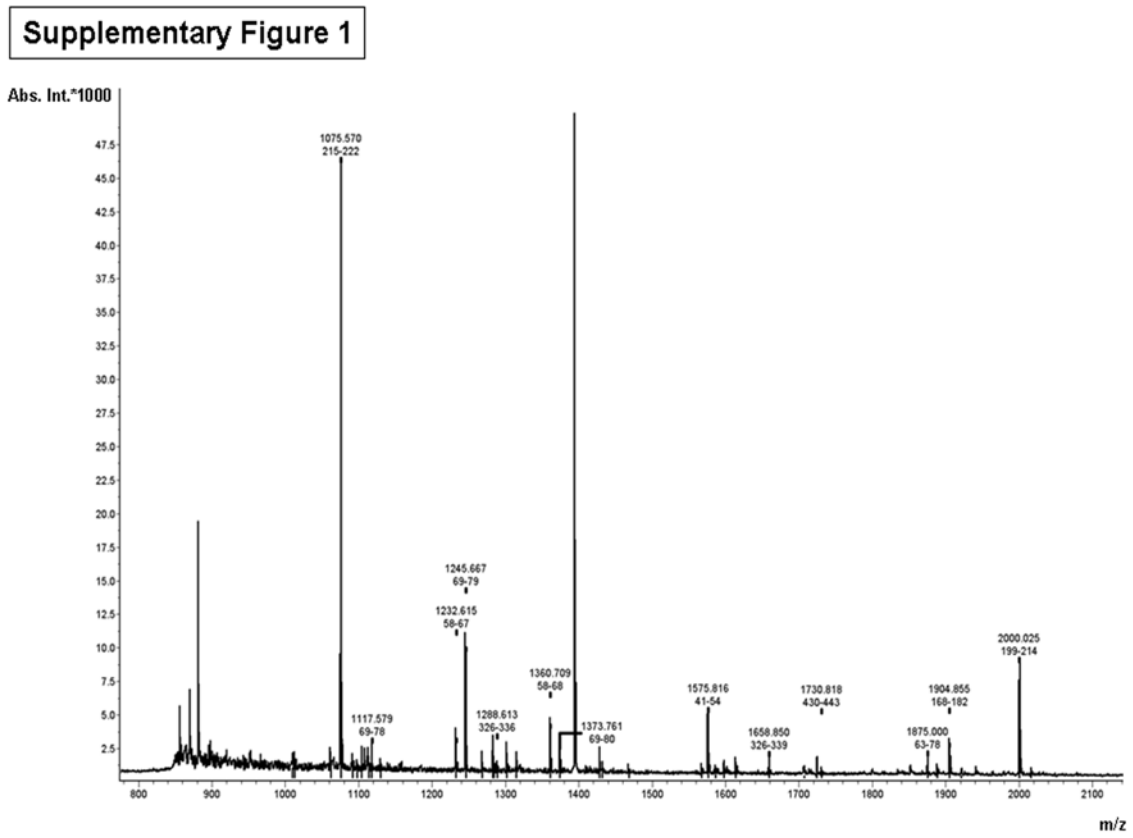
SUPPLEMENTAL MATERIAL



Supplementary Figure 1. Representative m/z spectrum of human ApoJ (P10909) identified by MALDI-TOF analysis. Peptides used for the identification are shown in the figure.

ARTÍCULO SEGUNDO

"Differential proteomic distribution of TTR (pre-albumin) forms in serum and HDL of patients with high cardiovascular risk"

Judit Cubedo [a, b, e], Teresa Padró [a, e], Rodrigo Alonso [f], Juan Cinca [c], Pedro Mata [f], Lina Badimon [a, b, d, e]

[a] Cardiovascular Research Center (CSIC-ICCC), [b] CIBERobn, [c] Cardiology Service, Hospital Santa Creu i Sant Pau, [d] Autonomous University of Barcelona, [e] Biomedical Research Institute Sant Pau (IIB-Sant Pau) of Barcelona, and [f] Jimenez Diaz Foundation of Madrid. Spain.

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Resumen resultados

Identificación en suero de la TTR: cambios en los pacientes AMI en función de los factores de riesgo

Mediante 2-DE en el suero se identifica la transtiretina (TTR) como un único punto de 42 kDa y un pI de 5.6 que se corresponde con la forma trimérica de la proteína (tTTR). En los pacientes con un AMI de nueva presentación, dentro de las 6 primeras horas desde el inicio del evento, la intensidad de la forma tTTR está inversamente relacionada con el número de factores de riesgo cardiovascular. Concretamente los pacientes con diabetes y dislipemia presentan los niveles más bajos de tTTR.

Relación de los cambios en la TTR en el AMI con un marcador de inflamación, la CRP

Los pacientes de alto riego en base a los niveles de CRP (>3mg/L) presentan valores de intensidad de tTTR significativamente más bajos. A las 72-96 horas tras el ingreso, momento en el que se detectan los niveles más altos de CRP, en la mayoría de los pacientes no se detecta la forma tTTR.

Validación cambios en el AMI y en una población de elevado riesgo cardiovascular

El análisis mediante ELISA de los niveles totales de TTR revela una disminución significativa en la fase temprana del AMI así como en una situación de alto riesgo cardiovascular como la de los pacientes con hipercolesterolemia familiar heterocigótica (FH). Los pacientes FH presentan niveles más elevados que los pacientes AMI. Estos cambios son independientes al tratamiento con estatinas. El seguimiento de los niveles de TTR desde el momento del ingreso en los pacientes AMI revela una disminución progresiva hasta las 72-96 horas.

Patrón de la TTR en las HDL de pacientes FH: asociación con el riesgo cardiovascular

El análisis de las HDL mediante 2-DE revela la presencia de la forma monomérica de la TTR (mTTR, 14kDa). Esta forma presenta una disminución de 1.9 veces en pacientes con FH que han tenido una manifestación clínica de patología cardiovascular previa. Además hay una relación inversa entre el nivel de riesgo según la escala de estratificación del riesgo Framingham (FRS) y la intensidad de la forma mTTR de las HDL, mientras que no hay relación entre el FRS y los niveles de CRP.

Distribución diferencial de las formas de TTR en el suero y las HDL

El análisis mediante WB en condiciones no reductoras revela la presencia de 4 formas de TTR en el suero (monómero, dímero, trímero y tetrámero) mientras que en las HDL sólo está presente el monómero de TTR. Específicamente, esta mTTR sólo se detecta en la fracción de HDL3.

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Differential proteomic distribution of TTR (pre-albumin) forms in serum and HDL of patients with high cardiovascular risk

Judit Cubedo[a,b,e], Teresa Padró[a,e], Rodrigo Alonso[f], Juan Cinca[c], Pedro Mata[f], Lina Badimon[a,b,d,e,*]

[a] Cardiovascular Research Center (CSIC-ICCC), Spain
[b] CIBERobn, Spain
[c] Cardiology Service, Hospital Santa Creu i Sant Pau, Spain
[d] Autonomous University of Barcelona, Spain
[e] Biomedical Research Institute Sant Pau (IIB-Sant Pau) of Barcelona, Spain
[f] Jimenez Diaz Foundation of Madrid, Spain

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ABSTRACT

Inflammation is a common condition contributing to cardiovascular disease progression which leads to clinical manifestations such as acute myocardial infarction (AMI). By applying a proteomic expression profiling approach we have investigated changes in transthyretin (TTR) in AMI-patients and its distribution patterns in HDL samples of patients with high cardiovascular risk, such as those with familiar hypercholesterolemia (FH).

Methods and results: The characterization by bidimensional electrophoresis (2-DE), followed by mass-spectrometry (MALDI-TOF) of serum samples revealed changes in the intensity of the TTR spot with a *pI* of 5.6 and a Mw of 42 kDa (tTTR) between AMI-patients in association to diabetic dyslipemia. Serum TTR levels, determined by commercial ELISA, were significantly lower ($p < 0.0001$) in AMI-patients ($n = 39$) and FH-patients ($n = 100$) than in healthy controls ($n = 60$). Western blot and 2-DE analysis showed a differential distribution profile of TTR forms between serum, where 3 TTR forms of 42 (tTTR), 28 (dTTR), and 14 kDa (mTTR) were detected, and HDL samples, where only mTTR was present.

Conclusions: Our results demonstrate alterations in TTR proteomic profile in relation to the clustering of risk factors which seems to highlight the implication of TTR in cardiovascular risk. The significant differences in TTR between serum (tTTR) and HDL (mTTR) underscore the importance of TTR-forms in the circulation and deserve further investigation to understand their function.

1. Introduction

Inflammation is a major risk factor for atherosclerosis which chronically progresses to acute coronary syndromes such as myocardial infarction [1]. In addition to inflammation, lipid levels, lipoprotein metabolism, and oxidative stress are well known risk factors for cardiovascular disease progression [2]. Serum levels of transport proteins are affected by stress situations, such as pro-inflammatory states and tissue necrosis, when the liver reprioritizes synthesis of serum proteins with an up-regulation of acute phase reactants and down-regulation of serum transport proteins [3]. Decreased concentrations of the so-called visceral or hepatic proteins, such as albumin, transferrin, and transthyretin, have been associated to inflammation, malignancy, or many other disorders [4]. Transthyretin (TTR) o prealbumin is involved in the blood transport of different molecules with binding capacity for thyroxin (T_4), triiodothyronine (T_3) and holo-retinol-binding-protein (RBP4 with retinol or vitamin A) [5–7]. Reduced TTR serum levels have been described in different pathological conditions such as Type 1 diabetes [8], lung cancer [9] and early death in elderly people [10]. TTR is transported by high density lipoproteins (HDL), in association with Apolipoprotein AI, and it has been suggested that the presence or absence of TTR affects the properties or stability of the HDL particles [11].

In the present study we have investigated TTR in two groups of patients. A group of high cardiovascular risk patients with heterozygous familiar hypercholesterolemia and a group of patients with acute manifestation of cardiovascular disease (myocardial infarction). Here we report that TTR forms change in both groups of patients showing a significant inverse correlation with high cardiovascular risk and clustering of risk factors.

* Corresponding author at: Cardiovascular Research Center (CSIC-ICCC), Hospital de la Santa Creu i Sant Pau, Av. S. Antoni M. Claret, 167, 08025 Barcelona, Spain. Tel.: +34 93 5565880; fax: +34 93 5565559.
E-mail address: lbadimon@csic-iccc.org (L. Badimon).

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2. Materials and methods

For detailed information see expanded material and methods supplemental data.

2.1. Study population

The study population comprised a group of patients with familiar hypercholesterolemia with and without a previous documented acute myocardial infarction (FH-CVD and FH-NoCVD, respectively) and a group of patients with an acute new-onset myocardial infarction (AMI-patients).

The FH group consisted in 100 patients with heterozygous familiar hypercholesterolemia (79 men and 21 women; mean age: 49 ± 1 years), 30 patients had had a previous documented acute myocardial infarction event (>3 years before) and 70 patients had not had any clinical episode of cardiovascular disease (CVD) before their recruitment.

The AMI group included 39 new-onset AMI patients (29 men and 10 women; mean age: 61 ± 2 years) who were admitted with chest pain and suspected of ACS at the Emergency Room of Santa Creu i Sant Pau Hospital. At the emergency department, routine diagnostic procedures were applied to establish the onset of symptoms as accurately as possible (*i.e.* description of chest pain, pulmonary edema, severe dyspnea, and syncope). In addition to the general patient history, clinical examination, 12-lead ECG, and laboratory tests were also run to characterize AMI-patients. All AMI-patients showed (1) typical chest pain lasting more than 30 min; (2) ST segment elevation >0.2 mV in at least 2 contiguous leads; (3) admission to the hospital within the first 6 h after chest pain onset; (4) normal serum CK and CKMB levels at admission; (5) negative troponin T at admission (excluding subacute myocardial infarction); (6) sinus rhythm. Exclusion criteria were a previous documented or suspected myocardial infarction and antithrombotic treatment because of the AMI onset before arriving to the emergency room and time of blood collection. Delayed contrast-enhanced (CE) CMR studies were performed within the first week after AMI, as previously described [12].

Patients included in the study did not present further comorbidities such as cancer, chronic infections, autoimmune diseases or thyroid hormones disorders.

Blood samples of AMI-patients were collected at the moment of admission within the first 6 h after the onset of the event (defined as time zero) and at different time points after the admission (8, 24 and 72–96 h).

The control group included sixty healthy individuals (45 men and 15 women; mean age: 62 ± 1 years) who attended to a routine health check.

The Ethics Committee of the Santa Creu i Sant Pau Hospital and the Jimenez Diaz Foundation of Madrid approved the project and the studies were conducted according to the principles of Helsinki's Declaration. All participants gave written informed consent to take part in the study.

Presence of metabolic syndrome in the three groups was considered when three from five of the following criteria were present: (1) obesity (BMI > 30); (2) diabetes; (3) HDL-cholesterol levels below 40 mg/dL for men and 50 mg/dL for women; (4) triglycerides levels above 150 mg/dL; (5) hypertension.

2.2. Blood collection and sample preparation

Venous blood samples were collected to prepare serum that was aliquoted and stored at −80 °C. For proteomic studies, serum samples were prepared as previously described [12]. Human HDL, HDL2 and HDL3 fractions were obtained by ultracentrifugation in density gradients.

Table 1
Study groups demographics and biochemistry.

	Control group	AMI group	FH group
n	60	39	100
Age (mean ± SEM)	62 ± 1	61 ± 2	49 ± 1
Females/males	15/45	10/29	21/79
Risk factors, *n* (%)			
Tobacco smoking	19	51	40
Hypertension	22	42	10
Dyslipemia	51	49	100
Diabetes mellitus	2	27	3
Metabolic syndrome	13	39	7
Background medication, *n* (%)			
ASA	3	10	22
ACEI	7	17	12
Statins	25	17	100
A2RA	3	5	5
B-blockers	2	7	13
Ca-Antagonists	2	10	7
NTG	0	2	6
OAD	2	17	1
CRP levels (mg/mL)[a]	–	7.6 ± 1.7	2.6 ± 0.4

ASA: acetylsalicylic acid; ACEI: angiotensin-converting enzyme inhibitors; A2RA: angiotensin 2 receptor antagonists; NTG: nitroglycerine; OAD: oral antidiabetic drugs.

[a] CRP levels: in AMI-patients, C-reactive protein values at admission.

2.3. Biochemical analysis

C-reactive protein (CRP), creatinine, GOT, GPT, lipids (Total-, HDL- and LDL-cholesterol) and T-troponin levels were measured by standard laboratory methods.

2.4. Proteomic and Western blot analysis

Serum and HDL extracts were separated by two-dimensional gel electrophoresis (2-DE). Spot patterns were analyzed for differences using PD-Quest 8.0 (BioRad). Protein spots of interest were excised for identification by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF). Sample extracts were also analyzed by western blot.

2.5. Quantification of TTR serum levels

Serum transthyretin was determined by a double antibody sandwich enzyme-linked immunosorbent assay (DunnLab).

2.6. Statistical analysis

Data are presented as median [IQR] unless stated. *N* indicates the number of subjects tested. Statistical analysis was performed with Stat View 5.0.1 software. Statistical differences between groups were analyzed by the non-parametrics Mann–Whitney or Kruskal–Wallis tests, or ANOVA single factor or including covariates, Student's *t*-test, as indicated. Correlations between variables were determined by single and multiple regression models. A p value ≤ 0.05 was considered significant.

3. Results

3.1. Clinical characteristics of the study population

The characteristics of AMI, FH and control groups are outlined in Table 1. There were no significant differences in age and sex between AMI-patients and controls. Lipid levels in control, AMI and FH groups are listed in Supplemental Table 1. No

significant differences were observed within AMI-patients and controls when comparing lipid parameters between individuals with and without statin and angiotensin-converting enzyme inhibitors (ACEI) treatment (Supplemental Table 2). All FH-patients were under statin treatment. AMI-patients showed significant higher levels of creatinine (mean ± SEM; AMI: 107.0 ± 5 μmol/L *vs.* Control: 81.3 ± 3 μmol/L; $p < 0.001$) and hepatic parameters as GOT (mean ± SEM; AMI: 69.0 ± 8 U/L *vs.* Control: 20.8 ± 1 U/L; $p < 0.001$), and GPT (mean ± SEM; AMI: 44.0 ± 5 U/L *vs.* Control: 22.2 ± 2 U/L; $p < 0.001$) when compared to controls. Metabolic syndrome was present in 13% of control individuals, in 39% of AMI-patients and in 7% of FH-patients.

3.2. TTR proteomic profile in AMI patients

Transthyretin (Swiss-Prot number: P02766) was identified in serum by 2-DE (p*I*: 4–7 and 10% SDS-PAGE gels) and MALDI-TOF mass spectrometry as a single spot with an apparent molecular mass of 42 kDa and a p*I* of 5.6, that corresponds to trimeric TTR (Supplemental Fig. 1). From the ACS patients studied with first new-onset AMI ($n = 27$; serum samples at the moment of admission within first 6 h after the onset of the event defined as time zero), the majority (58%) showed a 4.1-fold mean increase ($p < 0.05$) in tTTR intensity when compared to controls, whereas only 3 patients showed a 3.8-fold mean decrease ($p < 0.05$) in the intensity of the tTTR spot. The intensity of the tTTR spot showed important modifications among AMI-patients depending on the number of co-existing risk factors (Fig. 1A). Most of the patients showing high tTTR intensity (89%) had less clustering of risk factors (RF), while AMI-patients who depicted a decrease in tTTR intensity had ≥3 RF. AMI-patients with metabolic syndrome showed lower intensity levels of tTTR than AMI-patients without metabolic syndrome (median [IQR]; MS: 11,921 [7033–16,859] AU *vs.* no MS: 16,138 [6487–35,792] AU; p = NS; Fig. 1B). The lowest tTTR intensity in AMI-patients was detected in patients with both dyslipemia and diabetes ($p < 0.05$; Fig. 1C).

The intensity of the tTTR spot was significantly lower in AMI-patients having high-risk CRP levels (>3 mg/L) at the moment of admission (median [IQR]; CRP > 3 mg/L: 5777 [2513–13,043] AU *vs.* CRP < 1 mg/L: 19,680 [11,197–32,653] AU; $p < 0.05$; Fig. 2A; cut-offs of <1 mg/L and >3 mg/L, for low and high-risk patients, were used as previously suggested) [13]. There was a significant correlation between the ratio of tTTR spot intensity (AMI-patients *vs.* C) and CRP levels at the moment of admission ($p < 0.05$, $r = 0.123$; Spearman correlation).

At 72–96 h after the moment of admission the tTTR spot was undetectable in the majority of patients ($p < 0.0001$) except one that also showed a 10-fold decrease (Fig. 2B).

3.3. TTR serum levels in AMI, FH and control groups

As shown in Fig. 3A, total TTR serum levels were measured by a commercial ELISA in AMI-patients at time zero (defined as the time of admission within 6 h after the onset of the event), in the high cardiovascular risk group of FH-patients and in controls. When compared to the control group (Fig. 3AI), TTR levels were significantly lower in AMI-patients (median [IQR]; AMI: 170.1 [147.8–188.9] μg/mL *vs.* Control: 223.0 [203.3–250.1] μg/mL; $p < 0.0001$; Mann–Whitney). Similarly, patients with familiar hypercholesterolemia showed significantly lower TTR serum levels than controls (median [IQR]; FH: 200.1 [179.8–222.3] μg/mL *vs.* Control: 223 [203.3–250.1] μg/mL; $p < 0.0001$; Mann–Whitney) but significantly higher levels than AMI-patients ($p < 0.0001$; Fig. 3A). Within the FH-group, TTR serum levels of FH-patients with a previous AMI were reduced compared to those patients without clinical evidence of CVD (median [IQR]; FH-CVD: 196.1 [170.5–215.5] μg/mL *vs.* FH-NoCVD: 205.4 [183.4–224.6] μg/mL). The Framingham risk score (FRS) of the FH-population was not correlated to total TTR levels. AMI-patients showed significant difference in TTR levels in relation to sex (median [IQR]; AMI-patients Men: 174.0 [163.7–190.5] μg/mL *vs.* Women: 137.1 [112.4–156.9] μg/mL, $p < 0.05$; Mann–Whitney). No significant modifications in TTR serum levels were detected in relation to age. Drug treatment (statin and ACEI) did not exhibit any effect on TTR serum levels in the studied population (Control, AMI and FH groups; Supplemental Fig. 2A and B).

Interestingly when using a commercial ELISA to measure TTR levels in serum, unlike results obtained with proteomics, no differences were observed in diabetes and dylipemia (Figs. 3B and 1C). There were no differences either in TTR serum levels in patients with high-risk CRP levels.

TTR levels were followed in AMI-patients up to 96 h after admission (Fig. 3C) showing a progressive and significant decrease with time in the majority of patients (72% of patients) (median [IQR]; t = 72–96 h: 110.0 [86.4–132.3] μg/mL *vs.* t = 0: 168.5 [147.8–188.3] μg/mL, $p < 0.001$ and *vs.* t = 8 h: 157.8 [149.9–188.3] μg/mL, $p < 0.001$; and *vs.* t = 24 h: 146.5 [127.1–163.4] μg/mL, $p < 0.05$; Mann–Whitney). CRP levels were also measured showing significantly higher levels at 24 h and 72–96 h than at admission and 8 h post-admission (median [IQR]; t = 0: 1.0 [1.0–12.2] mg/L *vs.* t = 24 h: 24.7 [19.8–46.6] mg/L, $p < 0.0001$; *vs.* t = 72–96 h: 33.0 [14.6–69.8] mg/L, $p < 0.0001$; and t = 8 h: 7.3 [1.0–14.3] mg/L *vs.* t = 24 h, $p < 0.0001$; *vs.* t = 72–96 h, $p < 0.001$; Mann–Whitney). TTR and CRP serum levels were negatively and significantly correlated up to 72–96 h in AMI-patients ($r = -0.512$, $p < 0.05$).

There was an apparent discrepancy in the results obtained in the 2-DE analysis and the quantification of TTR by a commercial ELISA, therefore we decided to further investigate this discrepancy by analyzing the distribution of different forms of TTR.

3.4. TTR distribution in serum fraction

To expand the proteomic studies, proteins were separated by 2-DE in a p*I* range between 4.7 and 5.9. Under these conditions, besides the TTR spot detected at 42 kDa, we found a series of 3 spots at an apparent molecular mass of 14 kDa (that corresponds to TTR monomer) and a p*I* range between 5.0 and 5.6 (denoted as 1, 2, and 3 in Supplemental Fig. 3A) were detected. As shown in Supplemental Fig. 3B, western blot analysis for total TTR in serum samples analyzed by 1D-electrophoresis (15% PAGE-SDS gels), under reducing conditions and immunodetected with a monoclonal antibody against TTR, revealed three bands. One had approximately 14 kDa that corresponds to the monomeric form (mTTR), a second band had an apparent mass of 28 kDa, that agrees with expected molecular mass of the TTR dimmer (dTTR), and a third very faint band of approximately 42 kDa corresponding to trimeric TTR (tTTR). When samples were run under non-reducing conditions a faint band of 56 kDa, corresponding to the tetrameric form of TTR (ttTTR), was also detected. Dimeric, trimeric and tetrameric forms of the protein showed decreased intensities in AMI-patients, being in some cases nearly undetectable.

3.5. TTR distribution in HDL fraction

Because HDL transports TTR in serum we analyzed by 2-DE the total HDL fraction in subgroups of FH-patients with and without CVD and control subjects ($N = 10$/group). Monomeric TTR (mTTR) was identified (spot of 14 kDa and a p*I* of 5.6) in the HDL fraction of all the analyzed groups. In 60% of the cases a 1.9-fold decrease in the mTTR spot intensity was observed when FH-CVD patients were compared to controls (Fig. 4A). This decrease in mTTR intensity was

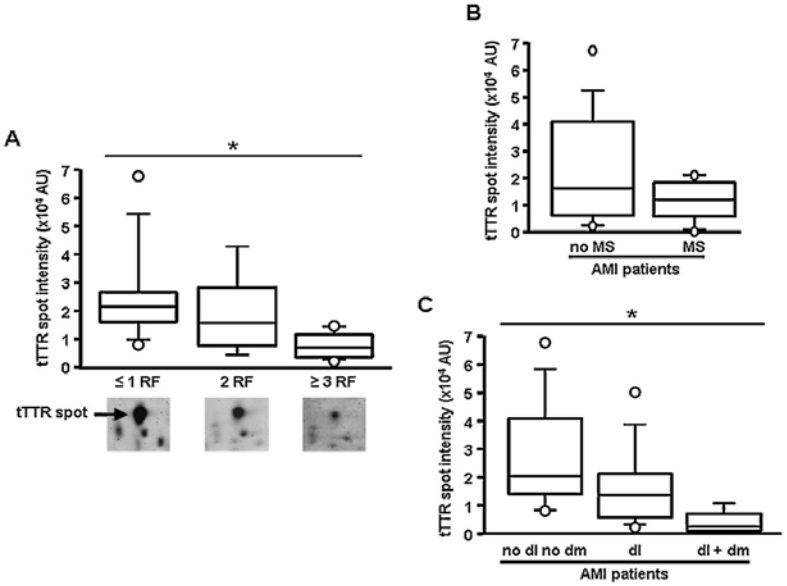

Fig. 1. (A) Representative image of TTR spot in AMI-patients and box plot diagram showing tTTR spot intensity in relation to the presence of different number of cardiovascular risk factors (RF) ($p < 0.05$ Kruskal–Wallis test). (B) Box plot diagram (median [IQR]) showing the tTTR spot intensity in AMI-patients with and without metabolic syndrome and (C) AMI-patients with dyslipemia (dl) and diabetes (dm) as cardiovascular risk factors (*$p < 0.05$ Kruskal–Wallis test).

associated with an increase in the Framingham risk score (FRS) in the FH-CVD patients when compared to controls, median [IQR] of FRS was for FH-CVD: 12 [10–19] and for controls: 6 [4–7] ($p < 0.05$; Mann–Whitney; Fig. 4B). No relationship was observed when the same analysis was performed with CRP levels (Fig. 4C).

Western blot analysis of the HDL fraction (control samples) confirmed the presence of only the 14 kDa band, mTTR (Supplemental Fig. 4A). The same results were obtained when samples were run under non-reducing conditions. In addition, when we analyzed separately the HDL2 and HDL3 fractions, mTTR was only detected in the HDL3 fraction. Similarly, by proteomic analysis mTTR was not detected in the HDL2 fraction, whereas the HDL3 fraction resolved three spots of 14 kDa when analyzed by 2-DE (Supplemental Fig. 4B).

4. Discussion

In this study we have investigated TTR in a population with acute manifestation of cardiovascular disease, *de novo* myocardial infarction, and in a high cardiovascular risk population with familiar hypercholesterolemia. We report that transthyretin shows important changes in both serum and HDL proteomes of AMI- and FH-patients when compared to controls. Here we demonstrate for the first time in the literature the inverse relationship between

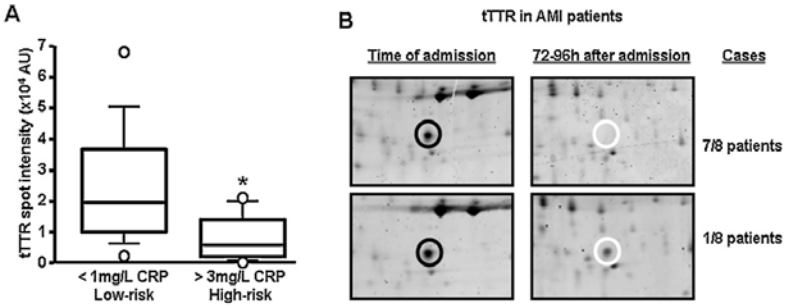

Fig. 2. (A) Box plot diagram (median [IQR]) showing (A) showing tTTR spot intensity in AMI-patients with CRP serum levels <1 mg/L and >3 mg/L at the moment of admission ($p < 0.05$ Mann–Whitney test). (B) Representative image comparing the intensity of tTTR in AMI-patients at the time of admission and 72–96 h afterwards. Notice that in most of the patients analyzed tTTR spot is not apparent after 72–96 h of the time of admission.

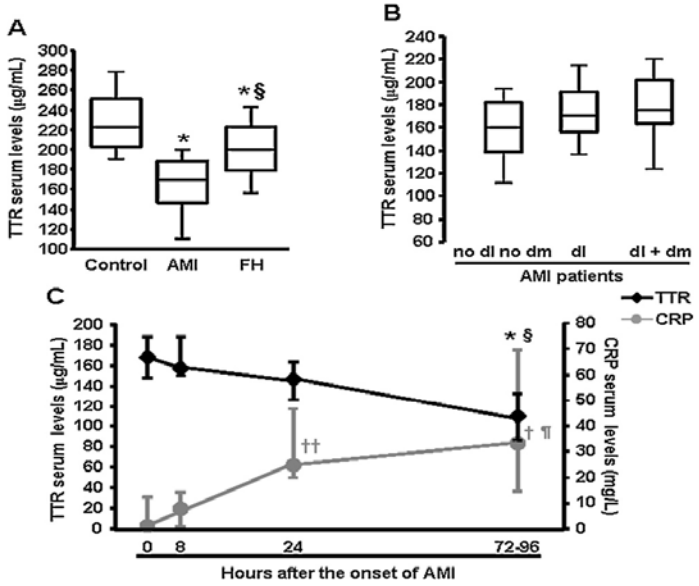

Fig. 3. Serum TTR levels (µg/mL) in AMI-patients and healthy donors measured by a commercial ELISA. (A) Box plot diagram (median [IQR]) showing serum TTR levels in the control, AMI (at the moment of admission), and FH groups (control: $n = 60$; AMI $n = 39$; FH $n = 100$). TTR levels are significantly decreased in AMI-patients within the first 6 h after the onset of the event and in FH-patients when compared to controls (*$p < 0.0001$; Mann–Whitney). TTR levels in FH-patients were significantly higher than in AMI-patients (§$p < 0.0001$; Mann–Whitney). (B) Box plot diagram (median [IQR]) showing serum TTR levels in AMI-patients with and without diabetes and dyslipemia. There were no significant variations between AMI-patients having or not dyslipemia and diabetes. (C) Time course diagram of serum TTR and CRP levels in AMI-patients between 0 and 96 h after the admission time. Values for each time point are given as median [IQR] (*$p < 0.001$ $t = 72$–96 h *vs.* $t = 0$ and *vs.* $t = 8$ h; §$p < 0.05$ $t = 72$–96 h *vs.* $t = 24$ h for TTR. ††$p < 0.0001$ $t = 24$ h *vs.* $t = 0$ and *vs.* $t = 8$ h; ¶$p < 0.0001$ $t = 72$–96 h *vs.* $t = 0$; †$p < 0.001$ $t = 72$–96 h *vs.* $t = 8$ h for CRP; Mann–Whitney).

TTR trimer levels after an AMI and the clustering of cardiovascular risk factors. Indeed, AMI-patients with metabolic syndrome show reduced levels of tTTR intensity in serum. The reduction in tTTR is particularly evident in patients with both, diabetes and dyslipemia, who show significant lower tTTR levels than the rest of AMI-patients. In our study the association of tTTR and cardiovascular risk factors highlights the relevance of the microheterogeneity of TTR and its implications in cardiovascular disease. In fact a specific form of TTR, S-sulfated TTR, has been related to myocardial infarction [14]. Besides the association between tTTR and the presence of cardiovascular risk factors, we have detected decreased intensity of tTTR in AMI-patients with higher levels of CRP at the moment of admission. The comparison with CRP, a well established inflammatory marker, was performed as a proof of concept and to validate the changes detected in tTTR. In fact, an association between TTR and CRP was not seen when total TTR serum levels were analyzed, highlighting the importance of the specific tTTR form rather than total TTR levels in the acute inflammatory response process. Even though TTR mainly exists as mTTR in serum, its biological activity as carrier of thyroid hormones and RBP4 is only achieved when it exists as homotetramer. In fact, thyroxine stabilizes the TTR tetramer, preventing the dissociation required to form the monomers and non-tetrameric oligomers [15,16]. The TTR homotetramer was not detected in the 2-DE analysis as experiments were carried out under reducing conditions.

Because of the detected changes in the TTR proteomic profile in association to cardiovascular risk factors we evaluated if available conventional assays, such as ELISA, were able to identify those TTR changes. Along with the detected changes in the TTR proteomic profile, we have found that total serum TTR levels are decreased early after an acute new-onset myocardial infarction. In agreement with the changes we have described within the first 6 h after the onset of the event, reduced total TTR levels have been related to acute phase response processes associated with inflammation [17] and in Type 1 diabetes [8]. Our study demonstrated that, after the early drop, AMI-patients show a progressive decrease in total TTR levels, reaching their lowest value 72–96 h after the time of admission. This decrease at 72–96 h agrees with the loss of tTTR spot in the serum proteome. In the time course analysis there is a concomitant increase in CRP and decrease in TTR serum concentrations. Indeed, the time point with higher levels of CRP comes along with the loss of tTTR spot, showing that changes in the specific trimeric form are representative of the inflammatory response process. Our present study design, however, does not allow risk prediction. In a previous study, the use of both CRP and TTR values has been postulated for the early diagnosis and follow-up of postoperative infection [18].

Although available ELISA depicted changes in TTR levels among the studied population, this method was not able to discriminate upon differences in cardiovascular risk factors, probably because of the lack of specificity in the analysis of TTR forms. Indeed by using proteomic approaches we are able to independently analyze different protein forms that are mixed when total levels are analyzed by available ELISA quantification methods.

In our study we have also found decreased TTR levels in high risk FH-patients when compared to controls. Interestingly, TTR levels in FH-patients are below control levels but higher than those found in AMI-patients, highlighting the fact that higher TTR changes are associated to higher risk and to the precipitation of an acute event.

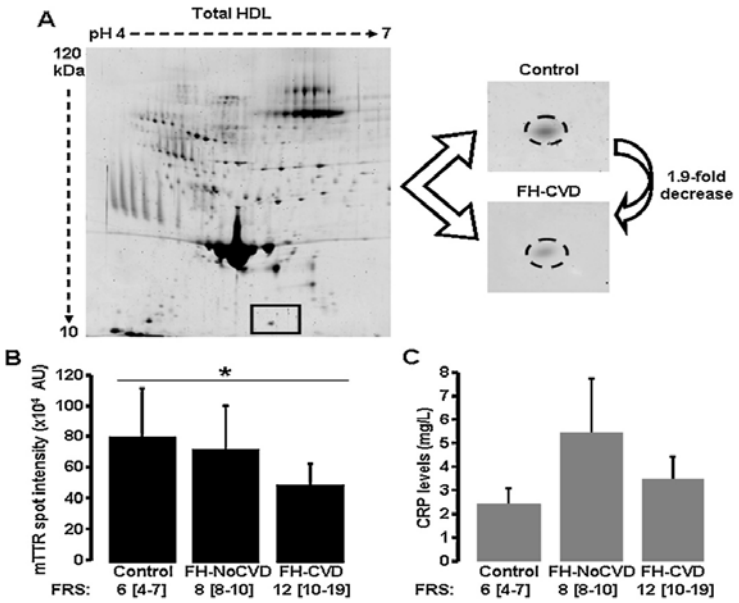

Fig. 4. (A) Representative 2-DE image of human HDL: *pI* range between 4 and 7, and 12% SDS-PAGE gels. Enlarged area of mTTR spot in control and FH-CVD. (B) Bar diagram of mTTR intensity in control, FH-NoCVD and FH-CVD (values expressed as mean ± SEM). There is a 1.9-fold reduction in mTTR intensity in FH-CVD patients. There is an inverse relation between mTTR intensity and Framingham risk score in FH-patients (*$p < 0.05$; Mann–Whitney). (C) Bar diagram of CRP levels (mg/L) in controls, FH-NoCVD and FH-CVD (values expressed as mean ± SEM).

The major known functional significance of TTR relates to the transport of RBP4 in blood and to the stability of the retinol-RPB4 complex [19]. It has been demonstrated that the retinol/RBP4 ratio is highly associated with the carotid intima media thickness (IMT), suggesting that the amount of retinol-free RBP4 (apo-RBP4) may have a specific role in the development of atherosclerosis [20]. To this respect, changes in circulating TTR levels might influence circulating retinol-free RBP4 and as consequence have an impact on cardiovascular risk. In addition, a decrease in TTR serum levels would affect thyroid hormone transport in blood and therefore result in an increase in the amount of circulating free thyroid hormones levels. In fact, decreased TTR levels have been related to increased levels of free thyroxine [21,22], which has been associated to a worse patients' survival after an AMI [23]. In addition, changes in thyroid homeostasis, the so called "euthyroid sick syndrome" have been related to severe illness such as myocardial infarction [24,25].

Interestingly, the HDL fraction transports the monomeric form of the protein (14 kDa) and decreased levels of this mTTR form are found in FH-patients with documented previous episode of cardiovascular disease when compared to controls. Moreover, a higher Framingham risk score was associated to the decrease in mTTR but not to total TTR levels quantified by ELISA, showing, once more, that one specific TTR form is more sensitive for cardiovascular disease than total TTR levels. This new finding indicates that in a high risk population there are changes in the composition of HDL particles that may contribute to a reduced cardiovascular protection. Ultracentrifugation methods for HDL isolation may have as a limitation the removal of the more weakly associated HDL proteins but as samples of different groups were processed at the same time and under the same conditions the loss of weakly associated proteins was proportional in all the groups. There are several studies that have associated changes in plasmatic levels of lipids and proteins within the first hours after an acute event and the assessment of patients' evolution. Indeed, persistent increased levels of plasma oxidized LDL after an AMI have shown to be a strong independent predictor of stent restenosis at a 6-month follow-up [26], and adiponectin levels after an AMI have shown to have a predictive value of future adverse cardiac events [27].

Our results indicate that HDLs carry mTTR and once in the plasma, various mTTR assemble together to form ttTTR in order to carry out its transport functions. Previous studies had reported that the presence of TTR on lipoproteins [28] represented approximately 1–2% of the total TTR in plasma [11]. Here we show that only mTTR is carried in HDL and that the HDL3 but not the HDL2 sub-fraction carries mTTR. Several studies associate differences in HDL particle remodelling and anti-atherogenic properties [29,30]. In fact, the potent anti-oxidative and anti-inflammatory activities of dense HDL3 particles are characterized by a proteome of distinct composition [31]. Therefore, the differential distribution of mTTR in the HDL fractions must be further analyzed in order to find out the possible implication of mTTR and its levels in the atheroprotective effects of HDL3 including anti-inflammatory, reverse cholesterol transport, and anti-oxidative functions. Even more, it is possible that lower TTR levels found in dyslipemia are due not to LDL levels but to the protein depletion of HDL particles, which would contribute to their differential properties against cardiovascular disease. Further studies comparing TTR levels in LDL particles and HDL2 and HDL3 fractions both in healthy controls and dyslipemic patients would help to prove this hypothesis.

In conclusion, in this study we have demonstrated that TTR values are reduced in patients with high cardiovascular risk. Changes in the specific trimeric form of TTR after an AMI, which are representative of the total serum levels of TTR, are highly significant. The inverse association of tTTR levels with the clustering of cardiovascular risk factors indicates the importance of TTR in cardiovascular risk. Besides the important reduction in tTTR in AMI-patients with diabetic dyslipemia, changes in mTTR levels in the HDL fraction of the high risk FH-patients also point to a protective effect of mTTR against cardiovascular disease.

The development of available tests to specifically measure ttTTR, tTTR, dTTR, and mTTR could indeed increase the specificity of cardiovascular risk evaluation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2012.02.024.

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SUPPLEMENTAL MATERIAL

Expanded materials and methods

Blood collection and sample preparation

Venous blood samples were collected to prepare serum that was aliquoted and stored at -80ºC. For proteomic studies, serum samples were prepared as previously described (1). Human HDL were obtained by ultracentrifugation from normocholesterolemic total serum fraction as described previously (2), HDL2 and HDL3 fractions were isolated with density gradients of 1.125 and 1.210, respectively. HDLs were analyzed to determine protein and cholesterol concentrations. We have used the ultracentrifugation isolation method in order to decrease the multitude of abundant plasma proteins that co-elute with HDL proteins with other methods such as gel filtration. The protein fraction was obtained by precipitation of 2mg of HDLs with pure ice cold acetone for 2 hours at -20ºC.

Biochemical analysis

C-reactive protein (CRP) levels were measured with a commercial assay (Roche) with a detection interval between 0.1 and 20 mg/L. When CRP levels were above the detection limit, samples were further diluted. Creatinine levels were analyzed using the alkaline picrate test. GOT and GPT levels were tested in Tris buffer by the measurement of Aspartate (>100 mM) and Alanine (>225 mM) concentration respectively. Creatinine, GOT, and GPT measurements were performed using a modular analytics D/P Hitachi (Roche). Lipids were measured by conventional enzymatic methods within 8 hours after the onset of the pain. The measurements within this time period are considered basal lipid levels. Total cholesterol levels were analyzed by the CHOD-PAP method and triglycerides by TOD-PAP (Boehringer-Mannheim). HDL- cholesterol levels were measured after manual lipoprotein precipitation with PEG 6000 method (Boehringer-Mannheim). If triglycerides were above 3.36 mmol/L, LDL- cholesterol levels were determined by centrifugation and if triglycerides were below 3.36 mmol/L, LDL-cholesterol levels were estimated by the Friedewald formula. T-troponin levels were measured with a commercial kit from Roche Diagnostics IVth generation and were analyzed with a Roche Diagnostics Elecsys 2010.

Proteomic analysis

Two-dimensional gel electrophoresis (2-DE): For analytical and preparative gels, respectively, a protein load of 120 µg and 300 µg protein of the urea/chaps/DTT serum extracts and urea/thiourea/chaps/DTT HDL extracts were applied to 18-cm dry strips (pH 4-7 linear range, GE Healthcare). Second dimension was resolved in 10% SDS-PAGE gels for serum and in 12% SDS-PAGE for HDL samples. 2-DE analysis of serum samples in AMI-patients was performed at two time points: the moment of admission (time zero) and 72-96 hours after the admission. In a subset of serum samples IEF was performed in a zoom of pH between 4.5 and 6.9 and 15% SDS-PAGE gels. Gels were developed by fluorescent staining (analytical gels) or comassie blue (preparative gels). For each independent experiment, 2-DE for protein extracts from controls and patients were processed in parallel to guarantee a maximum of comparability. Each 2-DE run was at least repeated twice. Analysis for differences in protein patterns was performed with the PD-Quest 8.0 (BioRad), using a single master that included all gels of each independent experiment. Each spot was assigned a relative value that corresponded to the single spot volume compared to the volume of all spots in the gel, following background extraction and normalization between gels.

Mass spectrometry analysis: Protein spots of interest were excised from 2-DE gels, washed (25mM Ambic), dehydrated (25mM Ambic/50% ACN followed by 100% ACN), dried, and enzymatic digested with one gel volume of sequence-grade modified porcine trypsin (Promega). Peptides from in-gel-trypsin digestion were desalted and concentrated by ZipTipU-C18 (Millipore) and mixed 1:1 with 5mg/mL α-cyano-4-hydroxy-cinnamic and spotted on a stainless steel mass spectrometry slide. Protein identification was performed by peptide-mass fingerprinting using an Ettan MALDI-TOF Pro (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer, GE-Healthcare) operated in delayed extraction/reflector mode. MALDI-generated mass spectra were internally calibrated using trypsin autolysis products, Ang III (angiotensin III), and ACTH (adrenocorticotropic hormone) peaks. The peptide masses were searched against the National Center for Biotechnology Information nonredundant mammalian database using ProFound™ and confirmed using a Mascot 2.3 search from Matrixscience selecting the SwissProt database. For the present study, protein identification was based on the measurement with a Mascot score higher than 55 and

minimum coverage of 20%. Minimal expectation for valid identification was < 0.005 and $p < 0.05$.

Western Blot Analysis

Sample extracts were resolved by SDS-PAGE, under reducing and no reducing conditions, and electrotransferred to PVDF (Polyvinylidene Difluoride) membranes in semi-dry conditions (Semi-dry transfer system, BioRad). Protein detection was performed using a rabbit polyclonal antibody (PAb) against TTR (FL-147, 1:1000 dilution, Santa Cruz Biotechnology) combined with the Dye Double Western Blot kit (Invitrogen). Band fluorescence was determined with Typhoon 9400 (GE Healthcare) and band quantification was performed using ImageQuant TL v7.01. software (GE Healthcare). Protein load was normalized using total protein fluorescent signal.

Quantification of TTR serum levels

Serum Transthyretin was determined by a double antibody sandwich enzyme-linked immunosorbent assay (DunnLab) with a detection limit of 3.13 ng/mL. Samples from AMI-patients were analyzed at the moment of admission (time zero) and at different time periods after the admission time (8, 24, 72 and 96 hours). The intra-individual variability of the assay assessed in 20 samples measured in three independent assays was of 9% ± 6%.

Statistical analysis

Data are presented as median [IQR] unless stated. N indicates the number of subjects tested. Statistical analysis was performed with Stat View 5.0.1 software. Statistical differences between groups were analyzed by the non-parametrics Mann-Whitney or Kruskal-Wallis tests, or ANOVA single factor or including covariates, Student´s t-test, as indicated. Correlations between variables were determined by single and multiple regression models. A p value ≤ 0.05 was considered significant.

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Legends to supplemental figures

Supplemental Figure 1. Representative 2-DE image of human serum sample after immunodepletion of six high abundant proteins (albumin, transferrin, haptoglobin, antytripsin, IgGs and IgAs) in a pI range between 4 and 7, and in 10% SDS-PAGE gels. The tTTR region is marked with a black circle, and an enlarged area of tTTR is shown.

Supplemental Figure 2. Serum TTR levels (μg/mL) in AMI-patients and healthy donors measured by a commercial ELISA. Box plot diagrams (median [IQR]) showing serum TTR levels in the control, AMI (at the moment of admission), and FH groups (control: n = 60; AMI n = 39; FH n = 100). TTR levels show no significant modifications between individuals with and without statin treatment (A) or with and without ACEI (B).

Supplemental Figure 3. (A) Representative 2-DE image of human serum sample after immunodepletion of six high abundant proteins (albumin, transferrin, haptoglobin, antytripsin, IgGs and IgAs) in a pI range between 4.7 and 5.9 and in 15% SDS-PAGE gels. Three spots identified as mTTR are marked with a black rectangle and shown in an enlarged image. (B) Representative western blot for TTR in controls and AMI-patients in total serum after immunodepletion of six high abundant proteins. Under reducing conditions three bands are detected, 14, 28 and, 42 kDa, corresponding to the mTTR, dTTR, and tTTR, respectively. If samples are run under non reducing conditions ttTTR is also detected (56 kDa), mainly in control samples.

Supplemental Figure 4. (A) Representative western blot for TTR in total HDL, HDL2, and HDL3 fractions of control samples. In total HDL and HDL3 fraction only the 14 kDa band was detected. In HDL2 no mTTR was seen. The same results are obtained under reducing and non reducing conditions. (B) Representative 2-DE images of total HDL, HDL2, and HDL3 fractions. In total HDL only one mTTR spot of 14 kDa is detected, while in HDL2 no mTTR spots are apparent. In HDL3 proteome three mTTR spots of 14 kDa are identified.

SUPPLEMENTAL TABLE 1. Lipid values in control, AMI and FH groups.

	Serum levels (mg/dL)			*p*-value			
	Control	AMI	FH	ANOVA	C *vs.* AMI	C *vs.* FH	AMI *vs.* FH
Total Cholesterol	215.5 ± 5.4	204.9 ± 7.7	251.9 ± 7.6	<0.0001	NS	0.001	<0.001
HDL-Cholesterol	49.0 ± 1.8	44.3 ± 2.8	43.7 ± 1.3	<0.05	NS	<0.05	NS
LDL-Cholesterol	141.2 ± 4.8	130.5 ± 6.9	188.5 ± 7.4	<0.0001	NS	<0.0001	<0.0001
Trigycerides	129.0 ± 11.7	169.6 ± 12.3	109.5 ± 5.7	<0.0001	<0.05	NS	<0.0001

NS = No significant.

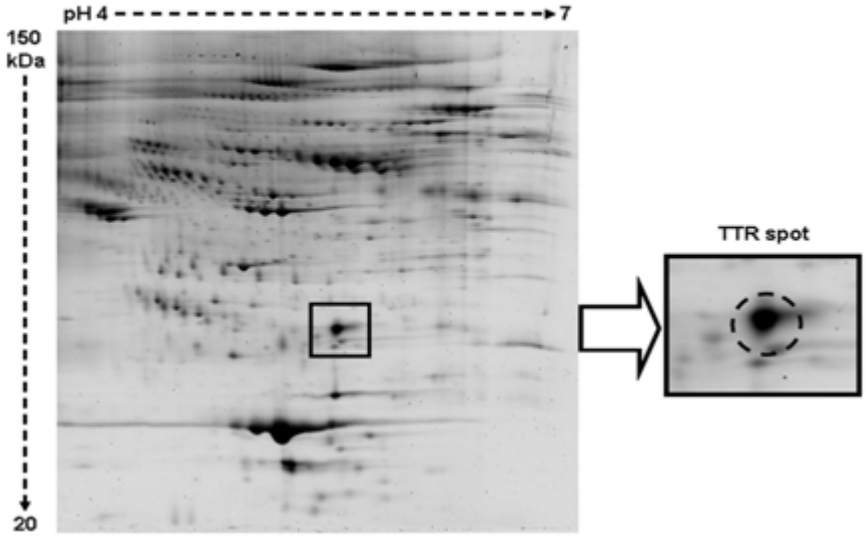
SUPPLEMENTAL TABLE 2. Effect of Statin and ACEI treatment in lipid parameters of AMI-patients and healthy donors.

	Control group			**AMI group**		
	No treated	Treated (statins ± ACEI)	*p*-value	No treated	Treated (statins ± ACEI)	*p*-value
Total Cholesterol*	219.1 ± 6.9	205.9 ± 7.9	NS	199.6 ± 8.1	214.5 ± 15.8	NS
HDL-Cholesterol*	49.4 ± 2.1	47.7 ± 3.8	NS	43.7 ± 2.6	45.3 ± 3.5	NS
LDL-Cholesterol*	146.2 ± 6.0	127.7 ± 6.3	NS	126.3 ± 7.7	136.8 ± 13.2	NS
Trigycerides*	118.3 ± 10.2	155.6 ± 31.7	NS	168.6 ± 17.8	171.2 ± 22.7	NS

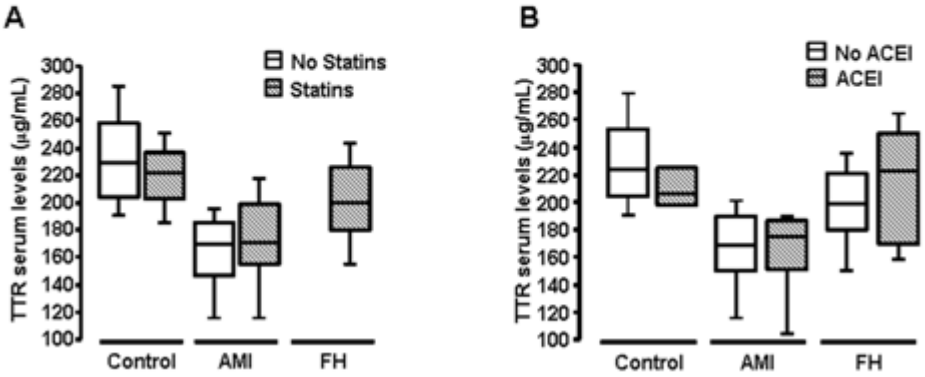
* Lipid levels given in mg/dL

ACEI = Angiotensin-converting enzyme inhibitors; NS = No significant.

Supplemental Figure 1



Supplemental Figure 2



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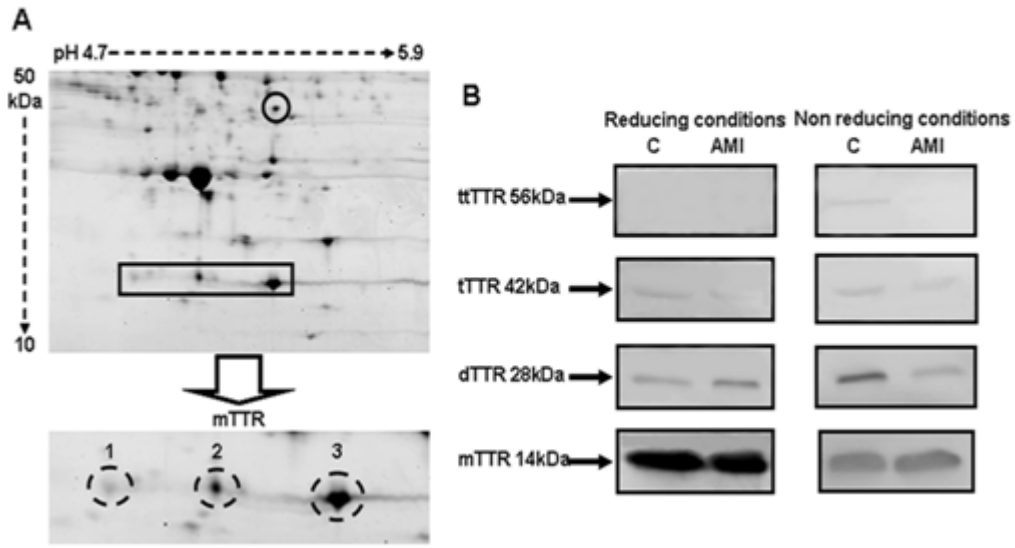
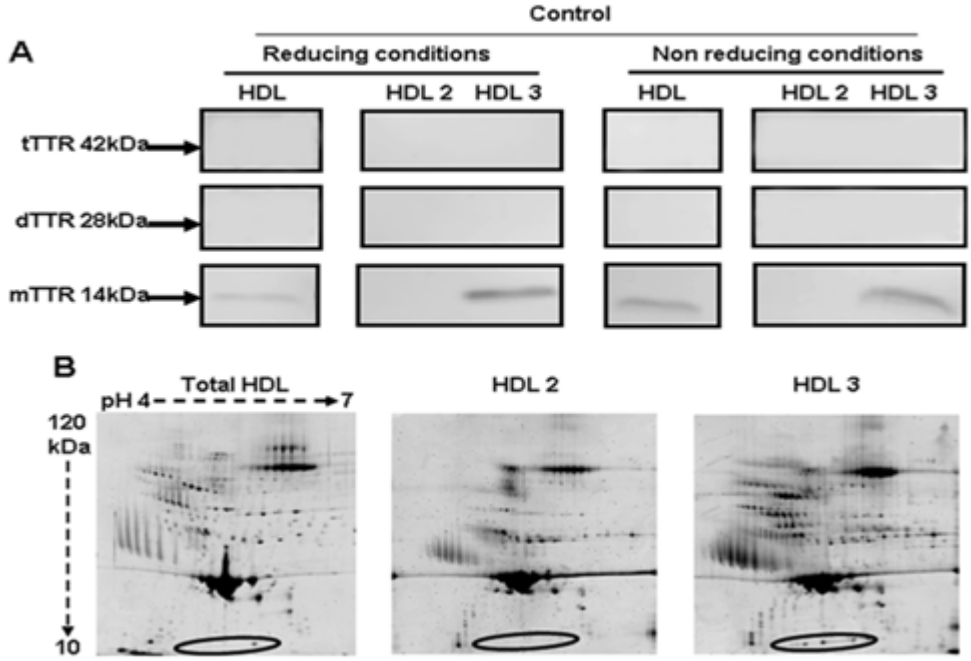


Supplemental Figure 4



ARTÍCULO TERCERO

"Lipocalin RBP-4 in acute myocardial infarction: effects on vascular function and inflammation"

Judit Cubedo * † ¶, Teresa Padró * ¶, Lina Badimon * † || ¶

*Cardiovascular Research Center (CSIC-ICCC), †CIBERobn, ||Autonomous University of Barcelona, ¶Biomedical Research Institute Sant Pau (IBB-Sant Pau). Barcelona, Spain.

Enviado

Resumen resultados

Cambios en el perfil sérico de la RBP4 en la fase temprana tras un AMI

Mediante 2-DE se identifica la RBP4 como dos formas con un pI de 5.4 y diferente masa molecular (23.01 y 22.78kDa). En la fase temprana tras el AMI se ve una disminución marcada en la intensidad total de las dos formas de RBP4, siendo esta disminución más marcada en la forma de 22.78kDa que llega a desaparecer en el 37% de los pacientes. A las 72-96h del ingreso se sigue viendo una disminución de los niveles de RBP4. Esta disminución en los niveles de RBP4 se valida mediante ELISA. Se ve una correlación positiva entre los niveles de RBP4 y los de su transportador, la TTR.

Efectos agudos de la RBP4 sobre la función vascular y la inflamación

El tratamiento con RBP4 induce un aumento en la expresión de eNOS en células endoteliales, pero no modifica la expresión de iNOS en células musculares. Además el RBP4 también induce un incremento dosis-dependiente en la expresión de COX2 en células musculares lisas lo que conlleva a un incremento en la liberación de prostaglandina I_2 (PGI_2).

Lipocalin RBP-4 in acute myocardial infarction: effects on vascular function and inflammation

Cubedo et al: RBP4 in acute vascular events

By

Judit Cubedo * † ¶, Teresa Padró * ¶, Lina Badimon * † || ¶

From

*Cardiovascular Research Center (CSIC-ICCC), †CIBERobn, ||Autonomous University of Barcelona, ¶Biomedical Research Institute Sant Pau (IBB-Sant Pau). Barcelona, Spain.

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Correspondence to:

Prof. Lina Badimon
Cardiovascular Research Center, c/Sant Antoni MªClaret 167, 08025 Barcelona, Spain.
Phone: (34) 935565880. Fax: (34) 935565559.
E-mail: ***lbadimon@csic-iccc.org***

ABSTRACT

Retinol binding protein 4 (RBP4), a member of the lipocalin family of proteins, is an adipokine mainly secreted by the liver and adipose tissue involved in the development of insulin resistance. Clinically, serum RBP4 levels have been shown to correlate with a cluster of cardiovascular risk factors accompanying insulin resistance as part of the metabolic syndrome. Here we have investigated by proteomic analysis RBP4 as a potential marker of vascular damage in the acute phase of myocardial infarction.

Methods and results: RBP4 identification and characterization by bidimensional electrophoresis (2-DE), followed by mass-spectrometry (MALDI-TOF) depicted two spots with the same pI (5.4) and different Mw (23.01 and 22.78 kDa), showing decreased intensities in AMI-patients. Determination of total serum RBP4 levels by ELISA showed that AMI-patients (N=68) had significantly lower levels than the control group (N=99; P <0.0001). To evidence the functional effects of RBP4 in vascular cells, human endothelial cells (HUVEC) and human vascular smooth muscle cells (VSMC) were treated with RBP4. HUVEC showed a significant increase in eNOS expression, and VSMC showed a significant increase in prostaglandin I_2 release.

Conclusions: We show both by proteomic and quantitative studies that serum RBP4 levels are significantly decreased in AMI-patients, being the RBP4 spot with the lower molecular weight the most sensitive to show changes. Moreover, RBP4 controls vasodilator function in vascular cells, therefore its decrease signifies an impairment of vascular cell function. In AMI-patients reduced RBP4 levels indicate a systemic vascular function affectation.

Key Words: acute-myocardial-infarction, RBP4, proteomic studies, vasodilatation, inflammation.

INTRODUCTION

Several studies have helped to establish the adipose tissue as a dynamic endocrine organ that is critical for regulating metabolism in both health and disease. The adipose tissue exerts its regulation through the adipocyte-derived secreted proteins (adipokines).[1] Adipose tissue expansion contributes to obesity and by metabolic regulation links obesity to several health problems including increased risk of type 2 diabetes, fatty liver, and cardiovascular disease.[2-7] The lipocalin retinol-binding protein 4 (RBP4) has been recently described as one of those adipokines.[8] The best known function of RBP4 is to deliver retinol (vitamin A) to tissues, since it is the only specific transporter of retinol in the circulatory system.[9] However, RBP4 has been associated to different pathological situations as to the modulation of systemic insulin resistance[8,10] with changes in serum levels in diabetic[11] and pre-diabetic patients,[12] the regulation of blood pressure in newly diagnosed hypertensive women,[13] and correlation with the degree of carotid intima-media thickness (IMT).[14] Our hypothesis was that low levels of RBP4 could be associated to clinical conditions that curse with impairment of vascular function. Up to now, RBP4 changes in the early phase of acute vascular events had not been investigated.

Here we report for the first time that RBP4 levels are significantly reduced in patients with an acute new-onset myocardial infarction and that RBP4 is involved in the regulation on vascular cell vasodilator function.

MATERIALS AND METHODS

Study population

The study population comprised 68 patients with an acute new-onset myocardial infarction (AMI-patients; 53 men and 15 women; mean age: 62 ± 2 years) and a control group including 99 apparently healthy individuals free of any cardiovascular event (74 men and 25 women; mean age: 55 ± 1 years) who attended to a routine health check.

The AMI group were admitted with chest pain and suspected of ACS at the Emergency Room of Santa Creu i Sant Pau Hospital and included in the study following the previously described criteria.[15,16] At the emergency department, routine diagnostic procedures were applied to establish the onset of symptoms (i.e. description of chest pain, pulmonary edema, severe dyspnea, and syncope). In addition to the general patient history, clinical examination, 12-lead ECG, and laboratory tests were also run to characterize AMI-patients. All AMI-patients showed (1) typical chest pain lasting more than 30 minutes; (2) ST segment elevation >0.2 mV in at least 2 contiguous leads; (3) admission to the hospital within the first 6 hours after chest pain onset; (4) normal serum CK and CKMB levels at admission; (5) negative troponin T at admission (excluding subacute myocardial infarction); (6) sinus rhythm. Exclusion criteria were a previous documented or suspected myocardial infarction and antithrombotic treatment because of the AMI onset before arriving to the emergency room and time of blood collection.

Patients included in the study did not present further co-morbidities such as cancer, chronic infections, autoimmune diseases or thyroid hormones disorders.

Blood samples of AMI-patients were collected at the moment of admission within the first six hours after the onset of the event (defined as time zero) and 72-96h after the admission time.

The Ethics Committee of the Santa Creu i Sant Pau Hospital approved the project and the studies were conducted according to the principles of Helsinki's Declaration. All participants gave written informed consent to take part in the study.

Blood collection and sample preparation

Blood samples obtention**:** Venous blood samples of AMI-patients, before starting any medication, and control individuals were collected to prepare serum that was aliquoted and stored at -80ºC. For proteomic studies, serum samples were prepared as previously described.[15,16] In brief, the six most abundant serum proteins were depleted using a specific affinity cartridge with binding capacity for albumin, IgGs, IgAs, transferrin, α1 antytripsin and haptoglobin (Multiple Affinity Removal Spin Cartridge, Agilent Technologies) as reported by the providers.

Proteomic analysis

Two-dimensional gel electrophoresis (2-DE): For analytical and preparative gels, respectively, a protein load of 120 µg and 300 µg protein of the urea/chaps soluble extracts was applied to 18-cm dry strips (pH 4-7 linear range, GE Healthcare). Second dimension was resolved in 10% SDS-PAGE gels. Gels were developed by fluorescent staining. For each independent experiment, 2-DE for protein extracts from controls and patients were processed in parallel to guarantee a maximum of comparability. Each 2-DE run was at least repeated twice. Analysis for differences in protein patterns was performed with the PD-Quest 8.0 (BioRad), using a single master that included all gels

of each independent experiment. Each spot was assigned a relative value that corresponded to the single spot volume compared to the volume of all spots in the gel, following background extraction and normalization between gels.

Mass spectrometry analysis: Proteins were identified after in-gel tryptic digestion and extraction of peptides from the gels pieces, as previously described,[15,16] by matrix - assisted laser desorption/ionization time-of-flight (MALDI-TOF) using an AutoFlex III Smartbeam MALDI-TOF/TOF (Bruker Daltonics). Samples were applied to Prespotted AnchorChip plates (Bruker Daltonics) surrounding the calibrants provided on the plates. Spectra were acquired with flexControl on reflector mode, (mass range 850 - 4000 m/z, reflector 1: 21.06 kV; reflector 2: 9.77kV; ion source 1 voltage: 19 kV; ion source 2: 16.5kV; detection gain 2.37x) with an average of 3500 added shots at a frequency of 200 Hz. Each sample was processed with flexAnalysis (version 3.0, Bruker Daltonics) considering a signal-to-noise ratio over 3, applying statistical calibration and eliminating background peaks. For identification, peaks between 850 and 1000 m/z were not considered as in general only matrix peaks are visible on this mass range. After processing, spectra were sent to the interface BioTools (version 3.2, Bruker Daltonics) and MASCOT search on Swiss-Prot 57.15 database was done (Taxonomy: Homo Sapiens, Mass Tolerance 50 to 100, up to 2 trypsin miss cleavages, Global Modification: Carbamidomethyl (C), Variable Modification: Oxidation (M)). Identification was accepted with a score higher than 56.

Quantification of RBP4 serum levels

Systemic total RBP-4 concentration was determined in serum by competitive sandwich enzyme-linked immunosorbent assay (AssayPro) using immobilized policlonal antibodies, as described by the providers. The detection limit of the assay was

0.195µg/ml. Samples from AMI-patients (N=68) were analyzed at the moment of admission and compared to the control group (N=99).

Cell culture and RBP4 treatment

Cryopreserved human umbilical endothelial cells (HUVEC) were grown in the recommended media (EBM-2) containing 2% FBS, hydrocortisone, hFGF-B, VEGF, R3- IGF -1, hEGF, heparin, ascorbic acid, gentamycin and amphotericin B, as supplied by the manufacturer (Lonza, UK) and experiments were performed in passages 3-6.

Primary human vascular smooth muscle cells (VSMC) were obtained by the explant technique from human non-atherosclerotic coronary arteries of hearts obtained from heart transplantation surgery and cultured as described previously.[17] VSMC were cultured in M199 medium containing 20% fetal bovine serum (FBS) and used at passages 6.

After the induction of quiescent status by growth factor/FBS withdrawal, HUVEC and VSMC were treated with/without RBP4 (5 and 7.5µg/mL) for 48h and 1.5h, 3h, and 24h respectively. Each experiment was performed six times.

NOS and COX2 expression

Cells were lysated and RNA was obtained using the RNeasy kit (QIAGEN). Cyclo-oxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) mRNA levels were analyzed by real-time PCR (7900HT Sequence Detection System of ABIPRISM; Applied Biosystems) in VSMC (COX-2 and iNOS) and in HUVEC (eNOS). TaqMan gene expression assays were obtained

from Applied Biosystems. Threshold cycle (Ct) values were determined and RNA amounts were normalized using the TATA binding protein (TBP) gene.

Quantification of prostaglandin I_2 in the culture media

Prostaglandin I_2 levels secreted by VSMC were analyzed with an EIA assay for 6-keto Prostaglandin $F_{1\alpha}$ (Cayman) in three different dilutions of the samples.

Statistical analysis

Data are expressed as median and interquartile range [IQR]. N indicates the number of subjects (proteomic and ELISA analysis) or experiments (cell culture experiments) tested. Statistical analysis was performed with Stat View 5.0.1 software. The Kruskal-Wallis test was used in multiple comparisons and Mann-Whitney test was used for comparison between groups. Wilcoxon test was used to analyze differences between serial measurements of RBP4 levels at different times after the AMI onset. A *P*-value $\leq$ 0.05 was considered significant.

RESULTS

Clinical characteristics of the study population

The characteristics of AMI and control groups are outlined in Table 1.

RBP4 proteomic profile in AMI-patients

RBP4 (P02753) was identified in serum by 2-DE and MALDI-TOF mass spectrometry as two spots with a pI of 5.4 and different apparent molecular masses, 23.01 and 22.78 kDa (Figure 1). Comparing with healthy volunteers AMI-patients showed an average reduction of 2-fold in the total RBP4 intensity (Median [IQR]; AMI: 16 [11-20] AU *vs.*

C: 33 [31-36] AU; $P < 0.05$; Figure 2A). The mean intensity of the spot with higher molecular mass (spot 1) showed a 1.8-fold decreased ($P < 0.001$) in AMI-patients when compared to the controls. The spot with lower molecular mass (spot 2) showed a 2.6-fold decrease ($P < 0.001$) in AMI-patients reaching undetectable levels in 37% of the cases (Figure 2B). At 72-96h after admission there was a 1.7-fold decrease in the intensity of both RBP4 spots (Figure 3), being significant the decrease in the spot number 1 ($P < 0.05$; Wilcoxon test).

RBP4 serum levels

To validate the proteomic findings total RBP4 levels were measured in serum by a commercial ELISA in both AMI-patients at time zero and in controls. Serum RBP4 levels were significantly decreased in AMI-patients (Median [IQR]; AMI: 29.7 [25.1-36.1] μg/mL *vs.* Control: 43.5 [39.5-47.1] μg/mL; $P < 0.0001$; Mann-Whitney; Figure 4). RBP4 levels were significantly and positively correlated with transthyretin levels, an acute phase marker with reduced levels due to cardiovascular risk[16] in the studied population (Spearman correlation r^2=0.476; $P < 0.0001$; Supplemental Figure 1).

Due to the age difference between AMI-patients and controls, differences in RBP4 levels within the control population were analyzed. No age related effects on RBP4 levels were detected (Table 2). No gender differences in RBP4 levels were detected in AMI and control groups. No significant modifications in RBP4 serum levels were detected in relation to the presence of risk factors.

RBP4 effects on HUVEC and VSMC

RBP4 induced an up-regulation of the vasodilator eNOS isoform in HUVEC (Median [IQR] fold change at 7.5μg/mL vs. 0μg/mL: 1.6 [1.3-2.1]; $P < 0.05$; Mann-Whitney;

Figure 5A). In VSMC, RBP4 did not induce any change in pro-inflammatory iNOS expression (Figure 5B); however, RBP4 induced an increase in the expression of the early gene COX2 in VSMC in a dose-dependent manner after 1.5h of treatment ($P <$ 0.05 Kruskal-Wallis; Median [IQR] fold change vs. 0μg/mL: 1.2 [1.2-1.3] and 1.7 [1.5-1.7] for 5μg/mL and 7.5μg/mL respectively $P < 0.05$ Mann-Whitney; for 7.5μg/mL *vs.* 5μg/mL $P < 0.05$ Mann-Whitney; Figure 6A), that induced a significant increase in the release of the vasodilator and antiplatelet eicosanoid PGI_2 ($P < 0.05$; Kruskal-Wallis; $P < 0.05$; Mann-Whitney at 7.5μg/mL *vs.* 0 and 5μg/mL; Figure 6B).

DISCUSSION

Several evidences support a potential role of RBP4 in pathways linking adiposity with atherosclerosis. RBP4 levels have been correlated with subclinical inflammation in childhood obesity,[18] and RBP4 mRNA expression in adipose tissue has been associated with inflammatory markers.[10] Over the last years, several studies have related this new adipokine with different cardiovascular risk factors such as type 2 diabetes, obesity, metabolic syndrome, or even advanced age.[19-22] Even more, RBP4 has been associated with components of the metabolic syndrome in men with coronary artery disease.[23] Measurement of RBP4 has been proposed to have a potential value as an acute phase reactant marker.[24,25] A recent study has revealed, both in, *in vitro* and *ex vivo* experimental procedures, a vasodilatory effect of RBP4 through an increase in nitric oxide, suggesting a relevant role of this adipokine on vascular regulation and function.[26] Therefore we hypothesized that RBP4 levels could be lower than normal in conditions of pathological vascular damage that usually curse with low levels of NO.

In this study we have found, both by proteomic analysis and by ELISA, that serum RBP4 levels are decreased in the early phase after an acute new-onset myocardial

infarction. To our knowledge this is the first study investigating RBP4 levels in patients in the early phase of acute myocardial infarction. Moreover, this reduction in the systemic circulation may have general effects in vascular cells since we also report for the first time that RBP4 induces eNOS expression on human endothelial cells, and PGI_2 release by smooth muscle cells without effects in iNOS. In the cell culture experiments physiological concentrations of RBP4 were used, similar to those used by Takebayashi and co-workers[26] who showed NO regulation by RBP4 *in vitro* by bovine aortic endothelial cells. Importantly, we have found that circulating levels of RBP4 change acutely under ischemic stress giving for the first time a clinical relevance to the vasodilator and anti-inflammatory RBP4 effects.

In our study, RBP4 levels in the control group are in agreement with previously reported values in large population studies,[27,28] levels that are considered to ensure an adequate vitamin A status.[29] However, there is some controversy on methodological approaches due to reports of very low levels in certain groups.[30] In agreement with our data on AMI-patients showing a decrease of RBP4 during stress, Basu et al[31] described decreased levels of RBP4 in type 1 diabetes patients and rightfully suggested that it may not be diabetes-specific but related to other mechanisms such as a response to stress, which can alter catabolic rates of retinol. In addition, reduced levels of RBP4 protein have been related to acute phase response processes associated with inflammation.[32]

Interestingly, our study demonstrates that a decrease in RBP4 is detected in the first six hours after the onset of an acute event. The serum proteome analysis has revealed the presence of two RBP4 forms that agree with the band profile of full-length RBP4 and C'terminal truncated RBP4 described in the western blot analysis of serum samples by Jaconi et al.[33] Interestingly, we have found that the RBP4 form with lower molecular weight is the most sensitive to changes due to acute AMI.

The main known function of RBP4 is the transport of retinol from the liver to the peripheral tissues.[34] To this aim RBP4 forms a complex with transthyretin (the RBP4-TTR complex) that abrogates glomerular filtration and the subsequent excretion through the kidney.[35] Recently, we have demonstrated that TTR levels are significantly decreased in the early phase after AMI.[16] Thus, the decrease in TTR comes along with a decrease in serum RBP4 levels probably due to an increase in free-RBP4 filtration through the kidney. Indeed, there was a significant correlation between RBP4 and TTR levels in the studied population (Supplemental Figure 1).

Because RBP4 is the specific transporter of retinol in blood to target tissues[34] a decrease in RBP4 levels may lead to a decrease in the efficiency of the retinol delivery to the tissues. Retinol is required to maintain immunity and epithelial turnover and therefore retinol deficiency could either directly disrupt epithelial integrity or indirectly increase susceptibility to oxidative stress.[36] In fact, decreased retinol plasma levels have been found during the first 48 hours after an AMI,[37] time window where we have found decreased RBP4 levels. This situation may lead to a decrease in the protection against stress of patients having lower levels of RBP4 in the early phase after the acute cardiovascular event, and even more to a deleterious effect of the decrease in RBP4 levels due to the loss of its vasodilatory properties.

In summary, we report that in the acute phase of AMI serum systemic levels of RBP4 are reduced and RBP4 proteomic profile altered. Because RBP4 shows a vasculoprotective homeostatic function the reduced systemic levels induced by an ischemic event have deleterious effects by predisposing to systemic vascular dysfunction. Whether this reduction in RBP4, in addition to a pathophysiological role in the vasculature, may serve as a biomarker of ischemia remains to be determined.

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Conflict of Interest: none declared.

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LEGENDS

Figure 1. Representative 2-DE images of human serum sample after immunodepletion of six high abundant proteins (albumin, transferrin, haptoglobin, antytripsin, IgGs and IgAs) in a pI range between 4 and 7, and in 10% SDS-PAGE gels. The RBP4 region is marked with a circle, and an enlarged area and a 3-D image of RBP4 spots are shown.

Figure 2. (A) Bar diagram (median [IQR]) of total RBP4 spot intensity in AMI-patients and control individuals. AMI-patients showed decreased total RBP4 spot intensity (* $P < 0.05$; Mann-Whitney). (B) Representative image of RBP4 spots (denoted as number 1 and 2). The intensity of spot 1 is decreased in 84% of AMI-patients, and the intensity of spot 2 is decreased in 89% of AMI-patients, reaching undetectable levels in 37% of the patients.

Figure 3. Representative images of RBP4 spots in AMI-patients at the moment of admission (t=0) and 72-96h after; and bar diagram (median [IQR]) showing the decrease in RBP4 spots intensity 72-96h after the moment of admission (* $P < 0.05$; Wilcoxon).

Figure 4. Box plot diagram (median [IQR]) of total RBP4 serum levels in AMI-patients and control individuals. AMI-patients showed decreased total RBP4 levels (* $P < 0.0001$; Mann-Whitney).

Figure 5. Line diagram (median [IQR]) of gene expression after treatment with/without different concentrations of RBP4 (0, 5 and 7.5μg/mL) showing changes in (A) eNOS expression in HUVEC (*P < 0.05 *vs.* 0μg/mL; Mann-Whitney) and (B) no significant changes in iNOS expression after VSMC treatment.

Figure 6. Bar diagram (median [IQR]) showing (A) changes in COX2 expression in VSMC after different times (1.5 and 3h) of RBP4 treatment and (B) line diagram showing the significant increase in PGI_2 levels secreted by VSMC (*P < 0.05 *vs.* 0μg/mL and †P < 0.05 *vs.* 5μg/mL; Mann-Whitney).

TABLE 1. Background description of AMI and control groups.

	Control	AMI
N	99	68
Age (mean ± SEM)	55 ± 1	62 ± 2
Females / Males	25 / 74	15 / 53
Risk Factors, N (%)		
Tobacco smoking	24	49
Hypertension	15	47
Dyslipemia	30	51
Diabetes Mellitus	1	22
Obesity	14	25

TABLE 2. RBP4 levels in age quartiles of healthy individuals.

	Age quartiles*			
	<48	48-55	55-62	62-79
RBP4 (μg/mL)	44.9 ± 1.5	45.1 ± 1.6	41.8 ± 1.7	45.0 ± 1.6

* No differences between quartile groups were observed.

Figure 1

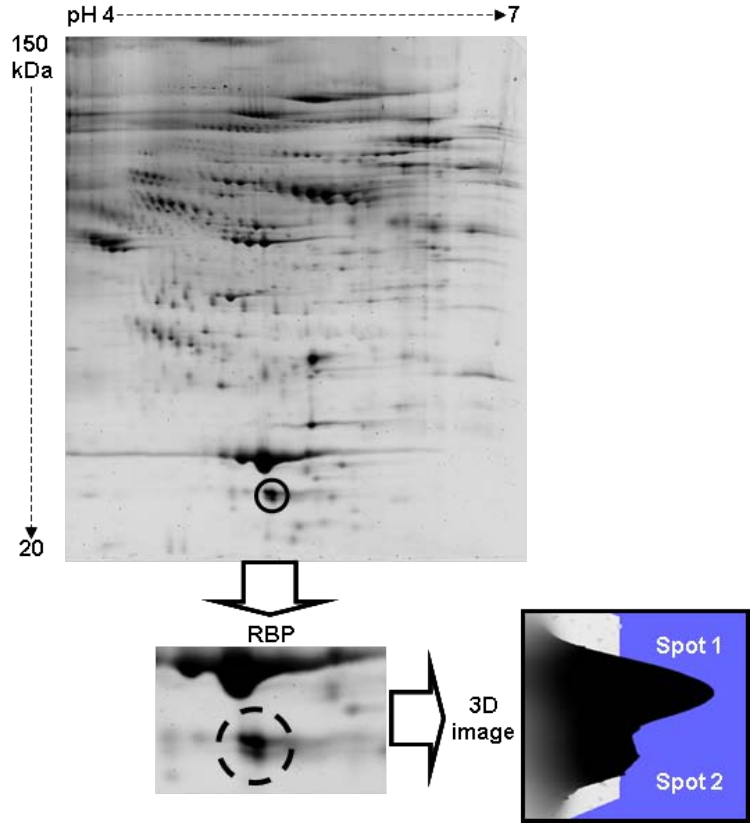


Figure 2

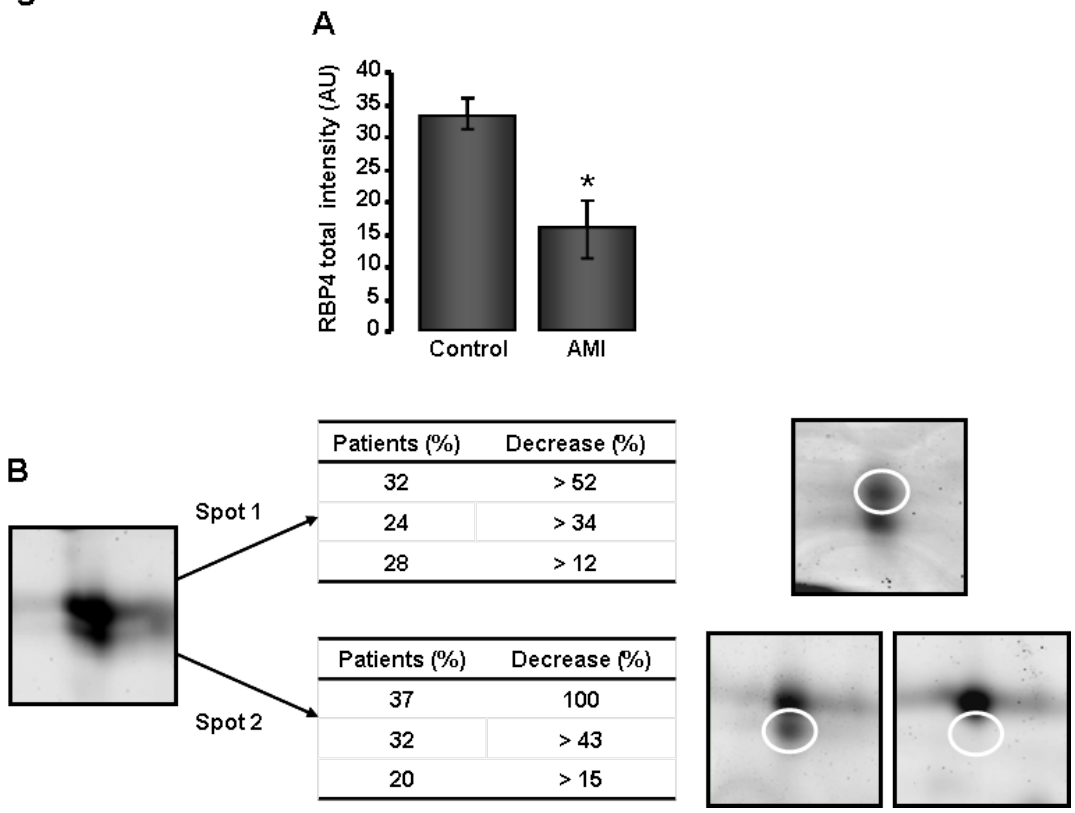

A
RBP4 total intensity (AU)
40
35
30
25
20
15
10
5
0
*
Control
AMI
B
Spot 1
Spot 2
Patients (%)
Decrease (%)
32
> 52
24
> 34
28
> 12
Patients (%)
Decrease (%)
37
100
32
> 43
20
> 15

Figure 3

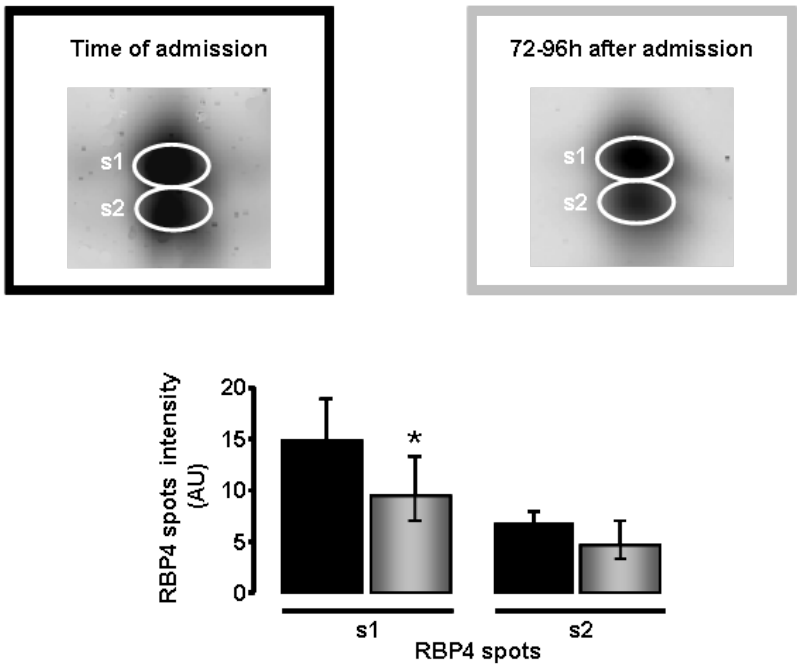

Time of admission
s1
s2
72-96h after admission
s1
s2
RBP4 spots intensity (AU)
20
15
10
5
0
*
s1
s2
RBP4 spots

Figure 4

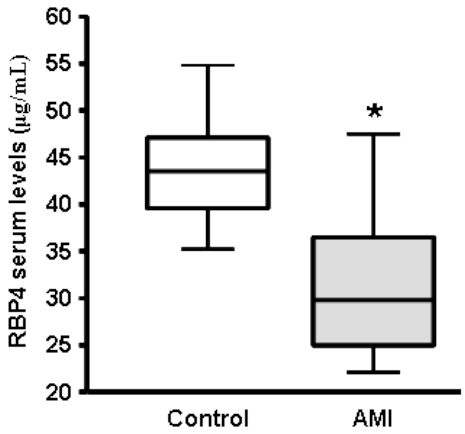


Figure 5

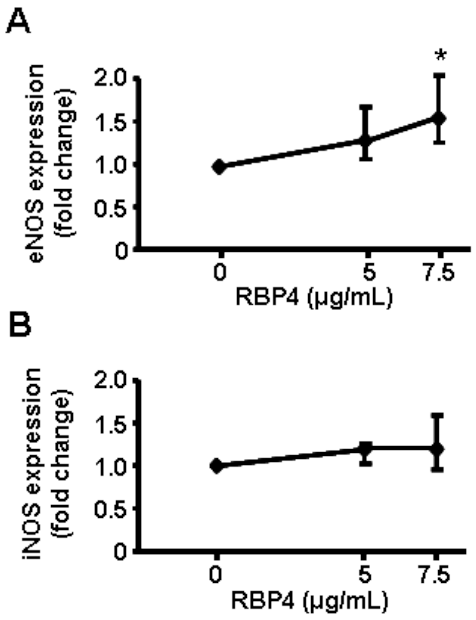
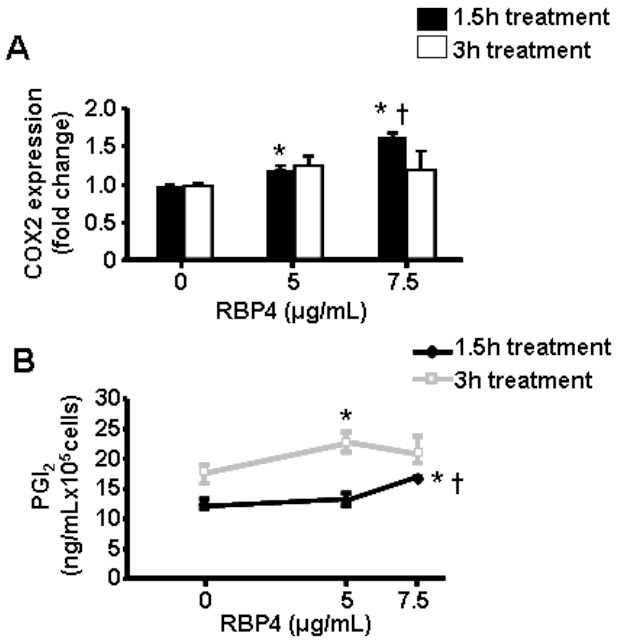
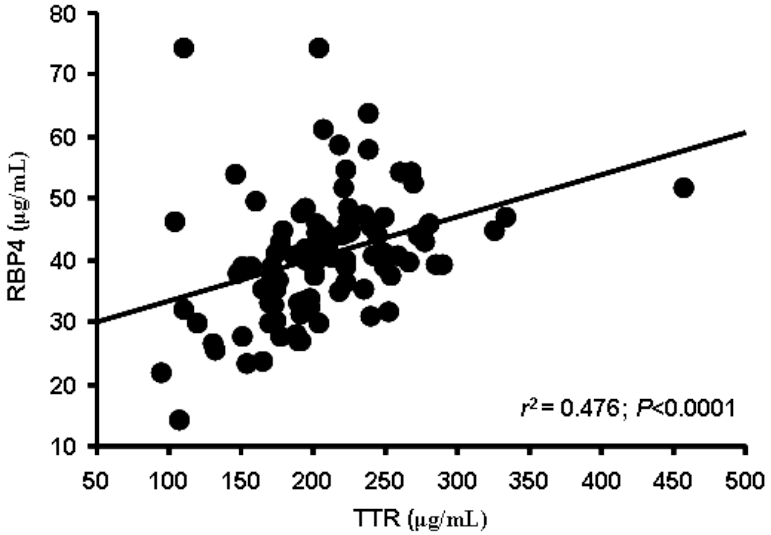

A
eNOS expression (fold change)
2.0
1.5
1.0
0.5
0
*
0
5
7.5
RBP4 (µg/mL)
B
iNOS expression (fold change)
2.0
1.5
1.0
0.5
0
0
5
7.5
RBP4 (µg/mL)

Figure 6


A
1.5h treatment
3h treatment
COX2 expression (fold change)
2.0
1.5
1.0
0.5
0
*
* †
0
5
7.5
RBP4 (µg/mL)
B
1.5h treatment
3h treatment
PGI2 (ng/mL x10^5 cells)
30
25
20
15
10
5
0
*
* †
0
5
7.5
RBP4 (µg/mL)

Supplemental Figure 1



Supplemental Figure 1. Correlation diagram between RBP4 and TTR serum levels in the studied population ($r^2 = 0.476$;$P < 0.0001$; Spearman correlation).

ARTÍCULO CUARTO

"Apolipoprotein A-I glycosylation changes in acute coronary syndromes"

Judit Cubedo * † ¶, Teresa Padró * ¶, Lina Badimon * † || ¶

*Cardiovascular Research Center (CSIC-ICCC), †CIBERobn, ||Autonomous University of Barcelona, and ¶ Biomedical Research Institute Sant Pau (IIB-Sant Pau). Barcelona. Spain

En preparación

Resumen resultados

Cambios en el perfil sérico de la Apo A-I a los 3 días del ingreso tras un AMI

En suero la forma mayoritaria de la Apo A-I se identifica por 2-DE como una serie de 5 puntos proteicos de 28 kDa y un pI entre 5 y 5.75. En el momento del ingreso tras un AMI no se observan cambios en los niveles de Apo A-I. Por el contrario a los 3 días del ingreso los pacientes AMI presentan una disminución significativa de todas las formas respecto al momento del ingreso, y de formas específicas respecto a los controles.

Los pacientes con síndrome metabólico presentan niveles más bajos de Apo A-I en el momento del ingreso que el resto de los pacientes. El análisis específico mediante 2-DE de las proteínas glicosiladas revela que 4 de las formas de la Apo A-I están glicosiladas.

Cuantificación valores séricos Apo A-I

El análisis mediante ELISA revela un aumento progresivo de los niveles de Apo A-I en los pacientes de AMI, presentando estos niveles una correlación negativa con los niveles medidos mediante 2-DE. Los pacientes con un aumento de Apo A-I a los tres días presentan valores más bajos de troponina T. Esta discrepancia se explica por una distribución diferencial de las formas de Apo A-I en las fracciones lipídicas y séricas.

Análisis distribución diferencial formas Apo A-I

Las formas de Apo A-I están distribuidas de una forma diferente entre las fracciones solubles y lipídicas del suero. En el análisis de la Apo A-I mediante WB se detectan dos formas, una de 28kDa (detectada por 2-DE) y otra de 45kDa que no se identifica en la 2-DE. Mientras que en el suero total la forma mayoritaria es la de 28kDa, en el suero libre de lipoproteínas la forma mayoritaria es la de 45kDa. En la fracción de HDL (total, 2 y 3) además de las formas de 28 y 45kDa, también se detectan otras dos de 35 y 55kDa respectivamente, siendo la de 28kDa la mayoritaria.

Mediante desglicosilación con PNGasa F de las muestras de suero se confirma que las dos formas séricas de la Apo A-I están N-glicosiladas. La forma de 45kDa está altamente N-glicosilada mientras que la de 28kDa está menos glicosilada. Al comparar el perfil de desglicosilación de pacientes AMI y controles, no se ven cambios en el momento del ingreso pero sí a los 3 días, momento en el que la forma de 45kDa presenta un mayor grado de glicosilación. Además la forma de 28kDa también está O-glicosilada.

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By

Judit Cubedo * † ¶, Teresa Padró * ¶, Lina Badimon * † || ¶

From

*Cardiovascular Research Center (CSIC-ICCC), †CIBERobn, ||Autonomous University of Barcelona, and ¶ Biomedical Research Institute Sant Pau (IIB-Sant Pau). Barcelona. Spain.

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Correspondence to:

Prof. Lina Badimon

Cardiovascular Research Center, c/Sant Antoni MªClaret 167, 08025 Barcelona, Spain.

Phone: (34) 935565880. Fax: (34) 935565559.

E-mail: ***lbadimon@csic-iccc.org***

ABSTRACT

The relevant role of HDL in atheroprotection is not only determined by its quality but also by the properties of its components. Indeed, there are several clinical and experimental evidences showing that during acute phase processes HDL particles loose proteins with anti-inflammatory and cito-protective properties. In this study we sought to investigate ApoA-I proteomic profile, as the main HDL component, in the early and late phase after an acute new onset myocardial infarction (AMI).

Methods: Characterization of serum ApoA-I in AMI-patients was performed by 2D-electrophoresis (2DE) followed by mass-spectrometry (MALDI-TOF/TOF) at two different time points: at the moment of admission within first six hours after the onset of the event (time zero) and 3 days after the admission time. The proteomic profile of AMI-patients was then compared to that of control individuals. Total ApoA-I levels were analyzed by ELISA. Deglycosylation assays were performed by PNGase F treatment followed by western blot (WB) analysis.

Results: ApoA-I characterization depicted a cluster of 5 spots (Mw: 28kDa; pI: 5-5.75) with no changes between AMI-patients at time zero and controls. Whereas 3 days after admission (late phase) a significant change in spot distribution was detected when compared to controls, with a strong decrease of spot number 4, non-glycosylated ApoA-I form. When ApoA-I distribution in AMI-patients 3 days after the admission time was compared to that at time zero a significant decrease in all spots was detected ($p<0.05$). ApoA-I total levels measured by ELISA depicted a progressive increase in AMI-patients after the admission time, and were negatively correlated with ApoA-I intensity in the 2-DE analysis (r^2=-0.525 $p<0.05$) due to a differential distribution of ApoA-I forms (45 and 28kDa) between lipoprotein-depleted serum and HDL. AMI-patients 3 days after admission showed a significant change in the deglycosylation profile of the 45kDa ApoA-I form.

Conclusions: Our results demonstrate that Apo A-I is both N- and O-glycosylated and a differential contribution of the HDL fraction and lipoprotein-depleted serum fraction to the total pool of plasma Apo A-I. Moreover we have described for the first time an increase in Apo A-I glycosylation in AMI-patients 3 days after the event which may have detrimental effects on ApoA-I properties.

Key Words: acute-myocardial-infarction, apo A-I, glycosylation, proteomic studies.

INTRODUCTION

High density lipoprotein (HDL) levels are inversely related to cardiovascular disease event presentation. Indeed HDL have well stablished anti-atherogenic properties **(1-3).** HDL structure and function are determined by the properties of its components thus, in addition to HDL quantity, HDL quality is a very important parameter in atheroprotection **(4).** In fact, modifications in the main protein of HDL Apolipoprotein A-I (Apo A-I) have shown to affect HDL properties **(5, 6).**

Different post-translational modifications have been described in Apo A-I resulting in different forms of the protein. A post-translational modification involving reversible phosphorilation of Apo A-I was described on a single serine residue present in the mature form of the protein **(7).** Lately, attention was focused on the impairing effects on HDL of non-enzymatic protein glycation and advanced glycation end product (AGE) formation **(8)** both associated with an increased risk for coronary artery disease **(9).** Additional Apo A-I modifications, such as deamidations and oxidations resulting in increased risk for vascular disease have been described **(10).** It has also been reported that after an acute myocardial infarction the HDL proteome shifts to an inflammatory profile **(11).** However the specific changes in ApoA-I during the evolution of an ischemic syndrome are not yet known.

In this study we hypothesized that the Apo A-I protein of HDL suffered changes in the late phase of an acute new-onset myocardial infarction (AMI) that could impair its atheroprotective properties.

MATERIALS AND METHODS

Blood collection and sample preparation

Freshly drawn venous blood samples were collected to prepare serum that was aliquoted and stored at -80º from patients and healthy individuals with informed consent. Procedures were approved by the Clinical Research Committee of the Hospital de la Santa Creu i Sant Pau.

For proteomic studies, serum samples, glycosylated serum fractions and HDL samples were prepared as previously described **(12, 13).** In brief, the six most abundant serum proteins were depleted using a specific affinity cartridge with binding capacity for albumin, IgGs, IgAs, transferrin, α1 antytripsin and haptoglobin (Multiple Affinity Removal Spin Cartridge, Agilent Technologies) as reported by the providers. The

glycosylated serum fraction was purified using a commercial kit (Qproteome Total Glycoprotein Kit, QIAGEN). Human HDL were obtained by ultracentrifugation of normocholesterolemic total serum and the protein fraction was obtained by precipitation with pure ice cold acetone.

The O-glycosylated serum fraction was purified using a commercial kit (Qproteome O-Glycan Glycoprotein Kit, QIAGEN). Lipoprotein-depleted serum was obtained after lipoprotein extraction.

Proteomic analysis

Two-dimensional gel electrophoresis (2-DE): For analytical and preparative gels, respectively, a protein load of 120 µg and 300 µg protein of the urea/chaps serum extracts were applied to 18-cm dry strips (pH 4-7 linear range, GE Healthcare). Second dimension was resolved in 10% SDS-PAGE gels. 2-DE analysis of serum samples in AMI-patients was performed at two time points: the moment of admission (t=0; N=27) and 72-96 hours after the admission (N=8). In a subset of samples IEF was performed in a zoom of pH between 4.5 and 6.9 and 15% SDS-PAGE gels. Gels were developed by fluorescent staining. For each independent experiment, 2-DE for protein extracts from controls and patients were processed in parallel to guarantee a maximum of comparability. Each 2-DE run was at least repeated twice. Analysis for differences in protein patterns was performed with the PD-Quest 8.0 (BioRad), using a single master that included all gels of each independent experiment. Each spot was assigned a relative value that corresponded to the single spot volume compared to the volume of all spots in the gel, following background extraction and normalization between gels.

Mass spectrometry analysis: Proteins were identified after in-gel tryptic digestion and extraction of peptides from the gels pieces, as previously described **(12, 13),** by matrix – assited laser desorption/ionization time-of-flight (MALDI-TOF) using an AutoFlex III Smartbeam MALDI-TOF/TOF (Bruker Daltonics). Samples were applied to Prespotted AnchorChip plates (Bruker Daltonics) surrounding the calibrants provided on the plates. Spectra were acquired with flexControl on reflector mode, (mass range 850 - 4000 m/z, reflector 1: 21.06 kV; reflector 2: 9.77kV; ion source 1 voltage: 19 kV; ion source 2: 16.5kV; detection gain 2.37x) with an average of 3500 added shots at a frequency of 200 Hz. Each sample was processed with flexAnalysis (version 3.0, Bruker Daltonics) considering a signal-to-noise ratio over 3, applying statistical calibration and eliminating background peaks. For identification, peaks between 850

and 1000 m/z were not considered as in general only matrix peaks are visible on this mass range. After processing, spectra were sent to the interface BioTools (version 3.2, Bruker Daltonics) and, with no further modifications, MASCOT search on Swiss-Prot 57.15 database was done (Taxonomy: Homo Sapiens, Mass Tolerance 50 to 100, up to 2 trypsin miss cleavages, Global Modification: Carbamidomethyl (C), Variable Modification: Oxidation (M)). Identification was accepted with a score higher than 56.

Quantification of Apo A-I serum levels

Apolipoprotein A-I serum concentration in control and AMI-patients samples (at the moment of admission defined as time zero and 3 days after) was determined by competitive sandwich enzyme-linked immunosorbent assay (AssayPro) using immobilized polyclonal antibodies, as described by the providers. The detection limit of the assay was 1.2 µg/ml and the intra-assay and inter-assay coefficients of variations were 4.6% and 7.3% respectively.

Western Blot Analysis and deglycosylation with Peptide-Nglycosidase F

Sample extracts were resolved by SDS-PAGE under reducing conditions and electrotransferred to PVDF (Polyvinylidene Difluoride) membranes in semi-dry conditions (Semi-dry transfer system, BioRad). Protein detection was performed using rabbit polyclonal antibody (PAb) against total Apo A-I (178422, 1:1000 dilution, Calbiochem) combined with the Dye Double Western Blot kit (Invitrogen). Band fluorescence was determined with Typhoon 9400 (GE Healthcare) and band quantification was performed using ImageQuant TL v7.01. software (GE Healthcare). Protein load was normalized using total protein fluorescent signal. Deglycosylation of Apo A-I was performed with the enzyme PNGase F (Sigma Aldrich) as previously described **(12).**

Study population

The study population comprised 39 patients with an acute new-onset myocardial infarction (29 men and 10 women; mean age: 61±2 years) who were admitted with chest pain and suspected of ACS at the Emergency Room of Santa Creu i Sant Pau Hospital. At the emergency department, routine diagnostic procedures were applied to establish the onset of symptoms as accurately as possible (i.e. description of chest pain,

pulmonary edema, severe dyspnea, and syncope). In addition to the general patient history, clinical examination, 12-lead ECG, and laboratory tests were also run to characterize AMI-patients. All AMI-patients showed (1) typical chest pain lasting more than 30 minutes; (2) ST segment elevation >0.2 mV in at least 2 contiguous leads; (3) admission to the hospital within the first 6 hours after chest pain onset; (4) normal serum CK and CKMB levels at admission; (5) negative troponin T at admission (excluding subacute myocardial infarction); (6) sinus rhythm. Exclusion criteria were a previous documented or suspected myocardial infarction and antithrombotic treatment because of the AMI onset before arriving to the emergency room and time of blood collection. Delayed contrast-enhanced (CE) CMR studies were performed within the first week after AMI, as previously described **(12).**

Patients included in the study did not present further co-morbidities such as cancer, chronic infections, autoimmune diseases or thyroid hormones disorders.

Blood samples of AMI-patients were collected at the moment of admission within the first six hours after the onset of the event (defined as time zero) and 3 days after the admission time.

The control group included 60 healthy individuals (45 men and 15 women; mean age: 62±1 years) who attended to a routine health check.

The Ethics Committee of the Santa Creu i Sant Pau Hospital approved the project and the studies were conducted according to the principles of Helsinki's Declaration. All participants gave written informed consent to take part in the study.

Presence of metabolic syndrome in the three groups was considered when three from five of the following criteria were present: (1) obesity (BMI>30); (2) diabetes; (3) HDL-cholesterol levels below 40 mg/dL for men and 50mg/dL for women; (4) triglycerides levels above 150 mg/dL; (5) hypertension.

Statistical analysis

For quantitative analysis (biochemical, lipid parameters and ELISA results) data are expressed as mean and standard deviation except when indicated. For semi-quantitative analysis (2-DE and WB analysis) data are expressed as median and interquartile range [IQR]. N indicates the number of subjects tested. Statistical analysis was performed with Stat View 5.0.1 software. Student's t-test and Mann Whitney testing were used for comparison between control and AMI groups for variables with and without parametric

distribution, respectively. The ANOVA test was used in analysis where the effect of variables acting as covariates was added to the model, and in multiple comparisons analysis using Fisher's PSLD as Post-hoc Test. Correlations between variables were determined by single and multiple regression models. A p value ≤ 0.05 was considered significant.

RESULTS

Apo A-I proteomic profile in AMI-patients

Proteomic analysis by 2-DE (pH ranges, 4-7 and 4.7-5.9) of serum samples revealed Apolipoprotein A-I (Apo A-I) as a cluster of five spots with the same apparent molecular mass of 28 kDa and a pI range between 5 and 5.75 **(Supplemental Figure 1).** The five spots were identified by MALDI-TOF analysis as Apo A-I in both AMI and control groups. The intensity of Apo A-I spots did not show any significant modification in the early phase after AMI **(compare Figure 1A with 1B, Figure 1D and Table 1).** Whereas 3 days after admission (late phase) a significant change in spot distribution was detected when compared to controls **(compare Figure 1A with 1C and Figure 1D).** Three days after admission spots 1 and 2 depicted significant decreased intensities (7-fold and 1.4-fold decrease, respectively) when compared to controls **(Figure 1D and Table 1).** Spot number 4 was minimally shown in a third (38%) of AMI-patients 3 days after the admission time. When Apo A-I distribution in AMI-patients 3 days after the admission time was compared to that of AMI-patients at time zero a significant decrease in all spots was detected **(compare Figure 1B and 1C, Figure 1D and Table 1).**

Total Apo A-I intensity **(Figure 2A)** that was not modified respect to control in AMI-patients at the moment of admission, was significantly reduced 3 days afterwards ($p < 0.05$). AMI-patients with metabolic syndrome showed significantly lower Apo A-I levels at the moment of admission than those without (Median [IQR]; AMI-MS: 263 [244-280] AU vs. AMI-NoMS: 306 [274-327] AU; $p < 0.05$; **Figure 2B**).

Glycoproteome of Apo A-I

The proteomic profile of glycosylated serum Apo A-I was studied after purification of glycosylated proteins through lectin binding. The glycoproteome and the non-

glycosylated-subproteome from AMI-patients with diabetes, AMI-patients without diabetes and control serums (pools of 10 individuals at time zero) were analyzed in triplicates. In the three groups the analysis of glycosylated Apo A-I serum fraction revealed the presence of spots 1, 2, 3, and 5, while spot 4 was not apparent **(Figure 3A).** This proteomic profile of glycosylated Apo A-I was very similar to the proteome profile of the total serum fraction of Apo A-I in AMI-patients 3 days after admission **(compare Figures 3A with 1C).** The non-glycosylated Apo A-I fraction at time zero revealed the presence of the five spots in the three groups **(Figure 3B).**

Apo A-I serum levels in AMI patients and control individuals

Apo A-I serum levels were measured (ELISA) in 39 AMI patients at time zero. When compared to the control group, Apo A-I levels were significantly higher in AMI-patients (Mean ± SEM; AMI: 271.4 ± 19.6 mg/dL *vs.* Control: 146.8 ± 5.9 mg/dL; $p < 0.0001$; **Figure 4A**). In the population under study (healthy donors and AMI-patients), serum Apo A-I levels were significantly correlated with the total cholesterol levels and triglycerides (total-c: $r^2 = 0.292$ $p < 0.05$ for control and $r^2 = 0.416$ $p < 0.05$ for AMI-patients; triglycerides: $r^2 = 0.384$ $p < 0.05$ for controls and $r^2 = 0.704$ $p < 0.001$ for AMI-patients). Apo A-I levels showed no significant variations in relation to sex and age in the studied population. Apo A-I levels were decreased in AMI-patients after adjustment for the effects of variables as total cholesterol and triglycerides ($p < 0.0001$). AMI-patients with metabolic syndrome showed significantly higher Apo A-I levels than those without metabolic syndrome (Mean ± SEM; AMI-MS: 363.3 ± 29.4 mg/dL vs. AMI-NoMS: 215.8 ± 18.0 mg/dL; $p < 0.0001$).

In 25 of the patients, Apo A-I levels were determined 3 days (72hours) after admission. In 44% of the patients, Apo A-I levels were still higher 3 days after compared to the time zero (Mean ± SEM; t=3d: 552.2 ± 55.1 mg/dL vs. t=0: 271.4 ± 19.6 mg/dL; $p < 0.0001$). These 44% of patients already showed higher Apo A-I levels at the moment of admission (Mean ± SEM; 44% AMI-patients: 386.2 ± 44.8 mg/dL *vs*. the rest of AMI-patients: 214.4 ± 23.2 mg/dL; $p < 0.05$). Apo A-I levels variation within 3 first days after the event was inversely and significantly correlated with troponin levels ($r^2 = -0.645$ $p < 0.05$; **Figure 4B**).

In AMI-patients Apo A-I levels measured by ELISA were significantly and negatively correlated with intensity of Apo A-I forms detected by 2-DE (r^2 = -0.525 $p < 0.05$; **Figure 4C**) implying that they do not measure the same.

Apo AI forms distribution in lipid-rich and soluble fractions

Comparison of Apo A-I in total serum and in lipoprotein-depleted serum samples (Western blot analysis analyzed by 1D-electrophoresis (15% PAGE-SDS gels) under reducing conditions) showed two bands of approximately 45 and 28 kDa, with a differential distribution profile ($p < 0.05$). In total serum the 28 kDa form was the 66.8% [64.3-71.03] of total Apo A-I, while in lipoprotein-depleted serum the main form was the 45 kDa form that represented the 66.3% [55.3-84.3] of total Apo A-I **(Figure 5)**. When HDL fractions (total, HDL2 and HDL3) where analyzed four Apo A-I forms were detected, the 45 and 28 kDa previously detected in serum samples, and two additional forms of 55 and 35 kDa. In all HDL fractions the 28 kDa form was the most abundant one (HDLt: 50.6% [41.4-62.1]; HDL2: 63.4% [46.6-65.6]; HDL3: 54.2% [51.0-57.6] **(Figure 5)**.

Apo AI glycosylation changes in the late phase after AMI

The N-glycosylation profile of Apo A-I in controls and AMI-patients was analyzed by PNGase F treatment. As shown in **Figure 6A**, western blot analysis for total Apo A-I revealed two bands of 45 and 28 kDa (bands A1 and A2 respectively in **Figure 6A**) in control and AMI samples (lanes 1, 3 and 5). After treatment with PNGase F (lanes 2, 4 and 6) 2 bands of approximately 32 and 24 kDa (bands D1 and D2 respectively in **Figure 6A**) were also apparent in controls and AMI-patients both, at time zero and 3 days after admission. There were no differences in the band distribution profile of Apo A-I after deglycosylation between controls and AMI-patients at time zero (**Figure 6B**). Whereas, AMI-patients 3 days after the admission showed an important change in Apo A-I glycosylation profile as depicted by the significant decrease of the A1 form after PNGase F treatment (AMI t=3d: 21.0 [18.5-22.7] *vs.* C: 36.9 [33.8-40.9] $p < 0.005$; and *vs.* AMI t=0: 33.8 [28.3-39.9] $p < 0.05$) and the significant increase of the D1 de-glycosylated form (AMI t=3d: 16.6 [14.8-20.4] *vs.* C: 5.8 [5.3-6.1] $p < 0.005$; and *vs.* AMI t=0: 4.5 [3.4-5.5] $p < 0.05$), demonstrating a higher glycosylation degree of Apo A-I in AMI-patients 3 days after the admission. Deglycosylation of transferrin was used as positive control of PNGase F treatment.

When only O-glycosylated proteins were analyzed by 2-DE, the same Apo A-I distribution profile as for total glycoproteins was observed **(Suplemental Figure 2A)** demonstrating that spots 1, 2, 3, and 5 also are O-glycosylated. The wash-out fraction resulting of the O-glycoprotein isolation showed the five Apo A-I spots **(Suplemental Figure 2B).**

Clinical characteristics of the patients

The characteristics of AMI and control groups are outlined in **Table 2**. Differences between groups in lipid parameters are described in **Supplemental Table I.**

DISCUSSION

In this study we have analyzed changes in serum proteome of Apo A-I in the early and late phase of an acute new-onset myocardial infarction. Specifically we have evaluated Apo A-I at the initial hours of ischemia and 3 days later MI, finding important changes in the Apo A-I glycosylation profile 3 days after the event. Until now several modifications had been reported in Apo A-I such as phosphorylation **(7),** fatty acid acylation **(14)** and oxidations **(15).** Here we report for the first time that within the 28kDa Apo A-I form, spots 1, 2, 3, and 5 represent glycosylated forms, and that these spots are both N- and O-glycosylated. Interestingly, we have detected in the late phase after AMI that serum Apo A-I is mainly present in its glycosylated forms loosing its non-glycosylated forms. Indeed, in 62% of AMI-patients the no-glycosylated spot was undetectable. The presence of glycosylated Apo A-I after an AMI may have affected its anti-inflammatory properties. In fact it has been reported in *in vitro* experiments that non-enzymatic glycation of Apo A-I with methylglyoxal impairs its anti-inflammatory properties **(5),** and that fructose-mediated Apo A-I glycation induces loss of several beneficial functions of Apo A-I and HDL **(16).** Interestingly glycosylation of Apo J, another HDL-transported protein, was found to be modified in acute new onset AMI patients **(12).**

It has been previously described that Apo A-I modifications lead to an acceleration of Apo A-I damage and therefore to an impairment of its activities **(10).** Moreover, there are several clinical and experimental evidences showing that during acute phase processes HDL particles loose proteins with anti-inflamatory and cito-protective properties **(17, 18).** Therefore, our results highlight a possible implication of Apo A-I glycosylation in the loss of HDL cardioprotective properties after AMI.

Surprisingly, there was a negative correlation between Apo A-I levels measured by ELISA and the intensity of Apo A-I forms detected in proteomic studies. Therefore, the ELISA technique seems to be quantifying different forms of Apo A-I all together without discrimination on specific post-translational modifications. To test the existence of different Apo A-I forms, lipid and soluble fractions of serum were analyzed for the presence of Apo A-I. Indeed, different Apo A-I forms were described recently in plasma samples of cardiovascular disease patients by 2DE analysis suggesting differential post-translational modifications associated to the disease **(19).** Our results demonstrate a differential contribution of the HDL fraction and lipoprotein-depleted serum fraction to the total pool of plasma Apo A-I. In fact, changes in Apo A-I distribution within different Apo A-I-containing fractions have been described in young AMI-patients, where 6 months after AMI Apo A-I was transported mainly in heavy HDL fractions isolated by ultracentrifugation **(20).** Specifically, lipoprotein-depleted serum fraction showed a predominance of high molecular weight form of Apo A-I (45 kDa). Interestingly, this Apo A-I isoform has revealed a higher glycosylation degree in AMI-patients 3 days after the event when compared to controls. A recent study has revealed an increase of an Apo A-I form of approximately 50 kDa in acute coronary syndrome patients proposing the presence of post-translational changes of Apo A-I as a new possible disease-associated marker of cardiovascular disorders **(19).** Our results show for the first time a significant higher degree of Apo A-I glycosylation after an acute coronary event.

Intriguingly, we have found that patients with higher total Apo A-I levels after an AMI had lower troponin T levels showing a possible protective role of the Apo A-I increase detected by ELISA. Previous studies have investigated Apo A-I changes after acute coronary syndromes reporting different results on total Apo A-I levels **(21, 22).** Moreover, one of those studies **(21)** specifically analyzed Apo A-I proteomic changes in AMI and unstable angina patients (UA) and found divergent results among both groups with and increase in UA-patients and a decrease in AMI-patients suggesting an antagonist response to the inflammatory reaction associated with the cardiac event in UA-patients.

In addition, we have found an association between Apo A-I and metabolic syndrome in AMI-patients. This is in line with previous studies where low levels of Apo A-I associate with metabolic syndrome in young adults **(23).**

In summary, despite the limitations of 2-DE approaches for the study of such a complex sample like serum, by careful use of biochemical analysis, we have obtained useful complementary information about post-translational modifications of Apo A-I. Thus, our results demonstrate that Apo A-I is both N- and O-glycosylated and moreover we have described for the first time an increase in Apo A-I glycosylation in AMI-patients 3 days after the event which may have implication in Apo A-I loss of beneficial effects.

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Disclosures

No conflicts to disclose.

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LEGENDS

Figure 1. Representative images of Apo A-I spots in controls (A) and AMI-patients at the moment of admission (B) and 3 days after (C). (D) Bar diagrams (median [IQR]) showing the intensity of each spot in the three conditions. None of the Apo A-I spots were modified in AMI-patients at the moment of admission. The five spots were decreased 3 days after the admission time when compared to the moment of admission (*p<0.05; Mann-Whitney test), and spots 1, 2, and 4 when compared to controls (§p<0.05; Mann-Whitney test).

Figure 2. Bar diagrams (median [IQR]) showing total intensity of Apo A-I spots (calculated as the sum of intensities of the five spots) showing (A) lower Apo A-I total levels in AMI-patients 3 days after the moment of admission (*p<0.05 *vs.* controls and §p<0.05 *vs.* t=0; Mann-Whitney test); (B) significantly lower Apo A-I levels in AMI-patients with metabolic syndrome (*p<0.05; Mann-Whitney test).

Figure 3. Representative 2-DE image of the Apo A-I cluster in (A) total glycosylated and (B) non-glycosylated serum fractions from controls, AMI-patients with diabetes (AMI dm) and AMI-patients without diabetes (AMI no dm).

Figure 4. Serum Apo A-I (µg/mL) in AMI-patients and controls measured by a commercial ELISA. (A) Bar diagram (mean ± SEM) showing serum Apo A-I levels in controls and AMI-patients (at admission and 3 days after). AMI-patients showed significantly increased Apo A-I levels at the moment of admission when compared to controls (* p<0.0001; Student´s t-test). Three days after admission AMI-patients showed a significant increase in Apo A-I serum levels when compared to controls (# p<0.0001; Student´s t-test). (B) Correlation graph showing the inverse negative correlation between Apo A-I serum levels variation (between time zero and 3 days after admission) and troponin T levels. (C) Correlation graph showing the inverse negative correlation between Apo A-I serum levels measured by ELISA and 2-DE in AMI-patients.

Figure 5. Representative WB image and bar diagram showing Apo A-I band distribution in serum samples, lipoprotein-depleted serum and HDL fractions. Serum and lipoprotein-depleted serum two bands were detected (45 and 28 kDa). Total HDL, HDL2 and HDL3 fractions depicted four bands (55, 45, 35 and 28 kDa). The 28 kDa band was the main form in HDL and total serum fractions. Whereas the 45 kDa form was the most representative one in lipoprotein-depleted serum fraction.

Figure 6. (A) Representative WB image of Apo A-I in serum samples of controls (lane 1) and AMI-patients at the time of admission (lane3) and 3 days after (lane 5) showing two bands of 45 and 28 kDa (bands A1 and A2 respectively). PNGase F treatment (lanes 2, 4 and 6) revealed the presence of two deglycosylated bands of 32 and 24 kDa approximately (bands D1 and D2 respectively). Transferrin was used as positive control of PNGaseF deglycosylation. (B) Bar diagram (median [IQR]) of the % of band intensity of total Apo A-I. AMI-patients 3 days after the event depicted a significant decrease in A1 intensity and a significant increase in D1 intensity after PNGase F treatment.

TABLE 1. Differences in Apo A-I spot intensity in AMI-patients when compared to controls.

	Median [IQR] of spot intensity (AU)			*p*-value *		
Apo A-I spot	Control	AMI t=0	AMI t=3d	AMI t=0 *vs.* C	AMI t=3d *vs.* AMI t=0	AMI t=3d *vs.* C
1	19 [13-26]	15 [11-21]	2.8 [2.6-3.7]	NS	< 0.001	< 0.05
2	59 [54-68]	50 [48-61]	43 [35-44]	NS	< 0.05	< 0.05
3	180 [143-187]	194 [155-217]	151 [126-167]	NS	< 0.05	NS
4	21 [15-31]	20 [15-30]	0 [0-3.4]	NS	< 0.0001	< 0.05
5	13 [11-14]	13 [11-16]	7.1 [6.4-10]	NS	< 0.05	NS
Total	299 [249-325]	285 [258-327]	197 [174-221]	NS	< 0.05	< 0.05

* Mann-Whitney test. NS = no significant.

TABLE 2. Background description and biochemical parameters of AMI-patients and healthy donors.

	Control group	AMI group
N	60	39
Age (mean ± SEM)	62 ± 1	61 ± 2
Females / Males	15 / 45	10 / 29
BMI	27.5 ± 0.5	28.2 ± 0.7
Risk Factors, n (%)		
Tobacco smoking	19	51
Hypertension	22	42
Dyslipemia	51	49
Diabetes Mellitus	2	27
Metabolic Syndrome	13	39

BMI = Body mass index.

Figure 1

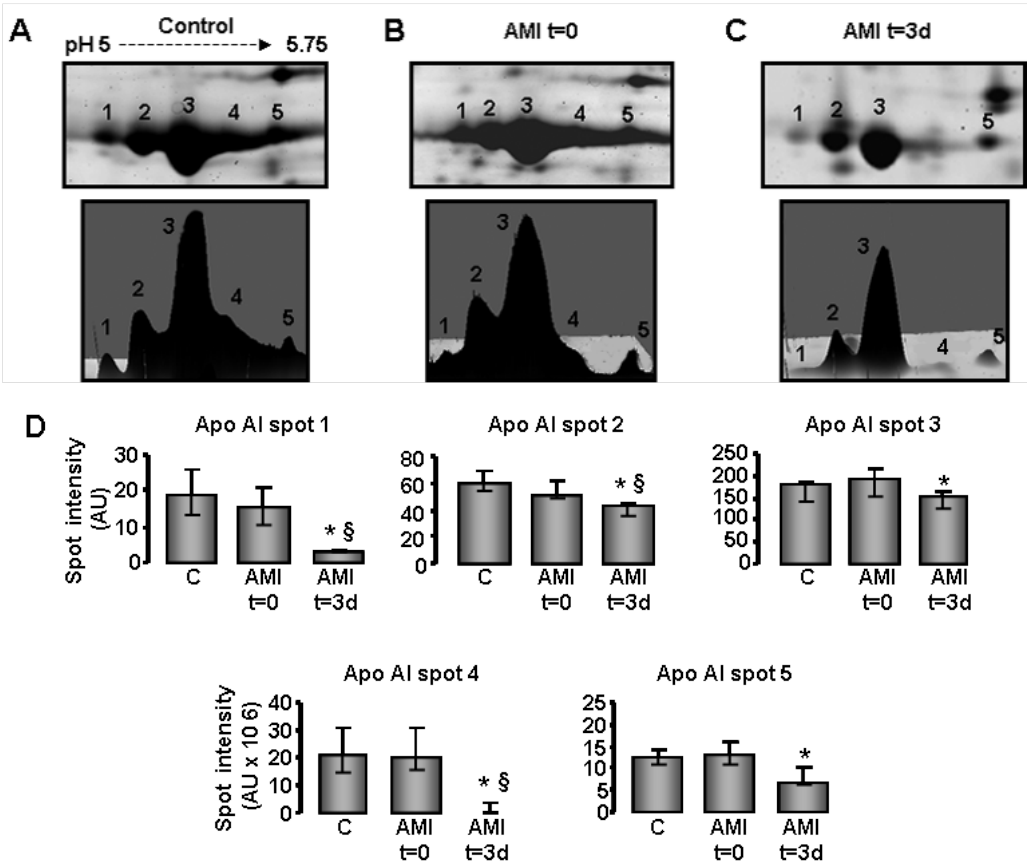

A
Control
pH 5
5.75
B
AMI t=0
C
AMI t=3d
D
Apo AI spot 1
Spot intensity (AU)
Apo AI spot 2
Apo AI spot 3
Apo AI spot 4
Spot intensity (AU x 10 6)
Apo AI spot 5
C
AMI t=0
AMI t=3d

Figure 2

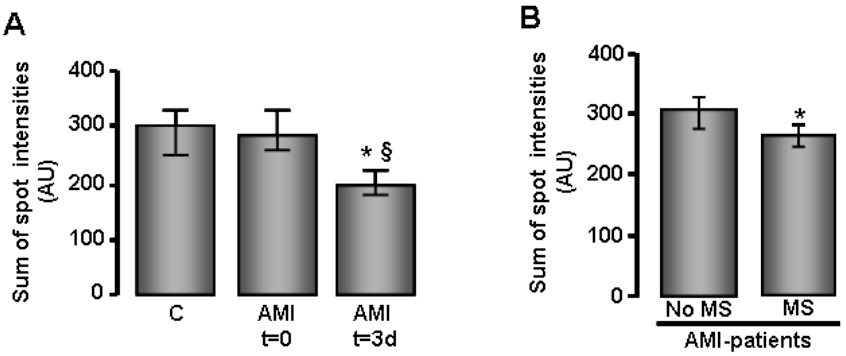

A
Sum of spot intensities (AU)
400
300
200
100
0
* §
C
AMI t=0
AMI t=3d
B
Sum of spot intensities (AU)
400
300
200
100
0
*
No MS
MS
AMI-patients

Figure 3

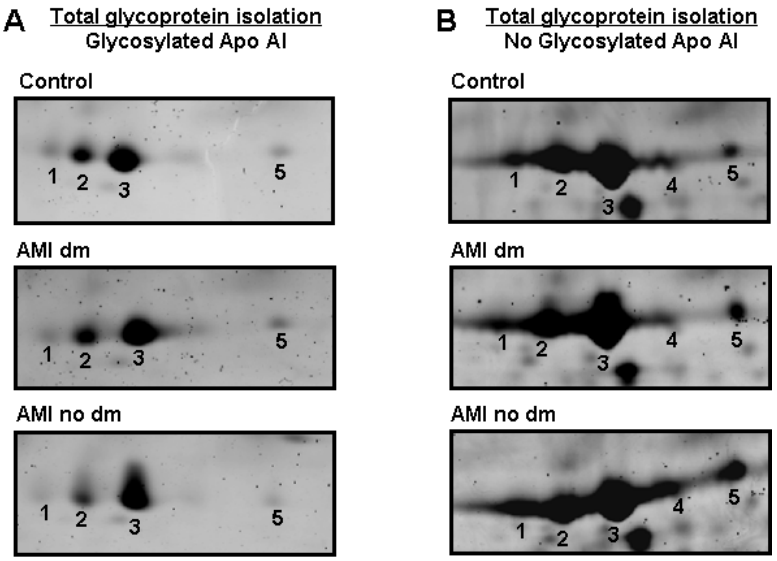

A
Total glycoprotein isolation
Glycosylated Apo AI
Control
1
2
3
5
AMI dm
1
2
3
5
AMI no dm
1
2
3
5
B
Total glycoprotein isolation
No Glycosylated Apo AI
Control
1
2
3
4
5
AMI dm
1
2
3
4
5
AMI no dm
1
2
3
4
5

Figure 4

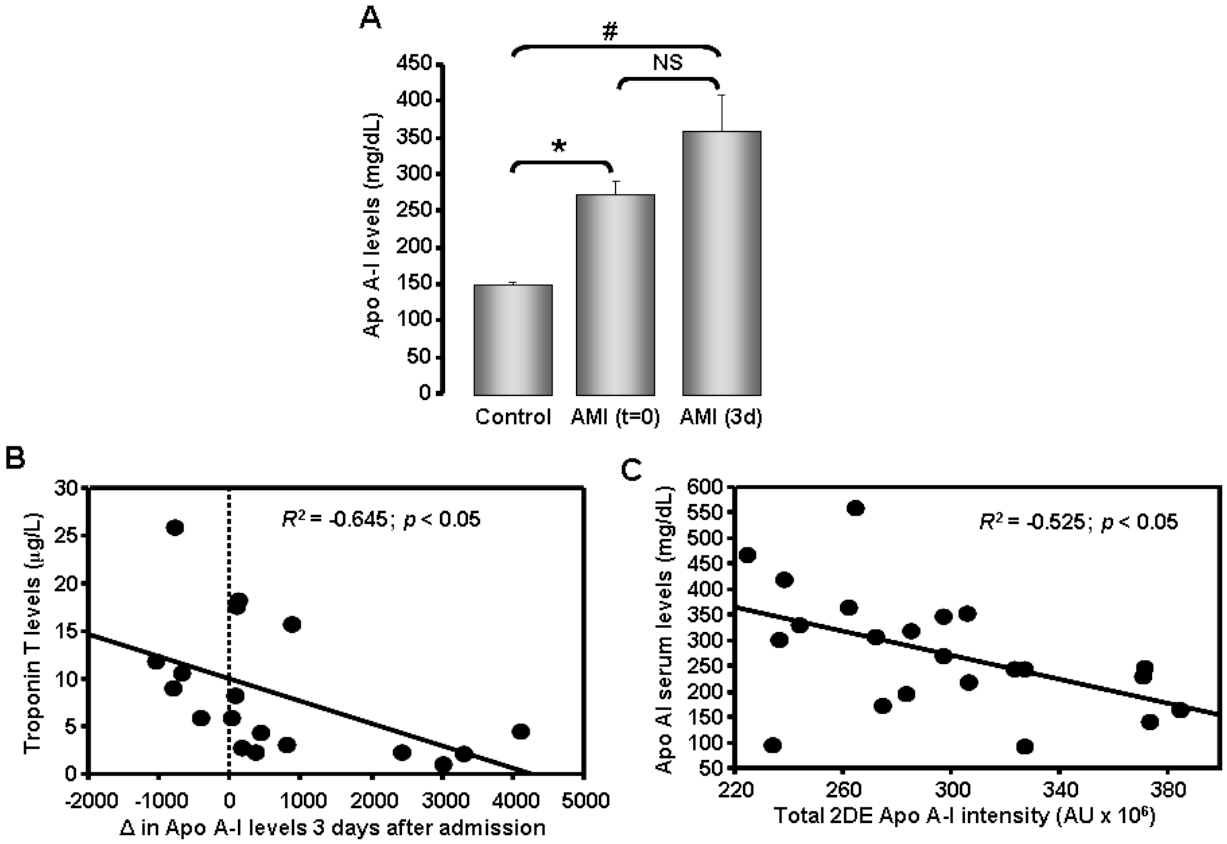
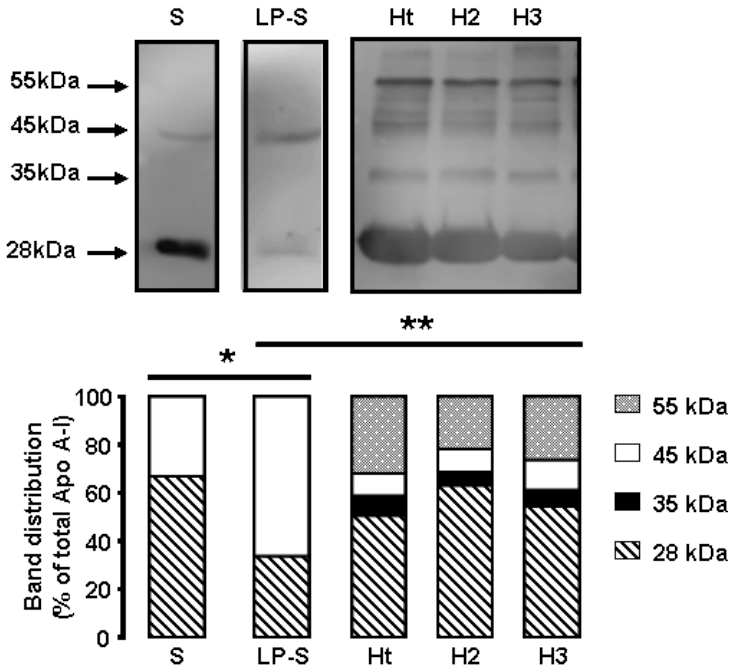
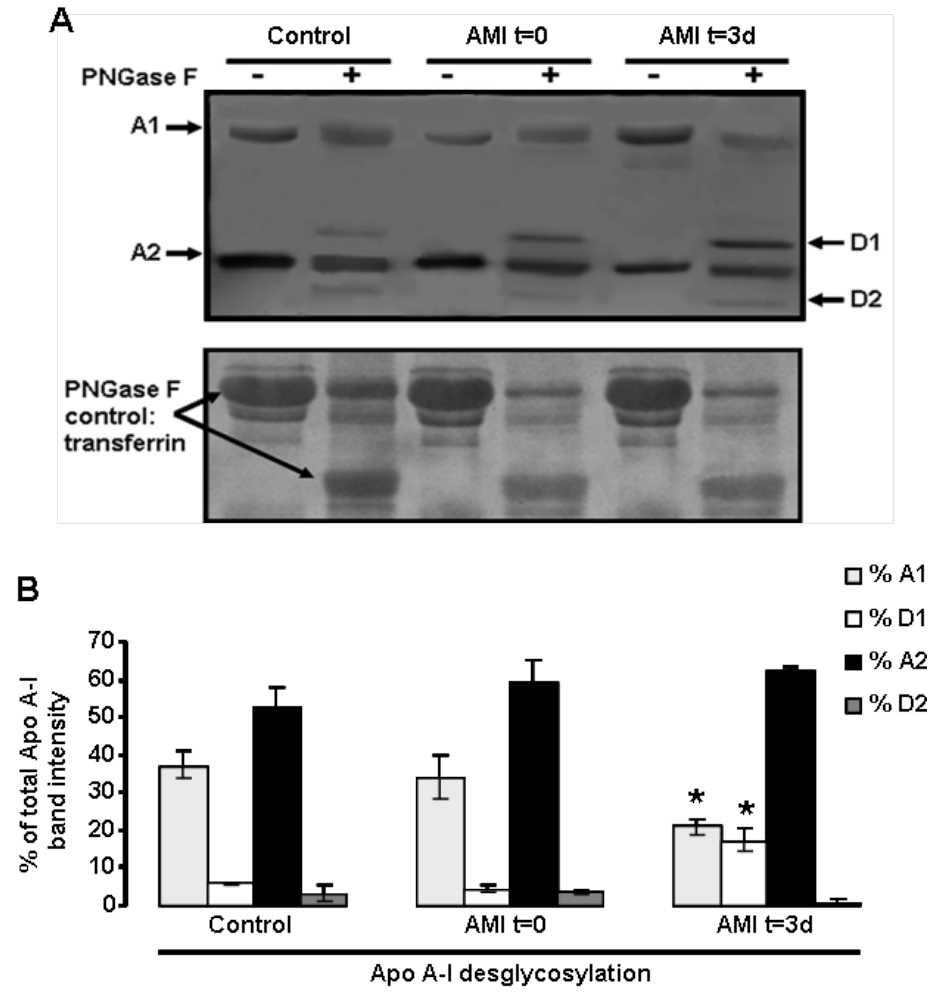

A
#
NS
*
Apo A-I levels (mg/dL)
450
400
350
300
250
200
150
100
50
0
Control
AMI (t=0)
AMI (3d)
B
Troponin T levels (µg/L)
30
25
20
15
10
5
0
R^2 = -0.645; p < 0.05
-2000
-1000
0
1000
2000
3000
4000
5000
Δ in Apo A-I levels 3 days after admission
C
Apo AI serum levels (mg/dL)
600
550
500
450
400
350
300
250
200
150
100
50
R^2 = -0.525; p < 0.05
220
260
300
340
380
Total 2DE Apo A-I intensity (AU x 10^6)

Figure 5



H: HDL(t=total, 2=HDL2, 3=HDL3)
S: complete serum
LP-S: lipoprotein depleted-serum

Figure 6


A
Control
AMI t=0
AMI t=3d
PNGase F
- + - + - +
A1
A2
D1
D2
PNGase F
control:
transferrin
B
% A1
% D1
% A2
% D2
% of total Apo A-I
band intensity
70
60
50
40
30
20
10
0
*
*
Control
AMI t=0
AMI t=3d
Apo A-I desglycosylation

SUPPLEMENTAL MATERIAL

LEGENDS TO SUPPLEMENTAL FIGURES

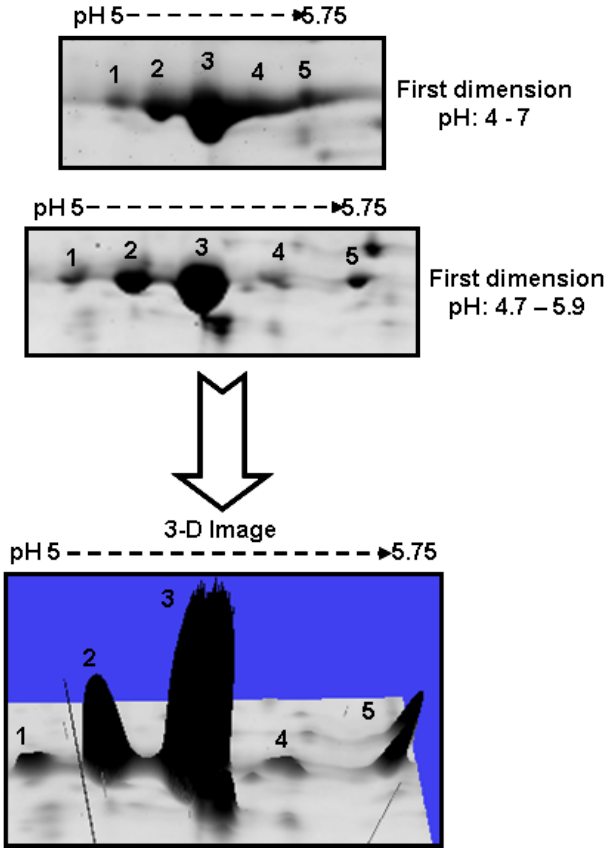
Supplemental Figure 1. Representative images of Apo A-I spots in serum samples analyzed by 2-DE in different ranges of pH and 3-D image showing the five Apo A-I spots detected, denoted from 1 to 5, from the acid to the more basic pH.

Supplemental Figure 2. Representative 2-DE image of the Apo A-I cluster in (A) O-glycosylated and (B) non-O-glycosylated serum fractions from control samples.

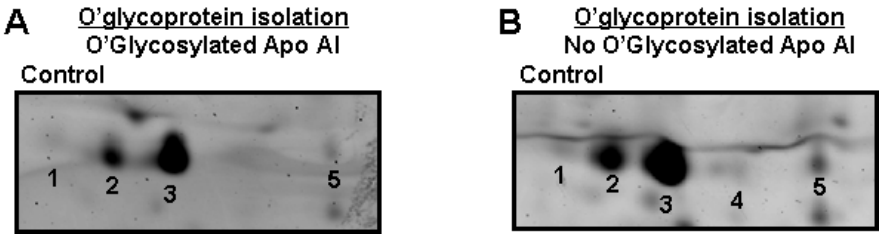
Supplemental TABLE I: Lipid parameters of the studied population.

	Serum levels		*p*-value
	Control group (N=60)	AMI group (N=39)	C *vs.* AMI
Total Cholesterol (mg/dL)	215.5 ± 5.4	207.5 ± 8.1	NS
HDL-Cholesterol (mg/dL)	49.0 ± 1.8	44.3 ± 2.1	NS
LDL-Cholesterol (mg/dL)	141.2 ± 4.8	130.5 ± 6.9	NS
Trigycerides (mg/dL)	129.0 ± 11.7	169.6 ± 12.3	< 0.05

Supplemental Figure 1



Suplemental Figure 2



ARTÍCULO QUINTO

"Identification of a novel ApoA-I truncated form transported by LDL and increased in diabetics"

Judit Cubedo * † ¶, Teresa Padró * ¶, Maisa García-Arguinzonis * ¶, Gemma Vilahur * † ¶, Jose María Pou #, Juan Ybarra #, Lina Badimon * † || ¶

*Cardiovascular Research Center (CSIC-ICCC), †CIBERobn, ||Autonomous University of Barcelona, # Endocrinology Department and ¶ Biomedical Research Institute Sant Pau (IIB-Sant Pau), Hospital de la Santa Creu i Sant Pau, Barcelona. Spain.

En preparación

Resumen resultados

Cambios en el patrón proteómico de la Apo A-I en el suero de pacientes diabéticos

Los pacientes diabéticos presentan un incremento significativo de la forma menos madura de la Apo A-I (Mw: 28kDa, pI:5.75). El análisis específico de las formas correspondientes a la Apo A-I glicosilada revela una correlación con los niveles de hemoglobina glicosilada.

Forma truncada de la Apo A-I

En la 2-DE se identifica una forma de Apo A-I de 26kDa y un pI de 5.75 que aumenta de forma significativa en pacientes diabéticos. El análisis mediante espectrometría de masas identifica esta forma de Apo A-I como una forma truncada en el extremo N-terminal a la que le faltan los aa 1-38 (forma que denominamos Apo A-I-Barcelona).

Análisis mediante 2-DE suero sin lipoproteínas y fracción lipoproteicas

El punto proteico correspondiente a la forma truncada detectada en el suero no se identifica ni en la fracción libre de lipoproteínas ni en la fracción HDL. En cambio, en la fracción LDL se detecta un punto proteico que coincide con en peso molecular y punto isoeléctrico con la forma truncada, y mediante WB de 1D y 2D se confirma que corresponde a la Apo A-I.

Identification of a novel ApoA-I truncated form transported by LDL and increased in diabetics

Cubedo et al: ApoA-I truncated form in diabetics

By

Judit Cubedo * † ¶, Teresa Padró * ¶, Maisa García-Arguinzonis * ¶, Gemma Vilahur * † ¶, Jose María Pou #, Juan Ybarra #, Lina Badimon * † || ¶

From

*Cardiovascular Research Center (CSIC-ICCC), †CIBERobn, ||Autonomous University of Barcelona, # Endocrinology Department and ¶ Biomedical Research Institute Sant Pau (IIB-Sant Pau), Hospital de la Santa Creu i Sant Pau, Barcelona. Spain.

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Correspondence to:

Prof. Lina Badimon

Cardiovascular Research Center, c/Sant Antoni MªClaret 167, 08025 Barcelona, Spain.

Phone: (34) 935565880. Fax: (34) 935565559.

E-mail: lbadimon@csic-iccc.org

ABSTRACT

Apolipoprotein A-I (ApoA-I) is the main protein of HDLs. In addition to its structural role, ApoA-I has a reverse cholesterol transport function to promote the efflux of cholesterol from peripheral cells into HDL, activates lecithin-cholesterol acyl transferase and shows anti-inflammatory properties. Therefore, ApoA-I modifications have a strong impact in HDL properties and in the regulation of the HDL particle size. By applying proteomic profiling we have investigated ApoA-I in serum and HDL samples and analyzed its changes in diabetic patients that very often have a pro-atherothrombotic phenotype.

Methods: Characterization of serum and HDL ApoA-I was performed by 2D-electrophoresis (2DE) followed by mass-spectrometry (MALDI-TOF/TOF). Serum proteomic profile of diabetic patients was compared to non-diabetic individuals.

Results: ApoA-I characterization depicted a cluster of 5 spots (Mw: 28kDa; pI: 5-5.75). In addition, one spot of 26kDa and a pI of 5.75 that was not present in HDL was found in serum samples. MALDI-TOF/TOF analysis revealed that the 26kDa spot is a truncated form of ApoA-I lacking aa 1-38 that we have named ApoA-I-Barcelona (aa 39-267). Diabetic patients showed a 46% increase intensity in the less mature Apo A-I form and a 64% increase in ApoA-I-BCN form ($p<0.05$). The Apo A-I-BCN is transported by LDL.

Conclusions: Our results demonstrate for the first time the presence in serum of a soluble truncated ApoA-I form (ApoA-I-BCN) that is also found in LDL particles. The increase of this truncated ApoA-I form in the diabetic patients may have effects in lipoprotein particle turnover and may contribute to their higher cardiovascular risk.

Key Words: diabetes, apo A-I, HDL, LDL, proteomic studies.

INTRODUCTION

Atherosclerotic cardiovascular disease is the leading cause of mortality in diabetic patients.[1] Indeed, patients with diabetes show two to three times higher coronary risk than non-diabetic patients.[2-4] Several large clinical trials have demonstrated the importance of plasma lipoproteins in the pathogenesis of coronary artery disease (CAD).[5] It is well known that high levels of LDL are considered a key contributor for the initiation and progression of atherosclerosis development,[6-8] being therefore one of the strongest predictors of CAD, in both diabetic and non-diabetic patients.[9] In contrast, elevated levels of high density lipoproteins (HDL) strongly correlate with reduced cardiovascular risk.[10-12] The structure and cholesterol transport ability of HDL particles are determined by the properties of their exchangeable apolipoprotein components.[13] Apolipoprotein A-I (Apo A-I) is the main protein in HDL representing the 70% of their total protein content and is central to HDL assembly, remodelling and metabolism.[14, 15] In addition to its structural role, Apo A-I has a functional role in reverse cholesterol transport by promoting the efflux of cholesterol from peripheral cells into HDL and activating lecithin-cholesterol acyl transferase (LCAT).[16] During reverse cholesterol transport, Apo A-I transitions from lipid-free proteins to spherical HDL particles through a two-step process.[17] First, lipid-free/lipid-poor Apo A-I acquires phospholipid and cholesterol from ATP-binding cassette transporter A1 (ABCA1)[18, 19] and generates nascent HDL. Second, cholesterol on nascent HDL is esterified by LCAT to yield cholesteryl ester[20, 21] and mature spherical HDL. In pioneering studies we reported that homologous high density lipoproteins, rich in Apo A-I (HDL3), injections in rabbits that did not modify HDL-cholesterol level standard biochemical measurements, could delay atherosclerotic plaque formation and induce atherosclerotic plaque regression.[22, 23] This effect was years latter shown in humans treated with Apo A-I Milano.[24] Apo A-I is also responsible for the recognition of HDL by liver SRB1 receptors.[25-27] Among all apolipoproteins, levels of Apo A-I correlate with the protective effect of HDL against atherosclerosis.[28, 29]

Apo A-I is a single polypeptide chain of 243 amino acid residues and a molecular weight of 28kDa. Besides the wide range of posttranslational modifications that Apo A-I protein has during processing, abnormal circulating Apo A-I variants have been identified. Recently, a C-terminal truncated form has been identified in a Japanese woman that expressed with reduced cholesterol esterification rate and decreased

lecithin-cholesterol acyltransferase activity.[30] It has also been described that chymase has the ability to cleave the C-terminus of Apo A-I at Phe225 reducing its ability to promote the cellular cholesterol efflux.[31]

In diabetic patients, the attention has been focused on non-enzymatic protein glycation and advanced glycation end product (AGE) formation. Both are related to hyperglycaemia in type 1 and type 2 diabetes where AGE adducts are associated to Apo A-I impairing its ability to activate lecithin:cholesterol acyltransferase (LCAT), the enzyme responsible for converting nascent HDL into mature HDL.[32] AGEs seem to accelerate the development of coronary artery disease.[33] In fact, decreased anti-oxidant properties have been reported in type 2 diabetic patients due to changes in HDL composition.[34]

In this study we have investigated Apo A-I forms by proteomic approaches and the influence of hyperglycaemia in the molecular changes of this protein.

METERIALS AND METHODS

Blood collection and sample preparation

Venous blood samples were collected to prepare serum that was aliquoted and stored at -80ºC. For proteomic studies, serum samples, glycosylated serum fractions and HDLs were prepared as previously described.[35, 36]

For LDL protein extraction, the method of Karlsson et al.[37] was used with minor modifications. Briefly, 1mg of LDL (1 g/l apoB) was delipidated by mixing with 14 ml of ice-cold tributyl phosphate:acetone:methanol (1:12:1), incubating for 90 min at -20°C, followed by centrifugation at 2800x g for 15 min. Protein pellets were washed sequentially with 1 ml of tributyl phosphate, acetone, and methanol, and then air dryed. Precipitates were boiled in solution containing 0.325M DTT, 4% chaps, and 0.045M Tris for 3 min, cooled at room temperature, diluted (1:15) in urea/thiourea/chaps solution and incubated at 35ºC for 15 min.

Lipoprotein-depleted serum (after lipoprotein extraction) was prepared for proteomic studies.

Proteomic analysis

Proteomic analysis was performed for complete serum (serum), lipoprotein-depleted serum, HDL and LDL fractions.

Two-dimensional gel electrophoresis (2-DE): For analytical and preparative gels, respectively, a protein load of 120 µg and 300 µg protein of the urea/chaps (for serum and lipoprotein-depleted serum extracts) and urea/thiourea/chaps (for HDL and LDL extracts) were applied to 18-cm dry strips (pH 4-7 linear range, GE Healthcare). Second dimension was resolved in 10% SDS-PAGE gels. In a subset of samples IEF was performed in a zoom of pH between 4.5 and 6.9 and 15% SDS-PAGE gels. Gels were developed by fluorescent staining. For each independent experiment, 2-DE for protein extracts from controls and patients were processed in parallel to guarantee a maximum of comparability. Each 2-DE run was at least repeated twice. Analysis for differences in protein patterns was performed with the PD-Quest 8.0 (BioRad), using a single master that included all gels of each independent experiment. Each spot was assigned a relative value that corresponded to the single spot volume compared to the volume of all spots in the gel, following background extraction and normalization between gels.

Mass spectrometry analysis: Proteins were identified after in-gel tryptic digestion and extraction of peptides from the gels pieces, as previously described,[35,36] by matrix – assited laser desorption/ionization time-of-flight (MALDI-TOF) using an AutoFlex III Smartbeam MALDI-TOF/TOF (Bruker Daltonics). Samples were applied to Prespotted AnchorChip plates (Bruker Daltonics) surrounding the calibrants provided on the plates. Spectra were acquired with flexControl on reflector mode, (mass range 850 - 4000 m/z, reflector 1: 21.06 kV; reflector 2: 9.77kV; ion source 1 voltage: 19 kV; ion source 2: 16.5kV; detection gain 2.37x) with an average of 3500 added shots at a frequency of 200 Hz. Each sample was processed with flexAnalysis (version 3.0, Bruker Daltonics) considering a signal-to-noise ratio over 3, applying statistical calibration and eliminating background peaks. For identification, peaks between 850 and 1000 were not considered as in general only matrix peaks are visible on this mass range. After processing, spectra were sent to the interface BioTools (version 3.2, Bruker Daltonics) and MASCOT search on Swiss-Prot 57.15 database was done (Taxonomy: Homo Sapiens, Mass Tolerance 50 to 100, up to 2 miss cleavage, Global Modification: Carbamidomethyl (C), Variable Modification: Oxidation (M)). Identification was accepted with a score higher than 56. Analysis of the truncated form was investigated using the Sequence Editor tool of BioTools. For theoretical digestion of Apo A-I, the sequence reported as P02647 in Swiss-Prot was used and the parameters for peptide

generation were the same as for MASCOT search. The peptides generated in Sequence Editor were then sent back to BioTools for comparison with the experimental data and MASCOT result.

Western Blot Analysis

LDL protein extracts were resolved by both, 1DE and 2DE under reducing conditions and electrotransferred to PVDF (Polyvinylidene Difluoride) membranes in semi-dry conditions (Semi-dry transfer system, BioRad). Protein detection was performed using rabbit polyclonal antibody (PAb) against total Apo A-I (178422, 1:1000 dilution, Calbiochem) combined with the Dye Double Western Blot kit (Invitrogen). Band fluorescence was determined with Typhoon 9400 (GE Healthcare) and band quantification was performed using ImageQuant TL v7.01. software (GE Healthcare). Protein load was normalized using total protein fluorescent signal.

Study population

The study population comprised a group of 12 diabetic patients with HbA1c > 6% (6 men and 6 women; mean age: 55 ± 3 years) and a control group of 6 healthy no diabetic individuals (3 men and 3 women; mean age: 51 ± 6 years) who attended to a routine health check.

The Ethics Committee of the Santa Creu i Sant Pau Hospital approved the project and the studies were conducted according to the principles of Helsinki's Declaration. All participants gave written informed consent to take part in the study.

Statistical analysis

Data are expressed as median [IQR] unless stated. N indicates the number of subjects tested. Statistical analysis was performed with Stat View 5.0.1 software. Statistical differences between groups were analyzed by the non-parametrics Mann-Whitney or Kruskal-Wallis tests for multiple comparisons. A p value ≤ 0.05 was considered significant.

RESULTS

Apo A-I proteomic profile in AMI and diabetic patients

Proteomic analysis by 2-DE of serum samples revealed Apolipoprotein A-I (Apo A-I) as a cluster of five spots with the same apparent molecular mass of 28 kDa and a pI

range between 5 and 5.75 (Figure 1A and 1B) in both DM and control groups. The Apo A-I proteomic profile in controls was compared with that of DM-patients (compare Figure 1A and 1B). Spot 5 showed a 46% increase ($p < 0.05$) in its intensity in DM-patients when compared to controls (Figure 1C). No differences were detected in total ApoA-I spot intensity between groups.

The proteomic profile of glycosylated serum Apo A-I was studied after purification of glycosylated proteins through its binding to lectins. The glycoprotein-subproteome revealed the presence of spots 1, 2, 3, and 5, while spot 4 was not apparent (Figure 2A). The no glycosylated Apo A-I fraction revealed the presence of the five spots (Figure 2A). The sum of Apo A-I spots corresponding to the glycosylated forms (spot 1, 2, 3, and 5) was significantly and positively correlated with HbA1c levels in DM-patients ($p < 0.05$; Figure 2B).

Apo A-I truncated form

MALDI-TOF analysis in diabetic samples revealed a spot with a pI of 5.75 and an apparent molecular mass of approximately 26 kDa (spot 6 in Figure 3A) that was identified as Apo A-I with a score of 108 (10/23 peaks matched, a sequence coverage of 34.8% and an intensity coverage of 60.5%). This spot showed a 64% increase in its intensity ($p < 0.05$) in DM-patients when compared to controls (Figure 3B).

Tryptic digestion of spots 1-5 of Apo A-I generated a peptide corresponding to aminoacids from residue 37 to 47 that was detected as a peak of 1235.65 m/z. This peak was not detected in the mass spectra obtained from the digestion of spot 6 (Figure 3C). As the molecular weight of spot 6 suggested a truncated form of Apo A-I, we performed an in silico analysis using the Sequence Editor tool of BioTools (Bruker Daltonics, BioTools 3.02) simulating the tryptic sequential digestion of the N-terminal amino acids from Apo A-I. In this analysis we obtained a theoretical set of peptides and their corresponding peak list (Supplemental Table 1) that were compared with the experimentally obtained spectra. The theoretical pick list obtained from a truncation from aa 1 to 38 contained a peak of 1422.735m/z corresponding to aa from 39 to 51 (Figure 4). This peak was included within the non-matched peak list of spot 6. Moreover, the absence of the 38 first amino acids corresponded with a molecular weight of 26.4kDa in agreement of the molecular mass observed in 2-DE gels of the spot 6 (Apo A-I-BCN).

When total serum, lipoprotein-depleted serum, HDL, and LDL samples were analyzed in a 2-DE zoom of pH between 4.7 and 5.9, this Apo A-I-BCN form was detected in both serum and LDL, but not in lipoprotein-depleted serum and HDL samples (Figure 5A). The presence of Apo A-I-BCN form in LDL extracts was confirmed by western blot analysis of both 1D and 2D electrophoresis (Figure 5B).

Clinical characteristics of the patients

Differences between groups in lipid parameters are described in Supplemental Table 2. Diabetic patients were treated as per guideline recommendations.

DISCUSSION

In this study we have analyzed the serum proteome profile of Apo A-I in diabetic patients and found important changes in the isoform distribution pattern. Interestingly, here we have described for the first time an Apo A-I truncated form (that we have called Apo A-I-BCN) that is significantly increased in well treated diabetic patients. Because Apo A-I is modified at a post-translational level, several modifications have been reported until now such as phosphorylation,[38] fatty acid acylation[39] and oxidations.[40] We have found that among the 28kDa Apo A-I forms, spots 1, 2, 3, and 5 represent glycosylated forms, and that the intensity of those glycosylated spots is correlated with the amount of glycosylated haemoglobin. It has been reported that *in vitro* non-enzymatic glycation of Apo A-I with methylglyoxal impairs its anti-inflammatory properties.[41] Specifically, fructose-mediated Apo A-I glycation has been demonstrated to induce the loss of several beneficial functions of Apo A-I and HDL.[42] Indeed, we have detected an increase of the less mature form of Apo A-I in diabetics, because the most basic forms of Apo A-I represent the less mature isoforms of the protein.[43]

Proteolysis is thought to play an important role in most types of amyloidoses, in atherosclerosis and neurodegenerative diseases. In fact, macrophage MMP degrade HDL-associated Apo A-I at both the N- and C-termini in coronary patients.[44] Intriguingly, we have identified a truncated form of Apo A-I lacking residues 1-38 (Apo A-I-BCN) that is significantly increased in serum samples of diabetic patients. Interestingly, we have detected this Apo A-I-BCN form in LDL but not in HDL samples. In vitro studies have demonstrated that the deletion of residues 1-43 from Apo A-I results in a less stable tertiary structure than full-length Apo A-I.[45] Moreover, the

deletion mutants lacking residues 1-41 and 1-59 showed altered lipid-binding ability compared to wild-type Apo A-I.[46] The N-terminal 44 aa of Apo A-I are predicted to be responsible for the stabilization of soluble Apo A-I, and recently, it has been demonstrated that residues 35-49 play a role in the adaptation of Apo A-I to the particle size of HDL.[17] Therefore, the lack of the 38 first amino acids may affect the transition from lipid-free Apo A-I soluble form to spherical HDL particles[47] (Supplemental Figure 1). Indeed, the N-terminal amino acids are necessary for formation of larger HDL complexes, and their absence leads to less stable HDL particles.[48] It has been described that Apo A-I treatment with artificial sweeteners such as aspartame or saccharin resulted in proteolitic cleavage of Apo A-I producing a truncated Apo A-I form that show much lower phospholipid binding activities.[49]

Moreover this truncation may lead to the incorporation of the Apo A-I-BCN form into LDL particles contributing to the already described modified LDL particles in diabetic patients. Several studies have reported different LDL modifications in diabetics[50] due to oxidations[51, 52] and glycosylations,[53] resulting in the presence of more atherogenic LDL particles in diabetic patients.[54, 55] Indeed, the increased cardiovascular risk of diabetic patients has been related to the presence of small, dense LDL particles, which have been shown to be more atherogenic and may explain, in part, the higher risk of those patients[56] even having similar LDL cholesterol plasma concentrations than control subjects.[57]

In summary, despite the limitations of 2-DE approaches for the study of such a complex sample like serum, by careful use of biochemical analysis, we have obtained useful complementary information about posttranslational modifications and the presence of protein variants in diabetes. Thus, we have described for the first time the increase of a 26kDa N-terminal truncated form of Apo A-I that we have named Apo A-I-Barcelona (Apo A-I-BCN) in diabetic patients which may have effects in lipoprotein particle turnover and further contribute to their higher cardiovascular risk. The presence of cleaved Apo A-I variants is closely related to the incidence of chronic degenerative disease as diabetes, atherosclerosis and aging.[49] Therefore the potential role of this truncated Apo A-I form in the pathogenesis of cardiovascular disease deserves further investigation.

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Disclosures

No conflicts to disclose.

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LEGENDS

Figure 1. Representative images of Apo A-I spots in controls (A) and DM-patients (B). (C) Box plot diagrams (median [IQR]) showing the intensity of each spot in both groups. Spot 5 was significantly increased in DM-patients (*p<0.05; Mann-Whitney test).

Figure 2. (A) Representative 2-DE image of the Apo A-I cluster in total glycosylated and non-glycosylated serum fractions. (B) Regression plot of the correlation between the sum of intensities of Apo A-I spots corresponding to glycosylated forms (spots 1, 2, 3 and 5) and HbA1c levels (p<0.05; Spearman correlation).

Figure 3. (A) Representative 2-DE image of the Apo A-I cluster including the Apo A-I spot number 6 identified at 26kDa. (B) Box plot diagram (median [IQR]) showing the truncated Apo A-I spot intensity in the studied groups. DM-patients showed a 2-fold increase in truncated Apo A-I when compared to controls (*p<0.05; Mann-Whitney). (C) MALDI-TOF spectra of the identification of human serum Apo A-I and enlarged image of the determinate region of the spectra where the absence of the 1235.65 m/z peak in the truncated Apo A-I is seen (top panel) when compared to 28kDa spots spectra (bottom panel).

Figure 4. Scheme showing the truncation of aa 1-38 in Apo A-I sequence and the fragments corresponding to the peaks detected in MALDI-TOF analysis. Apo A-I-BCN corresponds to aa from 39-267.

Figure 5. (A) Representative 2-DE image of the Apo A-I cluster in a pI zoom from 4.7 to 5.9 of serum, lipoprotein depleted serum, HDL, and LDL samples. Apo A-I-BCN is detected in both serum and LDL samples, but not in lipoprotein-depleted serum and HDL fractions. (B) Representative WB image of 1DE and 2DE of Apo A-I in LDL samples. In both images two forms of 28 and 26 kDa are apparent.

Figure 1

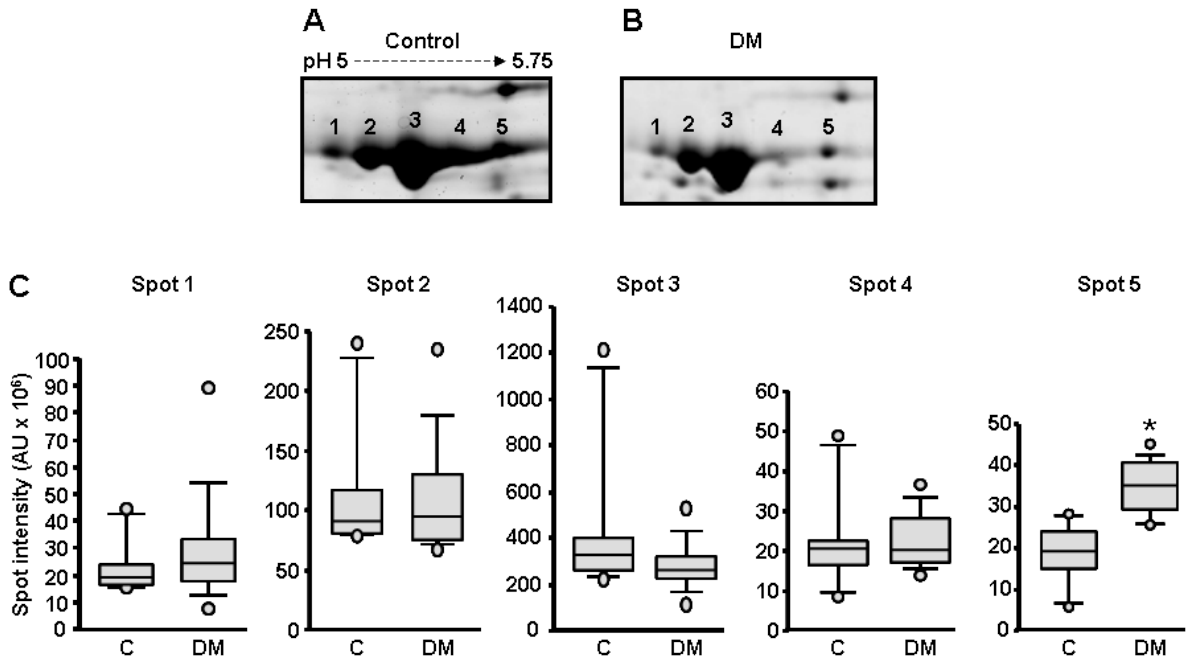

A
Control
pH 5
5.75
1 2 3 4 5
B
DM
1 2 3 4 5
C
Spot 1
Spot 2
Spot 3
Spot 4
Spot 5
Spot intensity (AU x 10⁶)
C
DM
*

Figure 2

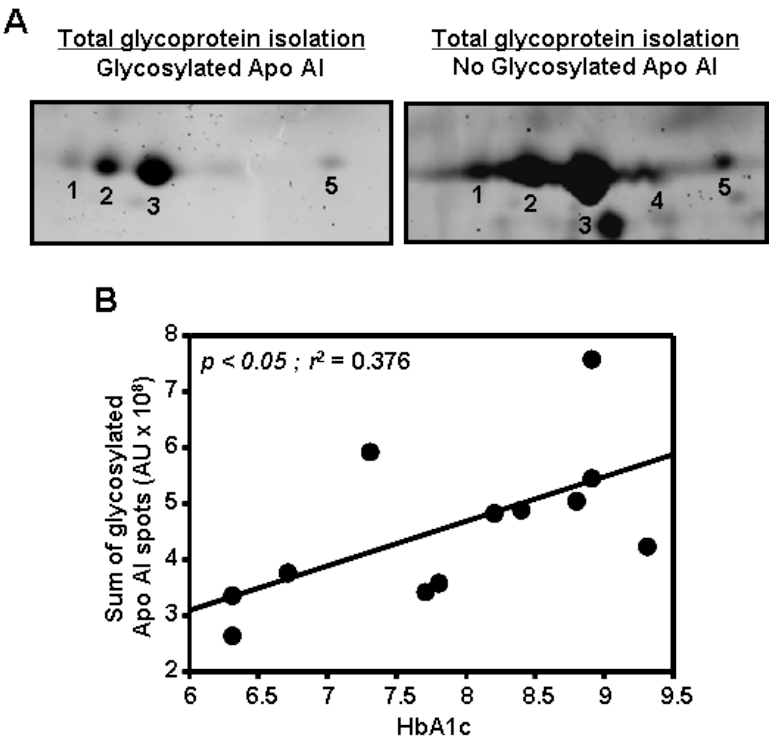

A
Total glycoprotein isolation
Glycosylated Apo AI
Total glycoprotein isolation
No Glycosylated Apo AI
1
2
3
5
1
2
3
4
5
B
p < 0.05 ; r² = 0.376
Sum of glycosylated Apo AI spots (AU x 10⁸)
HbA1c
2
3
4
5
6
7
8
6
6.5
7
7.5
8
8.5
9
9.5

Figure 3

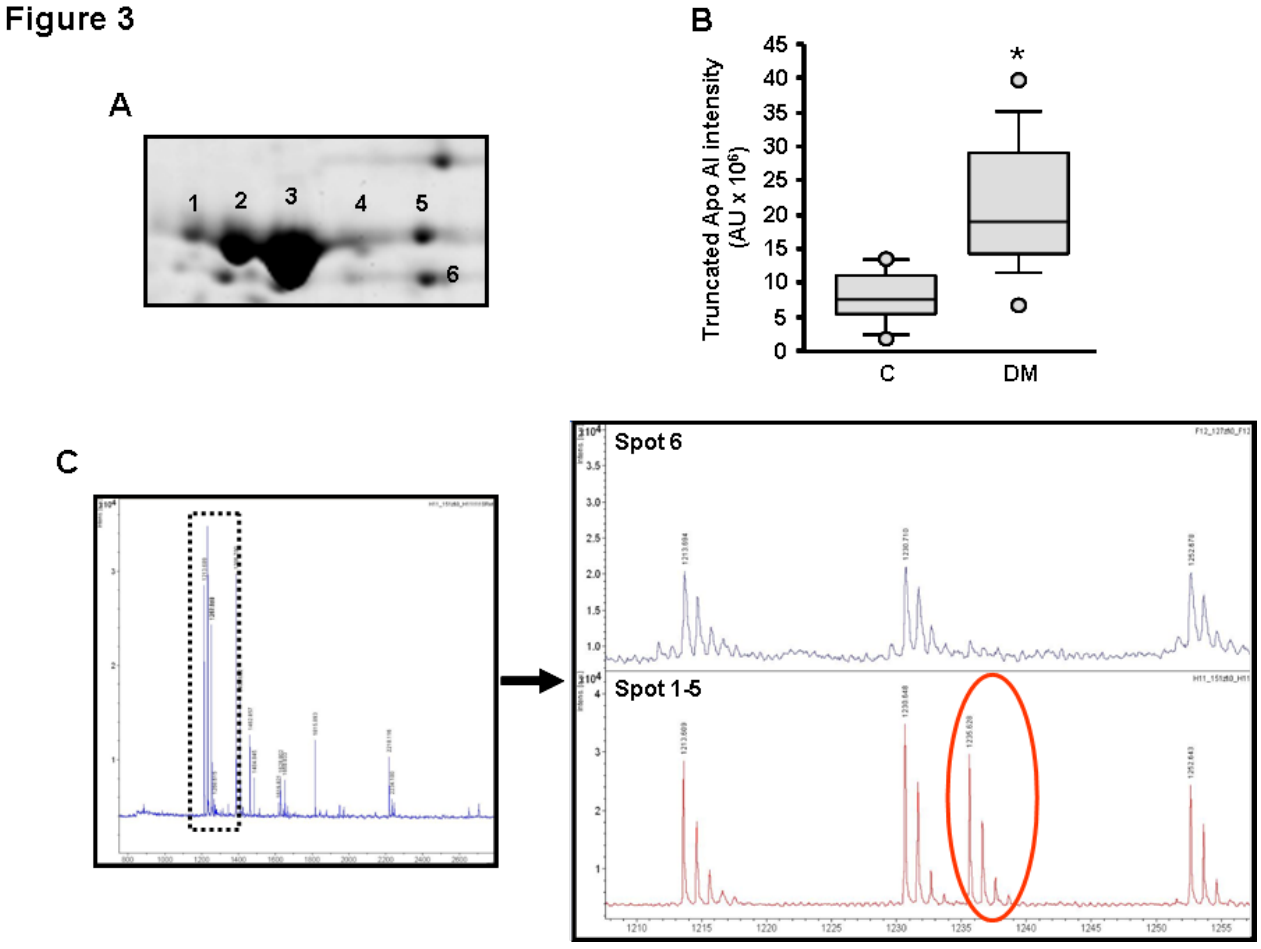

A
1
2
3
4
5
6
B
Truncated Apo AI intensity (AU x 10⁶)
45
40
35
30
25
20
15
10
5
0
*
C
DM
C
Spot 6
Spot 1-5
1210
1215
1220
1225
1230
1235
1240
1245
1250
1255

Figure 4

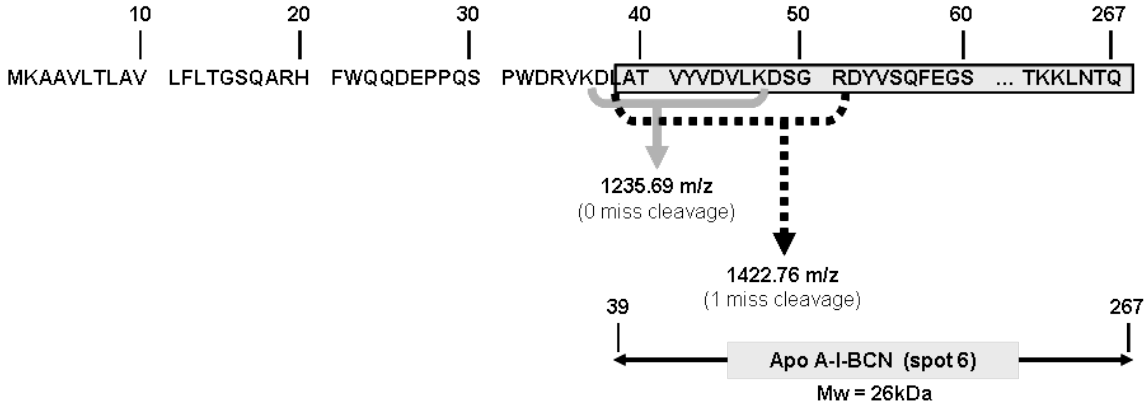
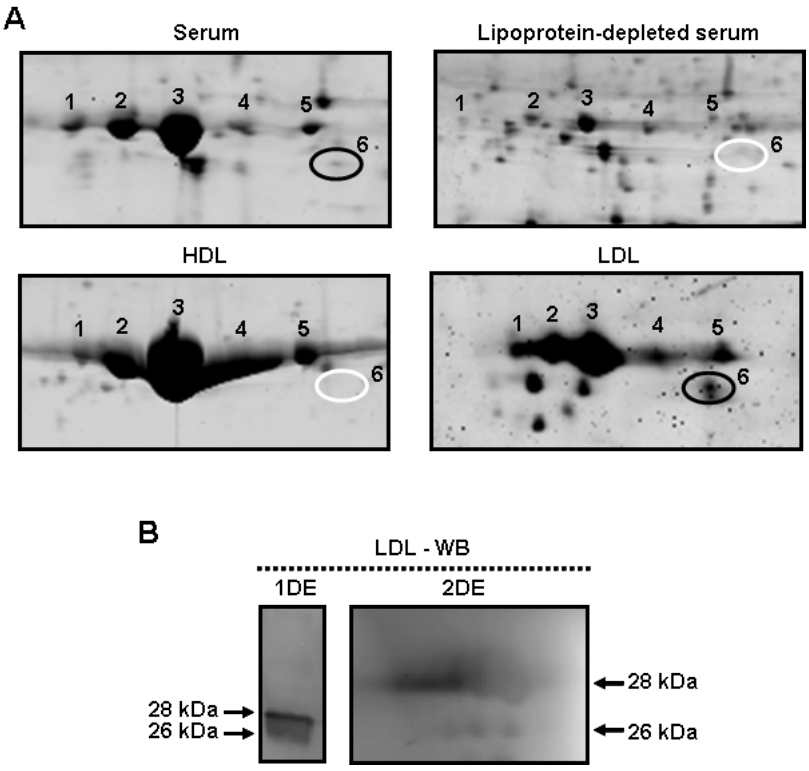

10
20
30
40
50
60
267
MKAAVLTLAV
LFLTGSQARH
FWQQDEPPQS
PWDRVKDLAT
VYVDVLKDSG
RDYVSQFEGS
... TKKLNTQ
1235.69 m/z
(0 miss cleavage)
1422.76 m/z
(1 miss cleavage)
39
267
Apo A-I-BCN (spot 6)
Mw = 26kDa

Figure 5


A
Serum
Lipoprotein-depleted serum
HDL
LDL
1
2
3
4
5
6
B
LDL - WB
1DE
2DE
28 kDa
26 kDa
28 kDa
26 kDa

SUPPLEMENTAL MATERIAL

Supplemental TABLE 1: Peak list obtained in the simulated tryptic digestion of Apo A-I using Sequence Editor tool of BioTools (Bruker Daltonics, BioTools 3.02)

No	Range	Mono	Partials	Sequence
44	[103-109]	868.513	1	QKVEPLR
49	[110-117]	872.435	0	AELQEGAR
55	[120-126]	895.476	0	LHELQEK
29	[75- 82]	930.502	1	DLEEVKAK
77	[160-168]	983.613	1	LAARLEALK
1	[1- 9]	1.006.570	0	ATVYVDVLK
65	[138-146]	1.007.562	1	ARAHVDALR
91	[193-201]	1.011.571	0	AKPALEDLR
58	[127-135]	1.030.512	0	LSPLGEEMR
50	[110-119]	1.128.589	1	AELQEGARQK
53	[118-126]	1.151.630	1	QKLHELQEK
80	[164-174]	1.156.620	1	LEALKENGGAR
88	[182-192]	1.214.614	0	ATEHLSTLSEK
94	[202-212]	1.229.702	0	QGLLPVLESFK
34	[83- 92]	1.251.614	0	VQPYLDDFQK
75	[158-168]	1.267.772	2	QRLAARLEALK
63	[136-146]	1.278.691	2	DRARAHVDALR
40	[94-102]	1.282.565	0	WQEEMELYR
70	[147-157]	1.300.641	0	THLAPYSDELR
59	[127-137]	1.301.640	1	LSPLGEEMRDR
23	[64- 74]	1.306.619	1	ETEGLRQEMSK
26	[70- 80]	1.334.639	1	QEMSKDLEEVK
35	[83- 93]	1.379.709	1	VQPYLDDFQKK
97	[213-224]	1.385.708	0	VSFLSALEEYTK
7	[14- 26]	1.399.662	0	DYVSQFEGSALGK
38	[93-102]	1.410.660	1	KWQEEMELYR
83	[169-181]	1.414.695	1	ENGGARLAEYHAK

No	Range	Mono	Partials	Sequence
2	[1- 13]	1.421.751	1	ATVYVDVLKDSGR
32	[81- 92]	1.450.746	1	AKVQPYLDDFQK
47	[105-117]	1.466.784	1	VEPLRAELQEGAR
98	[213-225]	1.513.803	1	VSFLSALEEYTKK
60	[127-139]	1.528.778	2	LSPLGEEMRDRAR
27	[70- 82]	1.533.771	2	QEMSKDLEEVKAK
41	[94-104]	1.538.719	1	WQEEMELYRQK
78	[160-174]	1.567.879	2	LAARLEALKENGGAR
33	[81- 93]	1.578.841	2	AKVQPYLDDFQKK
71	[147-159]	1.584.801	1	THLAPYSDELRQR
13	[32- 45]	1.611.778	0	LLDNWDSVTSTFSK
39	[93-104]	1.666.814	2	KWQEEMELYRQK
45	[103-117]	1.722.938	2	QKVEPLRAELQEGAR
48	[105-119]	1.722.938	2	VEPLRAELQEGARQK
5	[10- 26]	1.814.844	1	DSGRDYVSQFEGSALGK
14	[32- 47]	1.880.963	1	LLDNWDSVTSTFSKLR
56	[120-135]	1.907.977	1	LHELQEKLSPLGEEMR
19	[48- 63]	1.931.927	0	EQLGPVTQEFWDNLEK
81	[164-181]	1.969.038	2	LEALKENGGARLAEYHAK
99	[213-229]	1.970.036	2	VSFLSALEEYTKKLNTQ
8	[14- 31]	1.996.027	1	DYVSQFEGSALGKQLNLK
72	[147-163]	1.996.060	2	THLAPYSDELRQRLAAR
51	[110-126]	2.006.055	2	AELQEGARQKLHELQEK
24	[64- 80]	2.019.978	2	ETEGLRQEMSKDLEEVK
86	[175-192]	2.027.032	1	LAEYHAKATEHLSTLSEK
68	[140-157]	2.063.055	1	AHVDALRTHLAPYSDELR
42	[94-109]	2.133.068	2	WQEEMELYRQKVEPLR

No	Range	Mono	Partials	Sequence
30	[75- 92]	2.164.105	2	DLEEVKAKVQPYLDDFQK
54	[118-135]	2.164.131	2	QKLHELQEKLSPLGEEMR
57	[120-137]	2.179.106	2	LHELQEKLSPLGEEMRDR
17	[46- 63]	2.201.112	1	LREQLGPVTQEFWDNLEK
11	[27- 45]	2.208.143	1	QLNLKLLDNWDSVTSTFSK
89	[182-201]	2.208.175	1	ATEHLSTLSEKAKPALEDLR
92	[193-212]	2.223.263	1	AKPALEDLRQGLLPVLESFK
66	[138-157]	2.290.193	2	ARAHVDALRTHLAPYSDELR
69	[140-159]	2.347.215	2	AHVDALRTHLAPYSDELRQR
6	[10- 31]	2.411.208	2	DSGRDYVSQFEGSALGKQLNLK
12	[27- 47]	2.477.328	2	QLNLKLLDNWDSVTSTFSKLR
95	[202-224]	2.597.399	1	QGLLPVLESFKVSFLSALEEYTK
84	[169-192]	2.611.299	2	ENGGARLAEYHAKATEHLSTLSEK
20	[48- 69]	2.617.266	1	EQLGPVTQEFWDNLEKETEGLR
36	[83-102]	2.644.263	2	VQPYLDDFQKKWQEEMELYR
96	[202-225]	2.725.494	2	QGLLPVLESFKVSFLSALEEYTKK
3	[1- 26]	2.803.403	2	ATVYVDVLKDSGRDYVSQFEGSALGK
18	[46- 69]	2.886.451	2	LREQLGPVTQEFWDNLEKETEGLR

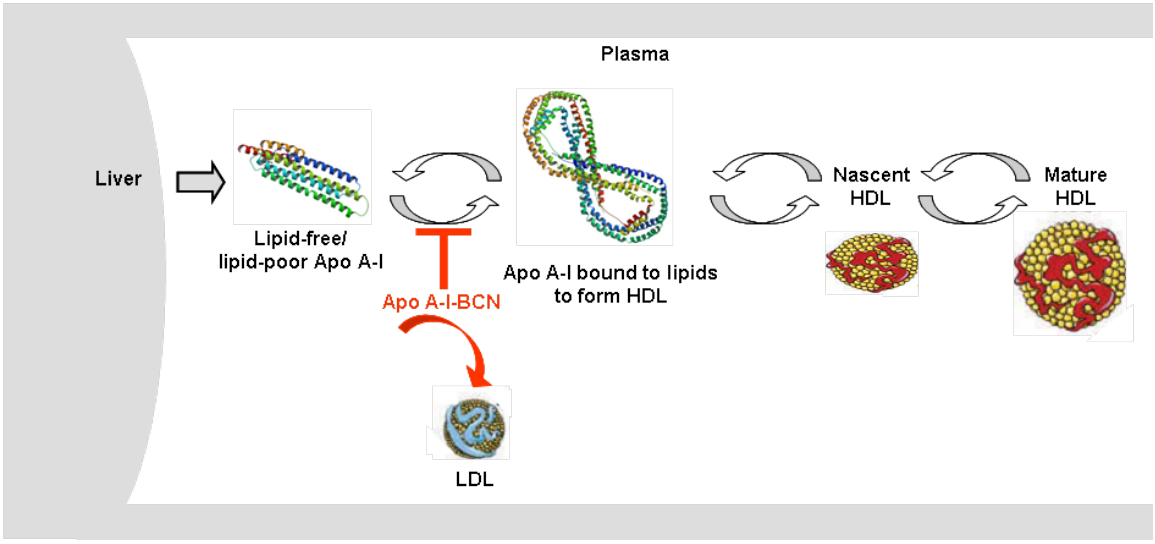
Supplemental TABLE 2: Lipid parameters of the studied population.

	Control (N=6)	DM (N=12)	*p*-value
Total-C (mg/dL)	219 ± 13.1	178 ± 8.9	< 0.05
HDL-C (mg/dL)	50 ± 3.9	59 ± 5.7	NS
LDL-C (mg/dL)	148 ± 10	105 ± 4.7	< 0.05
Trigycerides (mg/dL)	79 ± 5.6	158 ± 53.9	NS

LEGENDS TO SUPPLEMENTAL FIGURES

Supplemental Figure 1. Scheme of the conversion of Apo A-I molecule into mature HDL micelles. Proposed effect of Apo A-I-BCN in lipoprotein particle turnover.

Supplemental Figure 1



ARTÍCULO SEXTO

"Reperfusion triggered stress protein response in the myocardium is blocked by postconditioning. Systems biology pathway analysis highlights the key role of the canonical aryl-hydrocarbon receptor pathway"

Gemma Vilahur[1*], **Judit Cubedo**[1*], Laura Casaní,[1] Teresa Padró,[1] Manel Sabate-Tenas,[4] Juan J. Badimon[3], Lina Badimon[1,2]

*** both authors have contributed equally to this paper**

[1]Cardiovascular Research Center, CSIC-ICCC, Hospital de la Santa Creu i Sant Pau and CIBEROBN-Pathophysiology of Obesity and Nutrition - Barcelona, Spain. [2]Cardiovascular Research Chair, UAB, Barcelona, Spain; [3]AtheroThrombosis Research Unit, Mount Sinai School of Medicine, New York, NY, USA; [4] Cath Lab, Div.Cardioloy, Hospital Clinic, Barcelona, Spain

Enviado

Resumen resultados

El post-condicionamiento isquémico (IPost-Co) rescata el miocardio en riesgo por isquemia e isquemia/reperfusión.

El IPost-Co provoca una disminución del porcentaje del tamaño del infarto.

A nivel molecular IPost-Co induce una disminución de la apoptosis inducida por reperfusión tanto de la vía intrínseca (Fas, caspasa-8, NF-κB) como de la extrínseca (Bax, p53, caspasa-3 activa), y previene la activación de Akt/mTOR/$P70^{S6K}$.

Cambios en el patrón proteómico en proteínas cardíacas citoplasmáticas.

Los estudios proteómicos revelan que 23 proteínas miocárdicas citoplasmáticas están expresadas de forma diferencial entre los grupos control, isquemia, isquemia/reperfusión e IPost-Co. La isquemia y la posterior reperfusión inducen mayoritariamente una disminución en las proteínas citoplasmáticas, mientras que el

IPost-Co revierte el 43% de estos cambios e induce un incremento en 10 proteínas citoplasmáticas cardíacas, entre las cuáles se encuentran 2 proteínas de estrés, la Hsp90 y la endoplasmita, siendo la primera la que muestra una disminución más marcada.

Análisis *in silico* de las vías modificadas y validación de los resultados.

El análisis bioinformático de las proteínas diferenciales (*Ingenuity System Pathway Analysis*) revela que las proteínas citoplasmáticas diferenciales están relacionadas significativamente con la apoptosis. Concretamente la proteína de estrés Hsp90 está involucrada en la vía de señalización del receptor de hidrocarburo de arilo (AhR) que está relacionada con el daño celular. La implicación de la vía del AhR se valida mediante la disminución observada en los niveles de proteína de AhR y también en los niveles de RNA del factor de translocación nuclear del hidrocarburo de arilo (ARNT/Hif1β). Además el IPost-Co también normaliza la disminución inducida por I/R en la β-tubulina, proteína que se ha propuesto juega un papel en la vía del AhR impidiendo la translocación del ARNT al núcleo.

Reperfusion triggered stress protein response in the myocardium is blocked by post-conditioning. Systems biology pathway analysis highlights the key role of the canonical aryl-hydrocarbon receptor pathway.

By

Gemma Vilahur[1]*, **Judit Cubedo**[1]*, Laura Casaní,[1] Teresa Padró,[1]
Manel Sabate-Tenas,[4] Juan J. Badimon[3], Lina Badimon[1,2]

From:

[1]Cardiovascular Research Center, CSIC-ICCC, Hospital de la Santa Creu i Sant Pau and CIBEROBN-Pathophysiology of Obesity and Nutrition - Barcelona, Spain.
[2]Cardiovascular Research Chair, UAB, Barcelona, Spain; [3]AtheroThrombosis Research Unit, Mount Sinai School of Medicine, New York, NY, USA; [4] Cath Lab, Div.Cardioloy, Hospital Clinic, Barcelona, Spain;

*** both authors have contributed equally to this paper**

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Correspondence to:

Prof. Lina Badimon
Cardiovascular Research Center, c/Sant Antoni MªClaret 167, 08025 Barcelona, Spain.
Phone: (34) 935565880. Fax: (34) 935565559.
E-mail: lbadimon@csic-iccc.org

ABSTRACT

Aims: Ischemic post-conditioning (IPost-Co) exerts cardioprotection by diminishing ischemia/reperfusion injury. Yet, the mechanisms involved in such protection remain largely unknown. We have investigated the effects of IPost-Co in cardiac cells and in heart performance using molecular, proteomic and functional approaches.

Methods and Results: Pigs underwent 1.5h mid-LAD balloon occlusion and then were sacrificed without reperfusion (ischemia; n=7), subjected to 2.5 h of cardiac reperfusion and sacrificed (n=5); or subjected to IPost-Co before reperfusion and sacrificed 0.5 h (n=4) and 2.5h (n=5) afterwards. A sham-operated group was included (n=4). Ischemic- and non-ischemic myocardium was obtained for molecular/histological analysis. Proteomic analysis was performed by bi-dimensional electrophoresis followed by MALDI-TOF identification. Potential protein networks involved were identified by bioinformatics and Ingenuity Pathway Analysis (IPA). Cardiac function was assessed by echocardiography. IPost-Co diminished (up to 2.5h) reperfusion-induced apoptosis of both the intrinsic and extrinsic pathways whereas did not affect reperfusion-induced Akt/mTOR/P70S6K activation. Proteomic studies showed that IPost-Co reverted 43% of cardiac cytoplasmic protein changes observed during ischemia and ischemia+reperfusion. Systems biology assessment revealed significant changes in the aryl-hydrocarbon receptor (AhR) pathway (cell damage-related). Bioinformatic data were confirmed since the expression of HSP90, AhR, ANRT, and β-tubulin (involved in AhR-signalling transduction) were accordingly modified after IPost-Co. IPost-Co rescued 52% of the left ventricle-at-risk compared to reperfusion alone and resulted in a ≈30% relative improvement in LVEF (P <0.05).

Conclusions: IPost-Co improves cardiac function post-MI and reduces reperfusion-induce cell damage by downregulation of the AhR-signalling transduction pathway ultimately leading to infarct size reduction.

Key words: ischemic post-conditioning; proteomics; cell damage; AhR pathway

INTRODUCTION

Reduction of the burden of ischemia-reperfusion (I/R) injury is a major challenge of several treatments for cardiovascular diseases. Most of the detrimental effects of I/R are triggered within the first minutes following the reopening of the occluded artery and overall lead to irreversible cardiomyocyte death.[1, 2] Yet, apoptosis is also executed upon reperfusion likely contributing to the final extent of myocardial infarction.[3-5] Unlike many interventions that have proven to afford cardiac protection in animal models and failed to do so in humans, the phenomenon of ischemic preconditioning (brief periods of I/R before sustained ischemia) has been shown to protect the human myocardium in several clinical presentations of I/R including cardiac surgery, pre-infarction angina, and angioplasty.[6-8] However, the clinical utility of ischemic preconditioning is limited by the need to apply such intervention prior acute infarction. Interestingly, within the last decade, many experimental9 and proof-of-concept human studies[10, 11] have supported that the application of a similar regimen immediately after sustained ischemia (ischemic post-conditioning; IPost-Co) exerts cardioprotective effects limiting infarct size. IPost-Co-related cardioprotective mechanisms are currently under investigation. Emerging data are providing evidence that IPost-Co-triggered cytoplasmic effects mostly converge to inhibit the mitochondrial permeability transition pore (MPTP) in order to partly rescue jeopardized cardiomyocytes.[9, 12, 13] For instance, IPost-Co has shown to activate via membrane-receptor the pro-survival signal transduction pathway RISK preventing MPTP opening thus limiting irreversible cardiac injury.[14, 15] Nevertheless, whereas most studies in rodents and isolated ischemic hearts have clearly demonstrated that infarct size reduction by IPost-Co is RISK-dependent, recent studies in pigs have failed to implicate components of the RISK pathway as mediators of IPost-Co protection[13, 16-19] and have supported a causal role for STAT3 (signal transducer and activator of transcription 3) activation in mediating cardioprotection through ameliorating mitochondria function.[20] Such discrepancies have underscored the differences between experimental models (in vivo vs in vitro), species (rodents vs pigs) and/or protocols used (ischemia severity and duration, IPost-Co algorithms, etc).[21] More importantly, such observations support the need of further studies in clinically-relevant experimental approaches/models to unravel the alterations and signal transduction pathways that may account to the detected cardioprotection afforded by IPost-Co in the clinical scenario.[12, 13] As such, a recent study in rats has shown the capability of IPost-

Co to reduce the rate of myocyte apoptosis likely via RISK/mTOR-dependent mechanisms.[22] However, whether IPost-Co diminishes apoptosis in a human-resembling experimental model remains to be addressed. In this regard, we have recently reported in pigs that reperfusion is the main trigger of caspase-3 in the setting of I/R.[3] On the other hand, although IPost-Co has already shown to reduce infarct size, the effect of IPost-Co on cardiac performance and on the mechanisms underlying its protective effects remains largely unknown.

We sought to evaluate, in a pig model of I/R, the effect of IPost-Co in cardiac cell damage and on cardiac performance. To this end we have evaluated by proteomics, the coordinated changes that occur in myocardial-related cytoplasmic proteins upon IPost-Co and by echocardiographic assessment cardiac performance.

METHODS

For expanded and detailed materials and methods please refer to the **supplemental file.**

Experimental Model

Twenty-five swine were acclimated for 1week before any experimental procedure. Thereafter, twenty-one animals were randomized to one of the following four groups: I) closed-chest 1.5h left anterior descending (LAD) coronary occlusion with no reperfusion (n = 7); II) 1.5h LAD occlusion followed by 2.5h reperfusion (n = 5); III) 1.5h LAD occlusion followed by IPost-Co and 0.5h reperfusion (n = 4), and IV) 1.5h LAD occlusion followed by IPost-Co and 2.5h reperfusion (n = 5). The IPost-Co protocol was induced by 6 cycles of 20seconds of reperfusion and 20seconds of re-occlusion at the onset of reperfusion as previously reported.[18, 21] A sham-operated group of animals (n = 4) following the same operating procedure without ischemia was included.

The study protocol was approved by an institutional animal research committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Myocardial Ischemia/Reperfusion Model and Echocardiography

AMI was experimentally induced as we have previously described.[23, 24] Briefly, anesthesia was induced by intramuscular injection of zoletil® (7mg/Kg), domtor® (7mg/Kg), and atropine (0.03mg/Kg). Animals underwent endotracheal intubation, and

anesthesia was maintained by isofluorane inhalation (2%). Continuous infusion of amiodarone (300mg, 75mg/h) was initiated at the beginning of the procedure in all pigs as prophylaxis for malignant ventricular arrhythmias. These amiodarone doses do not alter haemodynamic parameters.24 Angiographic images were used to guide angioplasty balloon placement (below the first diagonal branch) and balloon occlusion was maintained for 1.5h. Thereafter, animals were distributed to one of the four groups described above. A sham-operated group was also performed.

Heart rate and ECG were monitored throughout the experimental procedure. We used 2D echocardiograms (Phillips iE33) to assess left ventricle ejection fraction (LVEF) in all animals before coronary occlusion (baseline), 1.5h post-ischemia (before reperfusion), and at the end of the reperfusion period (sacrifice). In order to reduce the variability echocardiographic examinations were all performed by the same professional trained in echocardiographic measurements and blind to experimental approaches.

Sample collection

Evan's Blue dye was injected in anesthetized animals to outline the area-at-risk (AAR) after which the animal hearts were arrested, rapidly excised. Hearts were sectioned so that consecutive slices were alternatively collected for infarct size analysis (TTC; see below) and cellular/molecular/proteomic studies of the ischemic (IM) and non-ischemic (NIM) myocardium.

Morphometric determination of infarct size by TTC

The impact of IPost-Co on limiting infarct size was evaluated by TTC staining that was performed in the excised myocardium of those animals allowed to be reperfused for 2.5h (with and without IPost-Co) to ensure an accurate infarct size assessment.[24]

Protein extraction from Myocardial Tissue

Frozen myocardial tissue samples from the IM and NIM of all animal groups were pulverized and homogenously distributed in different aliquots for RNA (Tripure® isolation reagent) and protein extraction (lysis buffer). For proteomic studies, pulverized tissue from the IM was homogenized in urea/thiourea buffer and protein concentration was measured with 2D-Quant Kit.

Mitochondrial extracts were obtained as previously described for apoptotic marker analysis.[25]

Molecular Analysis

Gene expression analysis: We analyzed by real-time PCR (Applied Biosystems and TaqMan RT-PCR) mRNA levels for: 1) apoptotic-related markers of both the extrinsic (Fas Receptor/CD95, caspase-8, and NFκ-B) and intrinsic (Bax, Bcl-2, and P53) pathways and the final irreversible executor caspase-3; 2) the RISK component PI3K; and 3) the aryl hydrocarbon nuclear translocator (ARNT or Hif1β).

Western Blot analysis: IM and NIM extracts were subjected to SDS-PAGE, blotted to nitrocellulose membranes and incubated with: 1) apoptosis markers (truncated-caspase-3 and p53 phosphorylated on Thr155); 2); RISK pathway (Akt/PKB phosphorylated on Ser473, Akt/PKB) and 3) myocyte hyperthrophy pathway (mTOR phosphorylated at Ser2448, and P70S6K phosphorylated at Thr389); 4) and aryl-hydrocarbon receptor (AhR). β-actin was used for protein loading control. Isolated mitochondrial extracts were processed similarly and membranes incubated against Bcl-2 phosphorylated at Ser87.

Caspase-8 activity assessment

Caspase-8 activity was also assessed in the IM following the manufacturer's instructions, read by optical density at 405 nm, and expressed as arbitrary units.

TUNEL staining

Apoptosis assessment in the IM was also performed by dUTP nick-end labeling (TUNEL) using an apoptosis detection kit according to the manufacturer's protocol (Chemicon Inc.). The number of TUNEL-positive cardiomyocytes was expressed as mean number/100 cells/microscopic field (mean 5 fields/animal). Around 140 cells were counted per field.

Proteomic analysis: two-dimensional gel electrophoresis and mass spectrometry analysis

Myocardial proteomic changes induced by ischemia and full reperfusion (2.5h) with respect to sham-operated animals were firstly investigated and then compared to that of IPost-Co. To that end, protein extracts from the IM of sham-operated animals, animals subjected to ischemia, ischemia+2.5h reperfusion and ischemia+IPost-Co+2.5h

reperfusion were separated by two-dimensional electrophoresis (2-DE). Three animals of each group were analyzed. Spot patterns were analyzed for differences using PD-Quest 8.0 (BioRad). Protein spots of interest were excised for identification by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF).[26, 27]

In-silico bioinformatic analysis

A systems biology approach was applied with Ingenuity System Pathway Analysis (IPA) software to determine the statistically significant neural networks and canonical pathways in which the identified proteins were involved.

Statistical Analysis

Because data were not normally distributed as observed by applying the Shapiro-Wilk test, a non-parametric statistical analysis was applied and results are reported as medians and interquartil range [IQR]. For independent factors (comparisons between groups) we performed Kruskal-Wallis and Mann-Whitney analysis; for repeated measurements (ischemic vs non-ischemic and different time-points) Wilcoxon and Friedman analysis. All statistical tests conducted were two-sided and $P < 0.05$ was considered significant. Statistical analysis was performed using SPSS (version 19.0).

RESULTS

IPost-Co limits infarct size

AAR was similar among the different animal groups achieving 34 [32-35] % of the LV. Infarct size was 87.9 %[86.8-90.6] of the AAR in 2.5h reperfused animals and 36.2 % [32.3-39.5] of the AAR in IPost-Co + 2.5h-reperfusion animals. IPost-Co, then, resulted in a 52% salvage of the left ventricle-at-risk as compared to reperfusion alone ($P < 0.05$).

Beneficial effect of IPost-Co on cardiac contractile function

All groups showed a similar mean heart rate throughout the procedure (Table 1A). As to cardiac performance, 1.5h of ischemia markedly and similarly diminished LVEF in all animals visualized by a marked akinesia. No changes in global cardiac function were detected in those subjected to reperfusion (Table 1B). In contrast, IPost-Co resulted in a 10% cardiac improvement (at 0.5h-reperfusion $P = 0.067$; at 2.5h-reperfusion $P < 0.05$),

which corresponds to an almost 30% relative increase in LVEF, respectively, with respect to all the other animals.

IPost-Co halts reperfusion-induced apoptosis execution

Real-Time PCR analysis: Gene expression levels in the NIM remained unaltered among the different animal groups. In the IM, IPost-Co prevented reperfusion-induced upregulation of several genes encoding for molecules downstream to the death receptor pathway and to the mitochondrial-related apoptosis pathway (Figure 1A and 1B, respectively) preventing caspase-3 increase (Figure 1C). Indeed, Fas-receptor/CD95, Caspase-8, NFκ-B, Bax, Bcl-2 and P53 expression levels remained unchanged in all IPost-Co animals with respect to animals subjected to ischemia alone and similar to that of NIM tissue.

Caspase-8 activity assessment: IPost-Co prevented the increase in caspase-8 activity within the IM detected in reperfused animals (0.31[0.29-0.35] AU) leaving, at 2.5h of post-reperfusion levels comparable to that of animals subjected to ischemia alone (0.10[0.08-0.13] vs. 0.10[0.07-0.11] AU).

Protein Analysis by Western Blot: The limited availability of anti-porcine-protein antibodies does not permit to analyze the protein expression pattern of all the evaluated genes. Yet, we could observe that mitochondrial expression of P-Bcl-2, protein levels of active P53 and activation of caspase-3 (Figure 2A) that were markedly enhanced in IM by reperfusion ($P < 0.05$), remained unchanged in all those animals subjected to IPost-Co ($P = 0.754$ vs ischemia alone) and with expression levels similar to those encountered in the NIM.

TUNEL staining: As shown in Figure 2B and in agreement with the detected levels of the apoptotic executor truncated caspase-3, IPost-Co resulted in low apoptotic cell counts within the IM identified by TUNEL staining as compared to I/R. The group of animals only subjected to ischemia, as expected, also displayed a low TUNEL detection.

IPost-Co effects on Mammalian target of rapamycin (mTOR) pathway activation

IPost-Co animals at 0.5h post-reperfusion showed an increase in PI3K and in Akt/mTOR/P70S6K phosphorylation that was comparable to that of animals subjected to 2.5h reperfusion without IPost-Co (Figure 3A, B). However, IPost-Co animals at 2.5h post-reperfusion showed reduced levels of protein phosphorylation. No changes were observed in NIM.

Effect of IPost-Co in swine myocardial proteome

Proteomic analysis of IM revealed that at least 23 cytoplasmic proteins were differentially expressed among the different groups (Supplemental Table 1 and Table 2). Spot intensity values were normalized by the intensity of albumin spot in each gel. The spot intensity in each group was calculated as the median [IQR] of the intensity obtained in the analysis of each independent animal (Supplemental Table 2). Ischemia induced the decrease in the intensity of 7 proteins being α-actin the only protein showing more than 1.7-fold increase in its intensity. I/R was associated with a reduction (1.7- to 3.3-fold) in the intensity of 13 cytoplasmic proteins to levels below sham-operated animals. A strong effect was detected in β-tubulin (3.3-fold decrease). Alpha-actin was still 1.8-fold above sham-operated animal levels after reperfusion.

IPost-Co induced an up-regulation of cytoplasmic proteins, thus the intensity of 10 proteins was more than 1.7-fold increased when compared with the sham-operated group, being 8 of these proteins also increased over 1.7-fold when compared with I/R group. The strongest change induced by IPost-Co referred to the CK-M type (Q5XLD3) that was 14-fold and 6-fold increased when compared with the I/R and the sham-operated groups, respectively. In addition, two stress-related cytoplasmic proteins were markedly down-regulated by IPost-Co. Those proteins were Hsp90 and endoplasmin with a 5- and a 1.9-fold decrease, respectively, as compared to the sham-operated group. After IPost-Co, 6 cytoplasmic proteins that were down-regulated in I/R were normalized reaching sham levels. β-tubulin levels were normalized after IPost-Co.

In-silico bioinformatic analysis: Involvement of AhR pathway

Bioinformatic analysis using the IPA software depicted that proteins showing changes due to ischemia were significantly involved in the molecular and cellular function pathway related to apoptosis ($P < 0.05$; Figure 4). Identified proteins were significantly

associated to the canonical pathway involved in aryl-hydrocarbon receptor (AhR) signalling ($P < 0.0001$; Figure 5A), with significant changes detected in the different groups of animals. Ischemia induced a down-regulation of Hsp90, an up-stream effector of the AhR pathway (Figure 5B) while reperfusion induced an up-regulation (Figure 5C).

When IPost-Co was compared to ischemia (Figure 5D) and I/R (Figure 5E) a strong down-regulation of Hsp90 was evident. If IPost-Co was compared to sham-operated animals Hsp90 was still under normal levels (Supplemental Figure 1A). Reperfusion induced up- and down-regulation of different subunits of Hsp90 (Supplemental Figure 1B) when compared to sham-operated animals.

AhR and ARNT changes in cardiac tissue

To experimentally validate the in silico findings on AhR involvement in IPost-Co, myocardial tissue extracts were specifically analyzed for AhR changes by WB (Figure 5F). I/R induced a 3-fold increase in AhR when compared to ischemia alone, and 6-fold increase when compared to sham-operated animals ($P < 0.05$). The strongest change was seen in IPost-Co where AhR band intensity was 21-fold and 16-fold reduced (for IPost-Co+0.5hR and IPost-Co+2.5hR respectively; $P < 0.05$) in comparison with I/R animals confirming the results obtained in the in-silico analysis. To further asses the role of the AhR signalling pathway, RNA expression of the aryl hydrocarbon nuclear translocator (ARNT) was analyzed (Figure 5G). IPost-Co prevented reperfusion-induced ARNT increase. Specifically, reperfusion was associated with a 4.4-fold change vs sham and 3.4-fold change vs ischemia ($P < 0.05$ for both).

DISCUSSION

Our data shows, in a pig model of 1.5h coronary ischemia and 2.5h reperfusion, that in contrast to fast-onset reperfusion, IPost-Co prevents cardiac extrinsic (Fas-induced) and intrinsic (mitochondrial) apoptotic pathway activation. Moreover, by proteomic approaches and in silico bioinformatic analysis we have shown for the first time that the cytoplasmic-related Hsp90/AhR signalling transduction pathway seems to be involved in the detected IPost-Co protective effects. The clinical evaluation of the hearts showed that the IPost-Co is associated with an acute amelioration in global cardiac performance ultimately limiting infarct size.

IPost-Co has shown to exert cardioprotection and reduce infarct size in both experimental and clinical studies.[10, 11] In fact, IPost-Co is the first method proven to reduce the final infarct size by about 50% in several in vivo models.[21] The benefit of IPost-Co on cardiac function/recovery in the setting of I/R has not been extensively studied. Studies in cats and rodents have reported the capability of IPost-Co to attenuate reperfusion-related ventricular arrhythmias following a short ischemic episode.[28, 29] We provide further evidence, by ultrasound assessment, that IPost-Co after severe ischemia exerts a beneficial effect on global cardiac performance indicating the potential of IPost-Co to attenuate post-ischemic myocardial stunning and favour the recovery of the left ventricle contractile performance.

The mechanisms and signal transduction pathways behind such cardioprotective effects remain largely unknown. Rodent studies have suggested the involvement of RISK and mTOR activation in the salvage of jeopardized myocardium. However, recent previously published data by Heush,[18] indicates that there seems to be no causal role for RISK and/or Akt/mTOR/P70S6K pathways activation in the cardioprotection afforded by IPost-Co in swine, an animal model of higher human resemblance than rodents. Authors report that RISK acutely increases within the first 30min and then declines to baseline levels, as we also find in our studies. Interestingly, they provide evidence that RISK blockade (with Wortmannin and U0126) does not affect IPost-Co-derived cardioprotection. Thereby, these results seem to indicate that RISK is not critical in IPost-Co. In fact, they have recently reported the involvement of STAT3 in mediating IPost-Co cardioprotection by preserving mitochondrial function.[20] We suggest that IPost-Co may also limit lethal reperfusion injury by preventing apoptosis execution. Indeed, I/R is a complex phenomenon that encompasses several changes in the cytoplasm that mostly converge in mitochondrial modulation of cell death. Our proteomic studies revealed important changes in the proteomic profile during ischemia and I/R mainly inducing a decrease in cardiac cytoplasmic proteins. Alpha actin was the only protein showing an important increase likely reflecting a compensatory effect in order to restore myocardial tissue loss. Interestingly, we report that IPost-Co seems to revert and/or prevent 43% of such detected changes. Moreover, in silico bioinformatic analysis showed a significant association between the differentially expressed cytoplasmic proteins and cell damage, being the most important canonical pathway involved the AhR signalling. We have found significant changes in Hsp90 which has been described to participate in the up-stream AhR signalling pathway. AhR resides in

the cytosol associated with an Hsp90 dimmer.[30-32] Upon ligand binding, AhR translocates to the nucleus where it dimerizes with the ARNT protein (also known as Hif1-β).[32, 33] It has been previously shown that reduction of Hsp90 level abrogates AhR function in a yeast model, probably because AhR is either improperly folded or destabilized in the absence of Hsp90.[34] Therefore, we can hypothesize that the detected reduction in AhR and Hsp90 induced by IPost-Co leads to a down-regulation of the AhR signalling pathway eventually blocking intrinsic and extrinsic pro-apoptotic downstream effectors. In fact, we have confirmed this hypothesis by demonstrating the decrease in ARNT gene expression levels after IPost-Co. Moreover, our proteomic analysis reveals that I/R induces a decrease in β-tubulin levels which is reverted by IPost-Co, supporting our proposed involvement of the AhR signalling pathway in IPost-Co cardioprotection. It has been previously suggested that β-tubulin plays a role in AhR signalling by preventing ARNT nuclear translocation.[35] Therefore, altogether, our results suggest that IPost-Co may exert cardioprotective effects by modulating the main effectors of the AhR/ARNT pathway, finally converging in apoptosis prevention (Figure 6). Indeed, the AhR pathway cross-talks with many cellular signal transduction cascades and ultimately leads cells in alternative directions of proliferation, cell cycle arrest, or apoptosis. Thus, apoptotic-related markers of both the extrinsic and the intrinsic pathways are involved in the AhR pathway. Specifically Fas/FasL and Bax, respectively, are down-stream effectors of AhR signalling pathway. In fact, polycyclic aromatic hydrocarbons-mediated activation of the AhR in germinal vesicle stage oocytes triggers transcriptional activation of the bax promoter in an AhR-dependent manner.[36] AhR has also shown to promote Fas-mediated apoptosis in hepatocytes in the absence of exogenous AhR agonists.[37] Here, we report that IPost-Co prevents reperfusion-triggered activation of both the death receptor (Fas/caspase-8) and mitochondrial apoptotic pathways eventually diminishing the detected amount of apoptotic cells in the jeopardized cardiac tissue as confirmed by TUNEL staining. Consequently, no changes were detected in the apoptotic marker Bcl2, a counterbalance pathway of apoptosis execution. Hence, taking into consideration that, on the one hand, the final extent of infarct size is not only determined by the severity of ischemia[38, 39] but also by the pathological processes initiated at reperfusion that provoke additional tissue injury,[5, 40, 41] and, on the other hand, that our data support that apoptosis is mainly a reperfusion-triggered phenomena[3, 42] IPost-Co antiapoptotic mechanisms should be regarded as a potential mechanism to partly explain the detected increase in salvaged

myocardium. The quantitative contribution of apoptosis to final extent of infarct size remains to be determined. Anversa's group reported, in human myocardial samples obtained from the peri-infarcted region of patients who have recently suffered an MI, that around 12% of myocytes showed apoptotic signs emphasizing a potential detrimental effect of apoptosis execution on the final size of infarction.4 Intravenous administration of a caspase inhibitor[43] or the transgene expression of the dominant-negative mutant of Fas-associated DD protein[44] have shown to significantly attenuate cardiomyocyte apoptosis and reduce infarct size. These observations not only suggest a link between cardiomyocyte apoptosis and the extent of myocardial injury but that modulation of the apoptotic signaling (i.e., IPost-Co) may mediate a meaningful anatomic and functional cardiac rescue following I/R injury. Indeed, some indirect beneficial effect on ventricular function could also be expected from infarct size limitation.[45] Yet, it cannot be overlooked that IPost-Co may also confer an immediate cardiac protection by ameliorating myocardial blood flow contributing to the final infarct size reduction.

Some potential limitations of our study deserve comments. As to the potential contribution of drug-related interferences in cardioprotection in our experimental setting, firstly, a post-conditioning effect of isofluorane should not be considered since its administration started much before the induction of ischemia (1.5h) in all animals. Regarding the well-reported volatile anaesthetic-induced pre-conditioning effect, this cannot be omitted, yet all animals were kept under the same conditions throughout the procedure. The same accounts to a potential protective effect of amiodarone on infarct size and the prevention of ischemic arrhythmias.[46] Our observations must also be interpreted in the context of this study design. As such, we ought to provide further understanding on the underlying mechanisms and signal transduction behind IPost-Co in an animal model with human resemblance in order to provide new potential therapeutic targets. This study, however, does not consider important clinical situations found in most STEMI patients. Indeed, the study includes juvenescent animals free from cardiovascular risk factors and hence, does not take into consideration the presence of certain co-morbidities and confounding factors (hyperlipemia, hypertension, diabetes, age, etc) as well as co-medications (adenosine, nitroglycerine, beta-blockers, statins, etc) that have shown to interfere and/or attenuate IPost-Co cardioprotective effects.[47] Finally, despite the small sample size, the behavior was similar between the different animals within each group.

In conclusion, here we show that IPost-Co not only limits tissue cardiac damage but also improves cardiac contractility acutely after MI. In addition we report for the first time that one of the mechanisms of by which IPost-Co may limit apoptotic reperfusion injury involves the AhR signalling pathway.

Conflicting results exist as to the beneficial effect of IPost-Co in acute MI patients, some reporting a reduction on infarct size[10] and others no protection.[48] These conflicting results which may derive from the presence of confounders (co-morbidities, co-medications, etc) raise concerns about the clinical translation of the IPost-Co phenomenon. Yet, also highlights that there is still an important lack of studies addressed towards elucidating and understanding the molecular basis and the mechanisms triggered in vivo upon IPost-Co in large animal models with translational impact. These studies will help to decipher cardioprotective signaling pathways and targets in a model suitable for faster translational impact into the clinical arena. Importantly, the benefits of IPost-Co are not only limited to patients with ACS but also to patients undergoing cardiac surgery, cardiac arrest and transplantation.[45, 49]

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LEGENDS

Figure 1. Effect of IPost-Co on gene expression of different components of the intrinsic (A) and extrinsic (B) apoptotic pathway and Caspase-3 (C) within the ischemic (IM; grey boxes) and non-ischemic (NIM; white boxes) myocardium.
* $P < 0.05$ versus the IM all other groups. † $P < 0.05$ versus NIM. Data are represented as box plot diagram [1.5h I (ischemia), n = 7; 1.5h I+2.5h R (reperfusion), n = 5; 1.5h I+Post-Co+0.5h R, n = 4; 1.5h I+Post-Co+2.5h R, n = 5]

Figure 2. Protein analysis and representative images of mitochondrial P-Bcl-2, P-p53 and truncated caspase3/total caspase3 (A). Apoptosis analysis in the ischemic cardiac region by TUNEL staining (apoptosis: red) and representative images; I: 1.5h I; II: 1.5h I+2.5h R; III: 1.5h I+Post-Co+0.5h R; IV: 1.5h I+Post-Co+2.5h R. (B).
* $P < 0.05$ versus the IM all other groups. † $P < 0.05$ versus NIM. Data are represented as box plot diagram [1.5h I (ischemia), n = 7; 1.5h I+2.5h R (reperfusion), n = 5; 1.5h I+Post-Co+0.5h R, n = 4; 1.5h I+Post-Co+2.5h R, n = 5].

Figure 3. Effect of IPostCo on PI3K gene expression (A) and activation of the Akt/ mTOR/P70S6k pathway (B) in the ischemic (IM; grey boxes) and non-ischemic (NIM; white boxes) myocardium.
* $P < 0.05$ versus the IM of 1.5hI and 1.5h I+Post-Co+2.5h. † $P < 0.05$ versus NIM. Data are represented as box plot diagram (1.5h I, n = 7; 1.5h I+2.5h R, n = 5; 1.5h I+Post-Co+0.5h R, n = 4; 1.5h I+Post-Co+2.5h R, n = 5)

Figure 4. Ingenuity Pathway Analysis network showing the relation between the identified cytoplasmic proteins in ischemic myocardial tissue. Red depict proteins up-regulated and green depict proteins down-regulated due to ischemia. Protein networks are significantly associated with apoptosis ($P < 0.0001$).

Figure 5. (A) Canonical pathway involved in aryl hydrocarbon receptor (AhR) signaling pathway obtained with the IPA software after the analysis of differential proteins in (B) I compared to sham-operated animals; (C) I/R compared with I; (D) IPost-Co compared with I; and (E) IPost-Co compared with I/R. Red: proteins up-

regulated. Green: proteins down-regulated. (F) Representative image of AhR WB analysis and box plot diagram showing band intensities. (G) Box plot diagram showing ARNT/Hif1β mRNA expression levels

* $P < 0.05$ vs Sham; † $P < 0.05$ vs IPost-Co+0.5hR and /or +2.5hR. I: ischemia; R: reperfusion; IPost-Co: ischemic post-conditioning.

Figure 6. Proposed AhR signaling transduction pathway involved in IPost-Co-related cardiac protection. I: ischemia; R: reperfusion; IPost-Co: ischemic post-conditioning. AhR: aryl hydrocarbon receptor; ARNT: aryl hydrocarbon nuclear translocator; Hsp90: heat-shock protein 90.

Table 1

A.

	Baseline (pre-I/R)	Sacrifice
I	75 [74 - 76]	78 [72 - 80]
I+2.5hR	73 [71 - 75]	70 [69 - 72]
I+IPost-Co+0.5hR	75 [73 - 77]	73 [72 - 74]
I+IPost-Co+2.5hR	76 [74 - 77]	70 [69 - 72]

B.

	Baseline (pre-I/R)	1.5h post-AMI	Sacrifice	%Reduction vs Baseline	%Reduction vs post-AMI
I	77 [71 - 80]	40 [39 - 47] *	40 [39 - 47] *	30 [29 - 35]	0 [0 - 0]
I+2.5hR	71 [69 - 72]	36 [33 - 38] *	36 [33 - 38] *	33 [32 - 36]	0 [0 - 0]
I+IPost-Co+0.5hR	71 [67 - 74]	35 [34 - 36] *	46 [43 - 49] *†	35 [32 - 37]	-12 [-13 - -9]
I+IPost-Co+2.5hR	66 [64 - 70]	35 [35 - 35] *	44 [44 - 45] *†	31 [27 - 35]	-9 [-10 - -9]

Table 1. Median [IQR] of heart rate **(A)** and cardiac global function (assessed by left ventricle ejection fraction: LVEF) (**B)** of all animals at the different tested time-points.

* $P < 0.05$ vs baseline; † $P < 0.05$ vs 1.5h Post-AMI. I= Ischemia; R=reperfusion; IPost-Co: ischemic post-conditioning.

Table 2

Protname	I/S	I+R/I	I+R/S	IPost-Co/I+R	Ipost-Co/S
Alpha actin	**↑1.9**	1.0	**↑1.8**	↑1.6	**↑2.8**
Annexin A1	↓1.1	**↓1.8**	**↓2.0**	ND Ipost-Co	ND Ipost-Co
Annexin A11	↓1.2	**↓1.8**	**↓2.0**	**↑5.3**	**↑2.5**
Annexin A2	↑1.5	**↓1.8**	↓1.3	**↑3.4**	**↑2.8**
Cardiac phospholamban	↑1.2	↓1.5	↓1.3	**↑2.6**	**↑2.1**
Cardiac troponin T isoform 1	**↓5.8**	**↑2.1**	**↓2.5**	**↑2.6**	↓1.1
Creatine kinase B-type	**↓2.8**	↑1.4	**↓2.0**	ND Ipost-Co	ND Ipost-Co
Creatine kinase M-type	**↓1.7**	↓1.3	**↓2.0**	**↑14.2**	**↑6.4**
Creatine kinase S-type	**↓2.4**	↓1.1	**↓2.5**	**↑5.1**	**↑1.9**
Cytosolic aspartate aminotransferase	1.0	↓1.6	**↓1.7**	**↑2.0**	↑1.3
Glutathione S-transferase P	↓1.3	↓1.1	↓1.4	**↑1.9**	↑1.4
Lactate dehydrogenase	↓1.4	↑1.3	↓1.1	↑1.4	↑1.2
Malate dehydrogenase	↓1.1	**↓1.8**	**↓2.0**	**↑2.7**	↑1.4
Myosin light chain 2v	**↓1.7**	↓1.1	**↓1.7**	**↑4.4**	**↑2.5**
Myosin light chain 3	↑1.3	**↓2.0**	↓1.4	**↑3.0**	**↑2.0**
PTEN	1.0	1.0	↑1.1	ND Ipost-Co	ND Ipost-Co
Rab GDP dissociation inhibitor beta	↓1.4	↓1.2	**↓1.7**	**↑3.4**	↑1.4
Spectrin beta chain, brain 4	1.0	↑1.4	↑1.3	↑1.6	**↑2.1**
Tubulin beta chain	**↓2.5**	↓1.1	**↓3.3**	**↑3.1**	1.0
T-complex protein subunit epsilon	↓1.3	↓1.6	**↓2.0**	**↑2.9**	↑1.4
Hsp 70kDa	↓1.2	**↓2.0**	**↓2.5**	**↑3.7**	↑1.6
Hsp 90kDa	**↓2.4**	**↑1.8**	↓1.4	**↓3.6**	**↓5.0**
Heat shock protein beta-2	↓1.3	↓1.2	↓1.4	**↑2.5**	**↑1.7**
Endoplasmin	↓1.3	↑1.4	↑1.1	**↓2.0**	**↓1.9**

TABLE 2. Median [IQR] of spot intensity in 2DE analysis of sham, ischemia, ischemia/reperfusion (I/R), and ischemic post-conditioning (IPost-Co) animals. ND = no detected.

Figure 1

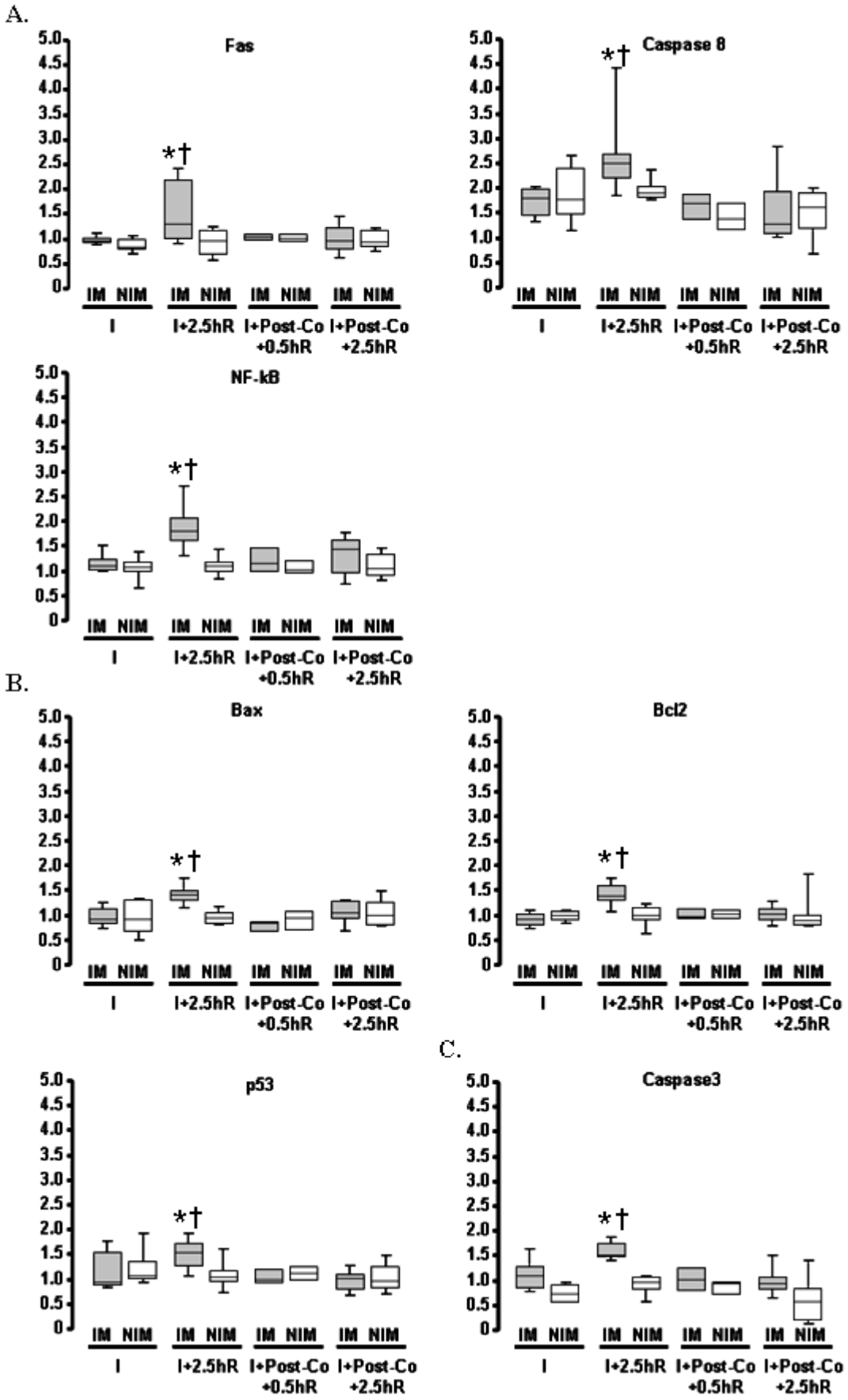
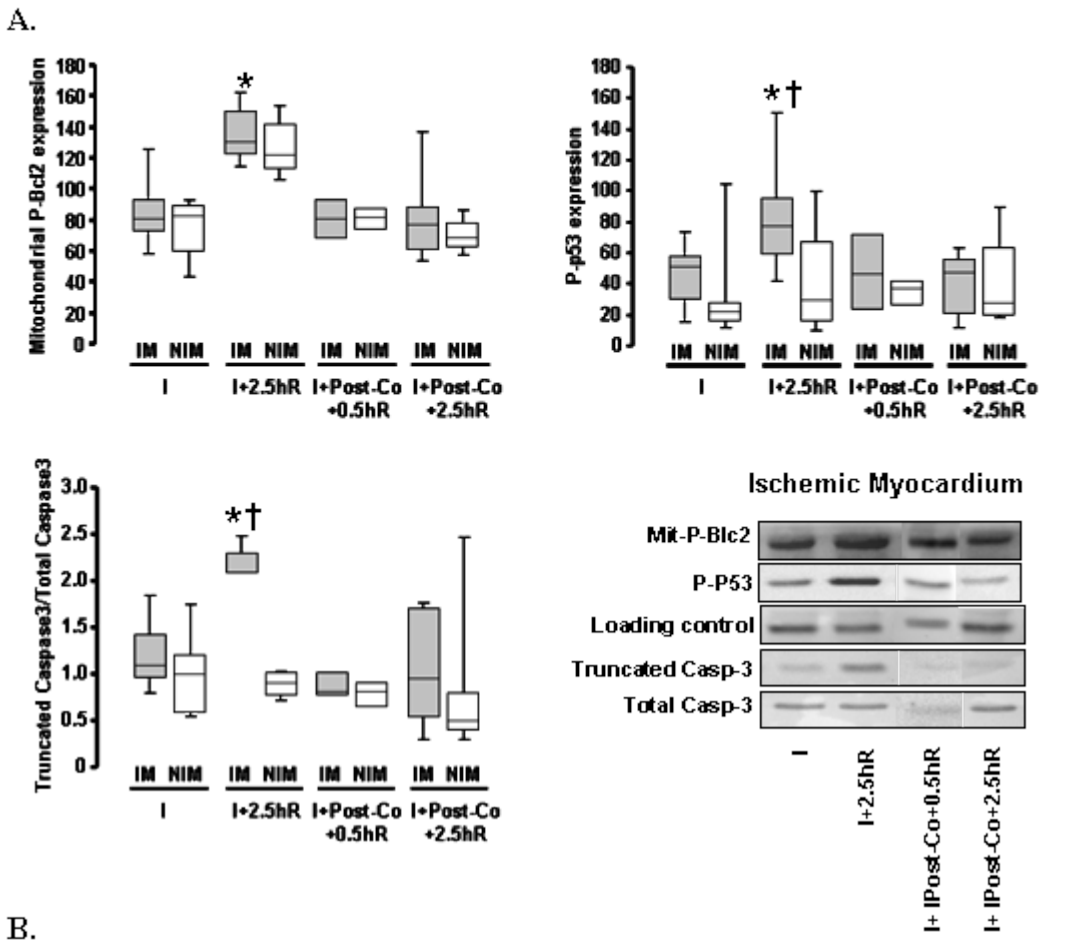

A.
Fas
Caspase 8
NF-kB
B.
Bax
Bcl2
C.
p53
Caspase3
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*†
IM NIM
I
I+2.5hR
I+Post-Co +0.5hR
I+Post-Co +2.5hR

Figure 2


A.
Mitochondrial P-Bcl2 expression
P-p53 expression
Truncated Caspase3/Total Caspase3
IM NIM
I
I+2.5hR
I+Post-Co +0.5hR
I+Post-Co +2.5hR
Ischemic Myocardium
Mit-P-Blc2
P-P53
Loading control
Truncated Casp-3
Total Casp-3
I
I+2.5hR
I+ IPost-Co+0.5hR
I+ IPost-Co+2.5hR

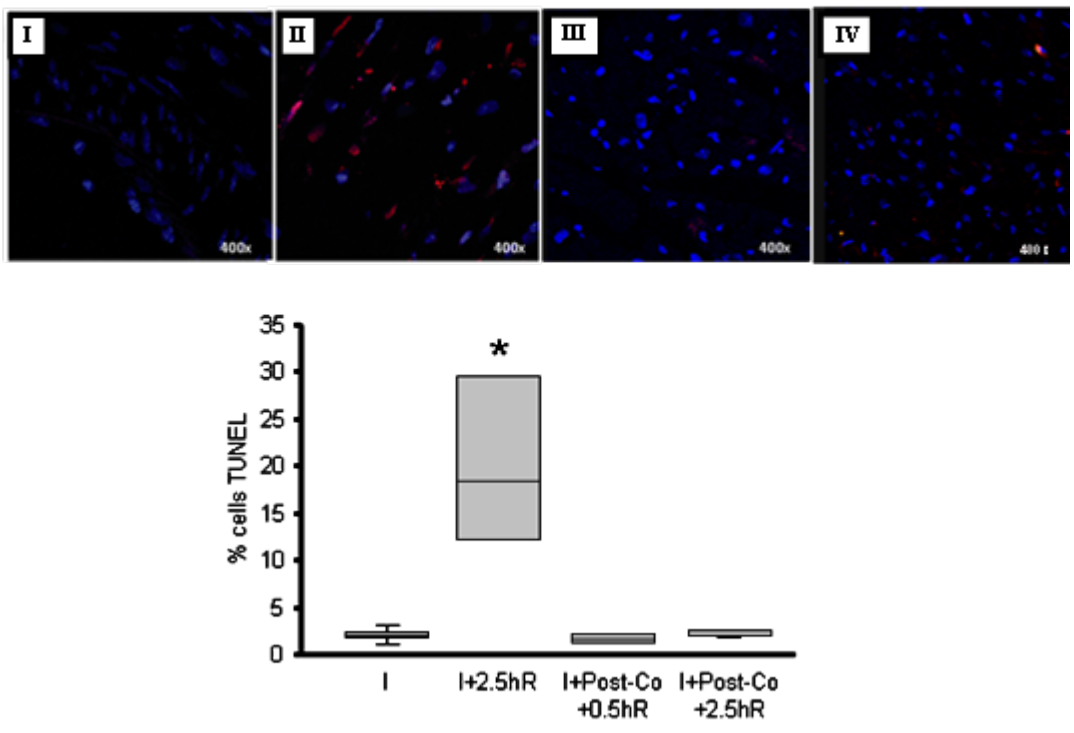

B.
I
II
III
IV
400x
% cells TUNEL
I
I+2.5hR
I+Post-Co +0.5hR
I+Post-Co +2.5hR

Figure 3

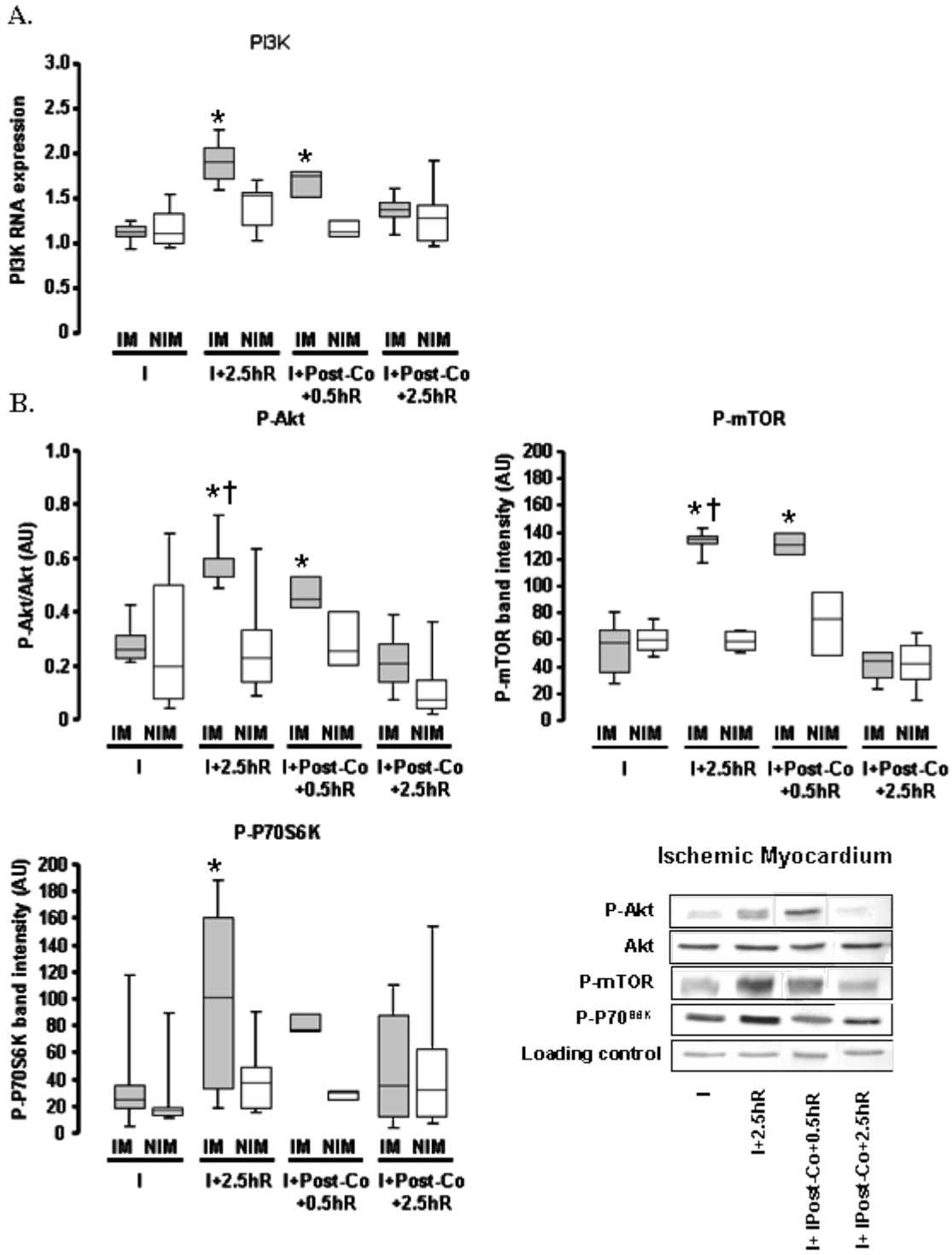

A.
PI3K
PI3K RNA expression
3.0
2.5
2.0
1.5
1.0
0.5
0
IM NIM
I
I+2.5hR
I+Post-Co +0.5hR
I+Post-Co +2.5hR
B.
P-Akt
P-Akt/Akt (AU)
1.0
0.8
0.6
0.4
0.2
0
P-mTOR
P-mTOR band intensity (AU)
200
180
160
140
120
100
80
60
40
20
0
P-P70S6K
P-P70S6K band intensity (AU)
Ischemic Myocardium
P-Akt
Akt
P-mTOR
P-P70 86K
Loading control
–
I+2.5hR
I+ IPost-Co+0.5hR
I+ IPost-Co+2.5hR

Figure 4

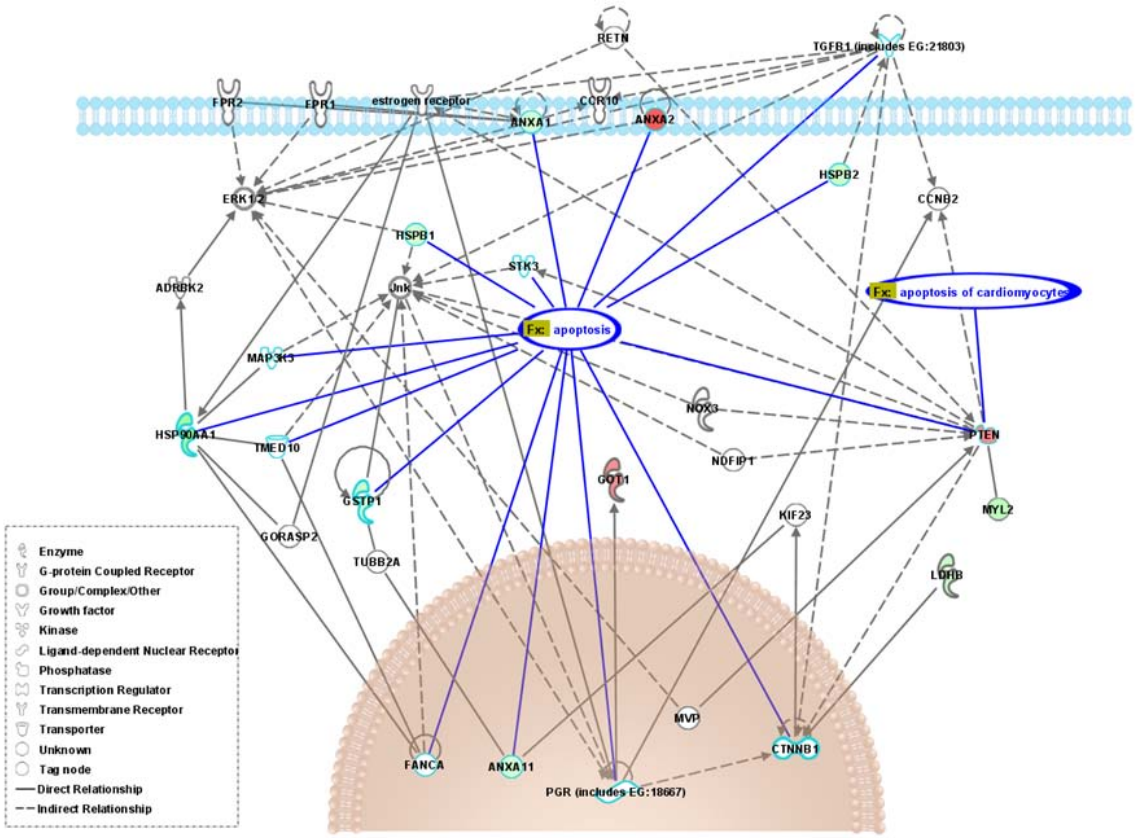

RETN
TGFB1 (includes EG:21803)
FPR2
FPR1
estrogen receptor
ANXA1
CCR10
ANXA2
HSPB2
CCNB2
ERK1/2
HSPB1
STK3
ADRBK2
Jnk
Fx: apoptosis of cardiomyocytes
Fx: apoptosis
MAP3K5
NOX3
HSP90AA1
TMED10
PTEN
NDFIP1
GOT1
GSTP1
KIF23
MYL2
GORASP2
TUBB2A
LDHB
MVP
FANCA
ANXA11
CTNNB1
PGR (includes EG:18667)
Enzyme
G-protein Coupled Receptor
Group/Complex/Other
Growth factor
Kinase
Ligand-dependent Nuclear Receptor
Phosphatase
Transcription Regulator
Transmembrane Receptor
Transporter
Unknown
Tag node
Direct Relationship
Indirect Relationship

Figure 5

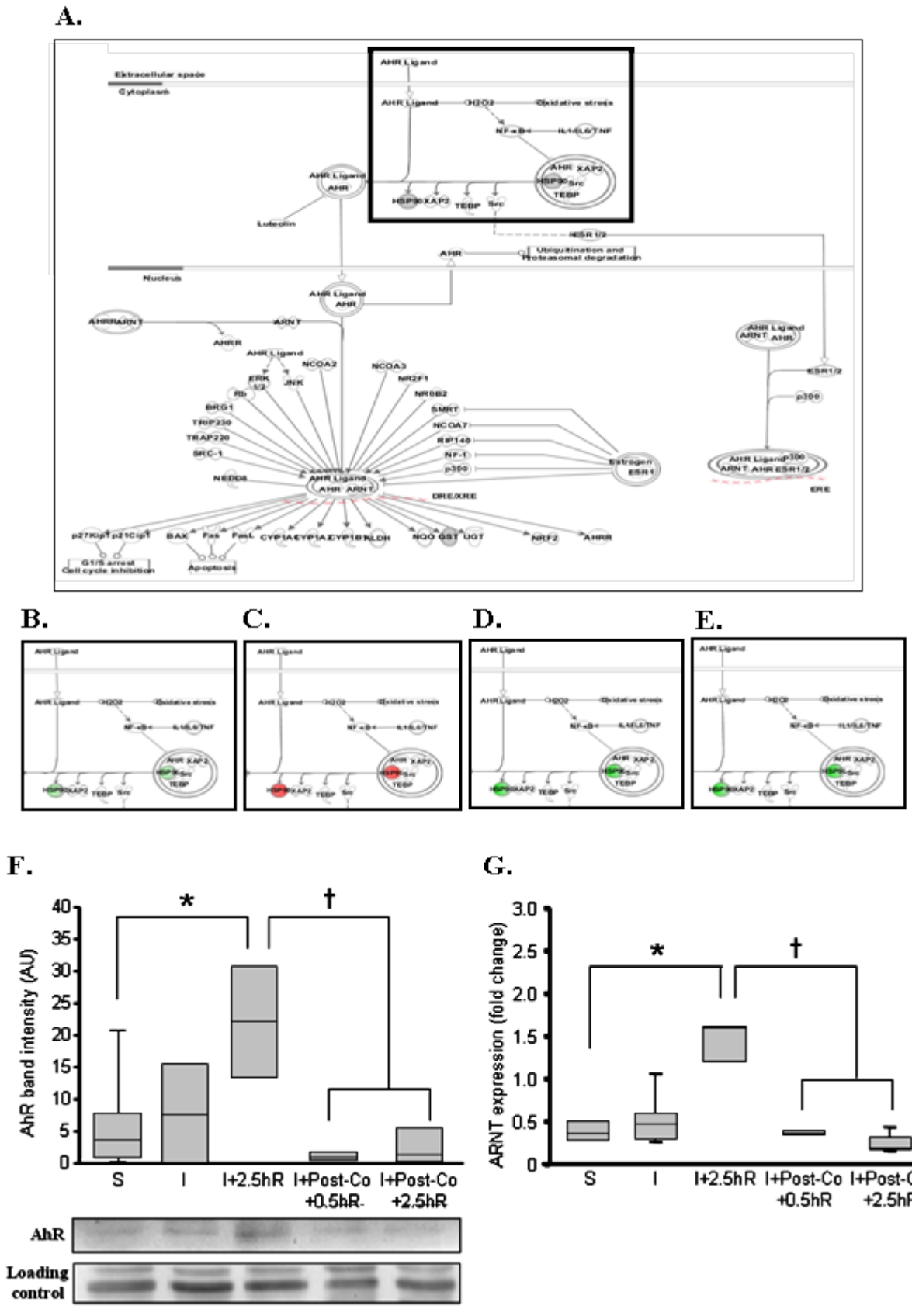
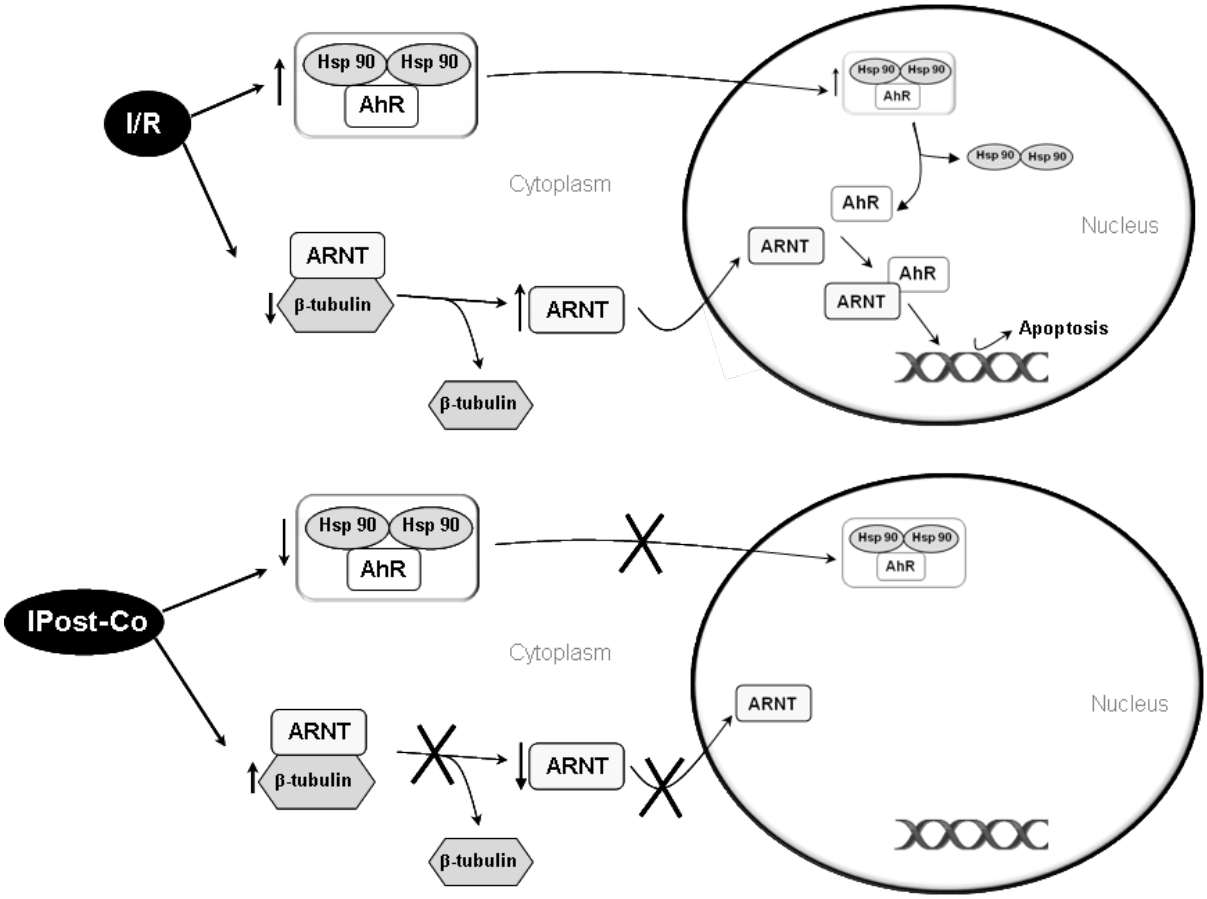

A.
B.
C.
D.
E.
F.
G.
AhR band intensity (AU)
ARNT expression (fold change)
S
I
I+2.5hR
I+Post-Co +0.5hR
I+Post-Co +2.5hR
AhR
Loading control

Figure 6


I/R
Hsp 90
Hsp 90
AhR
Cytoplasm
Nucleus
ARNT
β-tubulin
ARNT
β-tubulin
AhR
ARNT
Apoptosis
IPost-Co

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL MATERIALS AND METHODS

Experimental Model

Twenty-five swine were acclimated for 1week before any experimental procedure. Thereafter, twenty-one animals were randomized to one of the following four groups: I) closed-chest 1.5h left anterior descending (LAD) coronary occlusion with no reperfusion (n = 7); II) 1.5h LAD occlusion followed by 2.5h reperfusion (n = 5); III) 1.5h LAD occlusion followed by IPost-Co and 0.5h reperfusion (n = 4), and IV) 1.5h LAD occlusion followed by IPost-Co and 2.5h reperfusion (n = 5). The IPost-Co protocol was induced by 6 cycles of 20seconds of reperfusion and 20seconds of re-occlusion at the onset of reperfusion as previously reported.1, 2 A sham-operated group of animals (n = 4) following the same operating procedure without ischemia was included.

The study protocol was approved by an institutional animal research committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Myocardial Ischemia/Reperfusion Model and Echocardiography

12h prior to the experimental induction of acute MI (AMI) a loading dose of clopidogrel (150mg) was administered to all animals to avoid thrombotic complications due to catheter manipulation. AMI was experimentally induced as we have previously described.[3, 4] Briefly, anesthesia was induced by intramuscular injection of zoletil® (7mg/Kg), domtor® (7mg/Kg), and atropine (0.03mg/Kg). Animals underwent endotracheal intubation, and anesthesia was maintained by isofluorane inhalation (2%). Continuous infusion of amiodarone (300mg, 75mg/h) was initiated at the beginning of the procedure in all pigs as prophylaxis for malignant ventricular arrhythmias. These amiodarone doses do not alter haemodynamic parameters.[4] Angiographic images were used to guide angioplasty balloon placement (below the first diagonal branch) and balloon occlusion was maintained for 1.5h. Thereafter, animals were distributed to one of the four groups described above. A sham-operated group was also performed.

Heart rate and ECG were monitored throughout the experimental procedure. We used 2D echocardiograms (Phillips iE33) to assess left ventricle ejection fraction (LVEF) in

all animals before coronary occlusion (baseline), 1.5h post-ischemia (before reperfusion), and at the end of the reperfusion period (sacrifice). In order to reduce the variability echocardiographic examinations were all performed by the same professional trained in echocardiographic measurements and blind to experimental approaches.

Sample collection

Evan's Blue dye was injected in anesthetized animals to outline the area-at-risk (AAR) after which the animal hearts were arrested, rapidly excised. Hearts were sectioned so that consecutive slices were alternatively collected for infarct size analysis (TTC; see below) and cellular/molecular/proteomic studies (confocal microscopy, Western Blot, RT-PCR and proteomics) of the ischemic (IM) and non-ischemic myocardium (NIM).

Morphometric determination of infarct size by TTC

The impact of IPost-Co on limiting infarct size was evaluated by TTC staining that was performed in the excised myocardium of those animals allowed to be reperfused for 2.5h (with and without IPost-Co) to ensure an accurate infarct size assessment.4

Protein extraction from Myocardial Tissue

Frozen myocardial tissue samples from the IM and NIM of all animal groups were pulverized and homogenously distributed in different aliquots for RNA and protein extraction. Total RNA was obtained using the Tripure® isolation reagent, according to the manufacturer's instructions. Pulverized tissue aliquots from the IM and NIM were homogenized in lysis buffer, briefly sonicated, and total protein concentration was assessed by using the BCA method (Pierce) and used for western blot.For proteomic studies, pulverized tissue from the IM was homogenized in urea/thiourea buffer (7M Urea, 2M Thiourea, 2%Chaps), incubated 30min in ice and centrifuged at maximum speed for 15min at 4 ºC. Protein concentration was measured with 2D-Quant Kit (GE Healthcare). All processed samples were stored at -80 ºC until used. In addition, mitochondrial extracts were obtained as previously described[5] for apoptotic marker analysis. Briefly, tissue sample from the IM was homogenized in lysis buffer and centrifuged at 750g for 5min. The resulting supernatant was filtered and centrifuged at 10,000g for 10min yielding cytosolic extract (supernatant) and mitochondria[6]. The mitochondrial pellet was washed three times and resuspended in 1.0ml homogenization buffer/mg of left ventricle to a final protein concentration ranging from 5.0 to 5.6mg/ml, aliquoted, and frozen in liquid N2.

Molecular Analysis

Gene expression analysis: We analyzed by real-time PCR (RT-PCR; Applied Biosystems) mRNA levels for: 1) apoptotic-related markers of both the extrinsic (Fas Receptor/CD95, caspase-8, and NFκ-B) and intrinsic (Bax, Bcl-2, and P53) pathways and the final irreversible executor caspase-3; 2) the RISK component PI3K; and 3) the aryl hydrocarbon nuclear translocator (ARNTor Hif1β). TaqMan fluorescent RT-PCR primers and probes (6-FAM-MGB) were designed by use of Primer Express 2.0 software from Applied Biosystems and TaqMan RT-PCR was performed as previously described.[7] The specificity and the optimal primer and probe concentrations were previously tested. The threshold cycle (Ct) values were determined and normalized to the housekeeping gene 18SrRNA in order to adjust for equal amounts of RNA.

Western Blot analysis: IM and NIM extracts were subjected to SDS-PAGE and blotted to nitrocellulose membranes. Blots were blocked for 2h at room temperature and then incubated overnight with: 1) apoptosis markers [truncated-caspase-3 (StressGen) and p53 phosphorylated on Thr155 (Santa Cruz)]; 2); RISK pathway (Akt/PKB phosphorylated on Ser473 (P-Akt, Cell signaling), Akt/PKB (Santa Cruz) and 3) myocyte hyperthrophy pathway [mTOR phosphorylated at Ser2448 (Cell Signaling), and P70S6K phosphorylated at Thr389 (Cell Signalling); 4) and aryl-hydrocarbon receptor (AhR; Abcam). Membrane-bound secondary antibodies were detected using the SuperSignal chemiluminescence system (Pierce) and quantified with a ChemiDoc™ (BioRad). Protein expression was determined using a computerized software package (Quantity-One). β-actin was used for protein loading control. Isolated mitochondrial extracts were processed similarly and membranes incubated against Bcl-2 phosphorylated at Ser87 (Santa Cruz).

Caspase-8 activity assessment

Caspase 8 activity was also assessed in the IM following the manufacturer's instructions (Abcam). Caspase-8 activity was measured by reading the optical density at 405 nm and expressed as arbitrary units.

TUNEL staining

Apoptosis assessment in the IM was also performed by dUTP nick-end labeling (TUNEL) using an apoptosis detection kit according to the manufacturer's protocol (Chemicon Inc.). Hoechst (Molecular Probes) was performed for nuclear staining. Five photographs of each tissue section (0.1 μm) were taken. Images were acquired with the Leica inverted fluorescence confocal microscope (Leica TCS-SP2-AOBS). In parallel, we performed the proper controls to verify the veracity of our observations. The nuclei were viewed and counted by an observer blinded to the experimental conditions by using a computer-based quantitative colour image analysis system (Image J®). The number of TUNEL-positive cardiomyocytes was expressed as mean number/100 cells/microscopic field (mean 5 fields/animal). Around 140 cells were counted per field.

Proteomic analysis

Myocardial proteomic changes induced by ischemia and full reperfusion (2.5h) with respect to sham-operated animals were investigated. To that end, protein extracts from the IM of sham-operated animals, animals subjected to ischemia, ischemia+2.5h reperfusion and ischemia+IPost-Co+2.5h reperfusion were separated by two-dimensional electrophoresis (2-DE) and proteins were identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF).[8, 9] Three animals of each group were analyzed.

Two-dimensional gel electrophoresis: For analytical (120μg) and preparative (300μg) gels, the urea/thiourea/chaps soluble extracts were applied to 17-cm dry strips (pH 3-10 linear range, BioRad). Gels were developed by fluorescent staining (Flamingo, BioRad). For each independent experiment, 2-DE from each group was processed in parallel to guarantee a maximum of comparability and each run was at least repeated twice. Differences in protein patterns were analyzed with the PD-Quest software (version 8.0, BioRad), using a single master including all gels of each independent experiment. Each spot was assigned a relative value corresponding to the single spot volume compared to the volume of all spots in the gel, following background extraction and normalization between gels.

Mass spectrometry analysis: Protein spots were excised from 2-DE gels, washed, dehydrated, dried, and enzymatic digested with sequence-grade modified porcine trypsin (Promega), as previously described,[9] and analyzed by matrix – assited laser desorption/ionization time-of-flight (MALDI-TOF) using an AutoFlex III Smartbeam MALDI-TOF/TOF (Bruker Daltonics). Samples were applied to Prespotted AnchorChip plates (Bruker Daltonics) surrounding the calibrants provided on the plates. Spectra were acquired with flexControl on reflector mode, (mass range 850-4000 m/z, reflector 1: 21.06 kV; reflector 2: 9.77kV; ion source 1 voltage: 19 kV; ion source 2: 16.5kV; detection gain 2.37x) with an average of 3500 shots at a frequency of 200 Hz. Each sample was processed with flexAnalysis (version 3.0, Bruker Daltonics) considering a signal-to-noise ratio over 3, applying statistical calibration and eliminating background peaks. For identification, peaks between 850-1000 m/z were not considered (usually only matrix peaks are visible on this range). Processed spectra were sent to the interface BioTools (version 3.2, Bruker Daltonics) and MASCOT search on Swiss-Prot 57.15 database was done (Mass Tolerance 50 to 100, up to 2 miss cleavage, Global Modification: Carbamidomethyl (C), Variable Modification: Oxidation (M)). Identification was accepted with a mascot score >56.

In-silico bioinformatic analysis

A systems biology approach was applied with Ingenuity System Pathway Analysis 10 software (IPA) to determine the statistically significant neural networks and canonical pathways in which the identified proteins were involved

Statistical Analysis

Because data were not normally distributed as observed by applying the Shapiro-Wilk test a non-parametric statistical analysis was applied and results are reported as medians with interquartil range [IQR]. For independent factors (comparisons between groups) we performed Kruskal-Wallis and Mann-Whitney analysis; for repeated measurements (ischemic vs non-ischemic and different time-points) Wilcoxon and Friedman analysis. All statistical tests conducted were two-sided and $P < 0.05$ was considered significant. Statistical analysis was performed using SPSS (version 19.0).

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Supplemental Table 1

Swiss-Prot Nº	Protname	pI	Mw (kDa)	Function
P68137	Alpha actin	5.2	42	Cytoskeleton/ Structural protein
P19619	Annexin A1	6.4	39	Calcium / Phospholipid binding
P50995*	Annexin A11	7.5	54	Cell cycle / Cell division
P19620	Annexin A2	6.5	39	Calcium / Phospholipid binding
P61013	Cardiac phospholamban	9.1	6	ER / Calcium regulation
Q75ZZ6	Cardiac troponin T isoform 1	5.9	31	Calcium regulation
Q29594	Creatine kinase B-type	5.2	11	Energy transduction
Q5XLD3	Creatine kinase M-type	6.6	43	Energy transduction
P17540*	Creatine kinase S-type	8.5	48	Energy transduction
P00503	Cytosolic aspartate aminotransferase	6.7	46	Amino acid metabolism
P80031	Glutathione S-transferase P	8.0	23	Transferase activity
Q16082*	Heat shock protein beta-2	5.1	20	Stress response
P34934	Hsp 70kDa	5.5	42	Stress response
O02705	Hsp 90kDa	4.9	85	Stress response
P00336	Lactate dehydrogenase	5.6	37	Metabolism
P11708	Malate dehydrogenase	6.2	36	Metabolism
Q8MHY0	Myosin light chain 2v	4.8	19	Regulation of muscle contraction
P08590*	Myosin light chain 3	5.0	22	Regulation of muscle contraction
Q29092	Endoplasmin	4.7	92	ER / Stress response
B8XSI6	PTEN	5.9	47	Phosphatase activity
Q6Q7J2	Rab GDP dissociation inhibitor beta	6.3	50	GTP metabolism
Q9NRC6*	Spectrin beta chain, brain 4	6.2	416 §	Cytoskeleton
P02554	Tubulin beta chain	4.6	50	Cytoskeleton/ Structural protein
P48643*	T-complex protein subunit epsilon	5.4	60	Cytoskeleton / Molecular chaperone

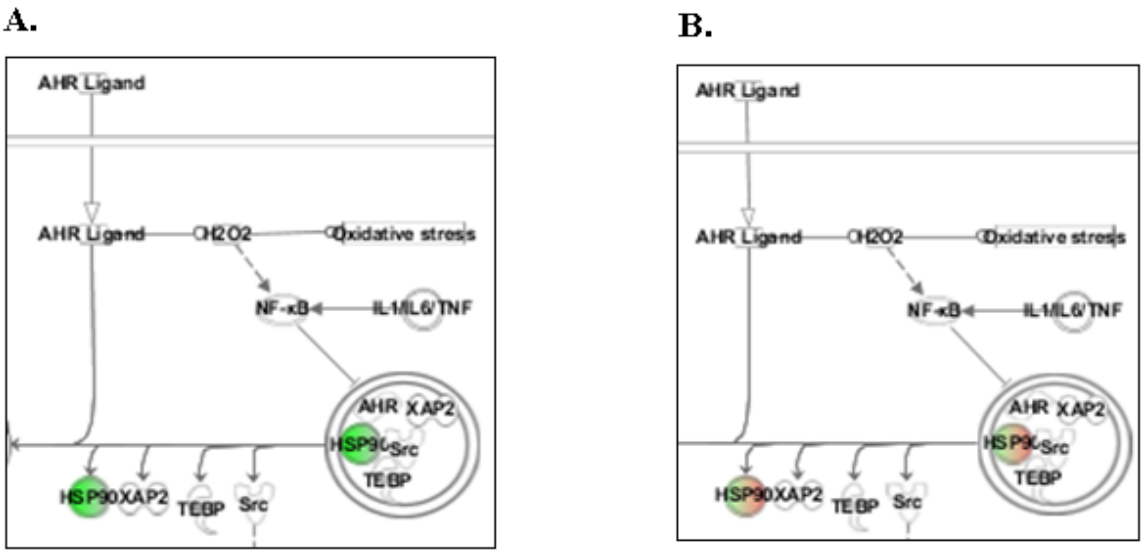
TABLE 2. Median [IQR] of spot intensity in 2DE analysis of sham, ischemia, ischemia/reperfusion (I/R), and ischemic post-conditioning (IPost-Co) animals. ND = no detected.

Supplemental Table 2

Protname	Sham	Ischemia	I/R	IPost-Co
Alpha actin	26.5 [14.0-40.2]	49.5 [41.2-70.8]	47.4 [26.7-52.9]	73.5 [72.7-87.3]
Annexin A1	4.0 [3.2-5.5]	3.7 [3.7-3.7]	2.0 [2.0-2.0]	ND
Annexin A11	29.0 [19.7-91.5]	24.9 [17.5-33.5]	13.6 [12.1-20.4]	71.7 [52.7-95.1]
Annexin A2	5.1 [4.5-12.8]	7.5 [6.4-7.7]	4.2 [3.8-5.4]	14.2 [10.8-17.2]
Cardiac phospholamban	4.3 [3.1-7.9]	5.3 [4.0-10.5]	3.5 [2.6-3.9]	9.2 [8.1-12.0]
Cardiac troponin T isoform 1	33.2 [23.1-53.5]	5.7 [4.9-8.5]	12.0 [7.8-14.2]	30.6 [25.4-36.4]
Creatine kinase B-type	6.5 [5.4-7.6]	2.3 [2.1-2.5]	3.2 [3.0-4.1]	ND
Creatine kinase M-type	24.5 [14.0-57.8]	14.7 [12.2-66.0]	11.1 [7.5-52.6]	157.3 [98.2-169.6]
Creatine kinase S-type	4.9 [4.3-5.1]	2.0 [2.0-2.0]	1.9 [1.9-1.9]	9.6 [6.9-13.3]
Cytosolic aspartate aminotransferase	76.0 [50.3-103.3]	76.5 [52.8-153.9]	48.4 [38.9-93.7]	96.2 [78.8-121.4]
Glutathione S-transferase P	6.6 [6.3-6.8]	5.1 [4.1-6.2]	4.9 [3.2-5.5]	9.2 [6.6-9.5]
Lactate dehydrogenase	39.2 [32.4-49.3]	27.6 [21.8-28.5]	35.0 [27.5-35.3]	48.3 [40.0-48.5]
Malate dehydrogenase	26.4 [17.8-35.0]	23.9 [21.8-50.1]	13.1 [12.2-13.9]	35.6 [28.7-42.6]
Myosin light chain 2v	51.6 [39.7-59.6]	30.3 [17.6-43.1]	29.0 [29.0-29.0]	126.7 [99.4-156.6]
Myosin light chain 3	92.9 [75.1-109.1]	124.6 [121.3-127.9]	63.0 [50.8-75.1]	186.6 [182.2-264.9]
PTEN	2.0 [1.7-4.2]	2.1 [1.4-2.1]	2.1 [1.7-4.3]	ND
Rab GDP dissociation inhibitor beta	4.6 [4.5-4.9]	3.4 [3.3-5.1]	2.7 [2.5-3.0]	6.6 [5.1-6.8]
Spectrin beta chain, brain 4	0.8 [8.3-13.6]	0.8 [0.8-0.8]	1.2 [0.9-12.1]	1.8 [1.6-2.0]
Tubulin beta chain	26.1 [24.4-32.2]	9.2 [5.4-18.5]	8.4 [7.3-9.6]	25.9 [19.0-32.8]
T-complex protein subunit epsilon	2.6 [2.3-3.2]	1.9 [1.8-2.5]	1.2 [9.7-15.1]	3.6 [3.2-3.9]
Hsp 70kDa	53.5 [44.3-59.5]	44.4 [34.3-46.7]	22.7 [22.2-27.4]	83.1 [67.3-120.7]
Hsp 90kDa	34.1 [26.2-55.0]	14.2 [13.6-46.3]	25.1 [16.6-25.5]	6.9 [6.6-7.2]
Heat shock protein beta-2	4.6 [3.7-5.1]	3.5 [3.5-3.5]	3.0 [2.1-3.9]	7.6 [5.9-9.3]
Endoplasmin	8.1 [7.6-10.4]	6.2 [5.2-13.4]	8.9 [6.0-10.5]	4.3 [3.7-5.1]

TABLE 2. Median [IQR] of spot intensity in 2DE analysis of sham, ischemia, ischemia/reperfusion (I/R), and ischemic post-conditioning (IPost-Co) animals. ND = no detected.

Supplemental Figure 1



Supplemental Figure 1. Canonical pathway involved in Aryl Hydrocarbon receptor signalling pathway obtained with the Ingenuity System Pathway Analysis software after the analysis of differential proteins in (A) post-conditioning compared to sham operated animals; (B) ischemia + reperfusion compared to sham operated animals. Proteins in green are down regulated and in red are up-regulated.

ARTÍCULO SÉPTIMO

"Ischemic post-conditioning affords a coordinated change in mitochondrial enzymes impaired by ischemia and reperfusion"

Judit Cubedo * †, Gemma Vilahur * †, Teresa Padró *, Lina Badimon * † ||

*Cardiovascular Research Center (CSIC-ICCC), †CIBERobn and ||Autonomous University of Barcelona. Spain.

En preparación

Resumen resultados

Análisis mediante 2-DE tejido cardíaco porcino.
El análisis mediante 2-DE del tejido de miocardio de cerdo revela que el 27% de los puntos proteicos detectadas corresponde a 25 proteínas mitocondriales no redundantes.

El post-condicionamiento isquémico revierte los cambios inducidos por la isquemia y reperfusión en las proteínas mitocondriales.
La isquemia y la posterior reperfusión del miocardio inducen la disminución de 14 proteínas mitocondriales, mientras que el post-condicionamiento normaliza el 57% de estos cambios induciendo mayoritariamente un incremento en las proteínas mitocondriales.

Análisis *in silico* de las vías modificadas.
El análisis bioinformático de las proteínas diferenciales ("*Ingenuity System Pathway Analysis*") revela que la cadena respiratoria mitocondrial y la vía de la fosforilación oxidativa están significativamente modificadas en la isquemia y la reperfusión. El post-condicionamiento aumenta las dos vías.

Ischemic post-conditioning affords a coordinated change in mitochondrial enzymes impaired by ischemia and reperfusion

By

Judit Cubedo * †, Gemma Vilahur * †, Teresa Padró *, Lina Badimon * † ||

From

*Cardiovascular Research Center (CSIC-ICCC), †CIBERobn and ||Autonomous University of Barcelona. Spain.

Total Word Count: 4900 words

Abstract Word Count: 231

Total Table and Figures: 6 Figures + 2 Tables (2 Supp Figures + 2 Supp Tables)

Correspondence to:

Prof. Lina Badimon

Cardiovascular Research Center, c/Sant Antoni MªClaret 167, 08025 Barcelona, Spain.

Phone: (34) 935565880. Fax: (34) 935565559.

E-mail: lbadimon@csic-iccc.org

ABSTRACT

Background: Ischemia induces important changes in cardiac metabolism and the following reperfusion contributes to the final extent of myocardial infarction leading to heart tissue injury. Ischemic post-conditioning (IPost-Co) has shown to limit infarct size by decreasing ischemia/reperfusion injury. Nowadays, it is not clear how IPost-Co exerts this cardioprotective effect. By using proteomic approaches we have investigated the coordinated changes in ischemia/reperfusion, and how IPost-Co affects cardiac metabolism.

Methods: Pigs (n=12) underwent 1.5h mid-LAD balloon occlusion and were then sacrificed (Group-I), allowed to reperfuse for 2.5h (Group-II) or subjected to IPost-Co before reperfusion (Group-III). A sham-operated group was included. Ischemic myocardium tissue extracts were analyzed by bi-dimensional electrophoresis (2-DE) followed by mass spectrometry (MALDI-TOF/TOF) identification. Potential networks with the differential-detected proteins were analyzed by Ingenuity Pathway Analysis.

Results: In the myocardial proteome 27% of the identified spots corresponded to 25 non-redundant mitochondrial-related proteins. Among them, ischemia and ischemia/reperfusion induced a decrease in 14 proteins, whereas IPost-Co normalized over 57% of those changes by inducing an increase in myochondrial-related proteins. Moreover, biosystems biology data revealed significant changes in the mitochondrial dysfunction and the oxidative phosphorylation pathways when comparing the three groups (p<0.001).

Conclusions: IPost-Co reverts and/or prevents the major mitochondrial-related coordinated metabolic changes that occur during ischemia and reperfusion, leading to the recover of mitochondrial function in the heart. This mitochondrial modulation is therefore an important effector mechanism of the ischemic post-conditioning cardioprotection.

Key words; ischemic post-conditioning; proteomics; mitochondria.

Abbreviations: IPost-Co, ischemic post-conditioning; 2-DE, bidimensional electrophoresis; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight.

INTRODUCTION

During ischemia important changes occur in cardiac energy metabolism due to the reduced oxygen availability that induce mitochondrial injury, increase ROS generation, and lead to oxidative DNA damage [1]. Early reperfusion is the current optimal way to rescue the heart, however, the process is associated with cellular damage by activation of deleterious signalling cascades which may cause the death of cardiac cells, and an increase in infarct size [2], the so-called ischemia/reperfusion injury. The imbalance between oxygen supply and consumption during ischemia and reperfusion induces modifications in cardiomyocyte structure and function through coordinated changes in gene and protein expression, and in the activity of a variety of proteins [3]. Mitochondria are the main source of ATP through oxidative phosphorylation via the electron transport chain. Because of its energy requirements the role of mitochondria in the heart is crucial, in fact it represents nearly one-third of its total mass. The correct maintenance of mitochondrial homeostasis is essential for cell survival as they are a potent source of free radicals and pro-apoptotic factors, but they can also diminish the detrimental effects of an excessive oxidative stress [4, 5]. During myocardial ischemia the electron transport chain is damaged leading to an increase in cardiomyocyte death during reperfusion. Experimental approaches have demonstrated that chemical blockade of electron transport during ischemia inhibits the opening of the mitochondrial permeability transition pore decreasing cardiomyocyte damage during reperfusion [6]. Ischemic post-conditioning (IPost-Co) has revealed as a powerful tool that activates intrinsic prosurvival signalling cascades to limit reperfusion injury [7]. There are several studies on changes in specific pathways during IPost-Co such as the activation of Reperfusion Injury Salvage Kinases (RISK) [8-10] or the prosurvival Survivor Activating Factor Enhancement (SAFE) [11]. Moreover, the cardioprotective effect of IPost-Co has shown to be at least in part due to the modulation of mitochondrial oxidative metabolism [12].

Acute injury induces rapid posttranslational modifications of proteins thereby altering protein levels [13] that can be detected by differential proteomic analysis of the affected organ. The study of the coordinated changes of several proteins as accomplished by proteomics may afford a better understanding of the whole ischemia/reperfusion process, and an overall view of how IPost-Co affects cardiac metabolism.

In the present study, proteomic tools were used to determine changes in protein expression in the ischemic heart after ischemia and ischemia/reperfusion, and to investigate whether IPost-Co might modify these changes.

Our studies have been conducted in a swine model of experimentally-induced ischemia/reperfusion that reasonably resembles the clinical setting. In one hand, swine human-like cardiovascular system allows to fulfil many important requirements for further translation into the clinical setting. Furthermore, our standardized pig model [14, 15] offers the advantage of precisely selecting the site of coronary occlusion under fluoroscopy guidance and the possibility of controlling both ischemia duration and reperfusion resulting in reproducible and consistent development of ischemic and reperfusion damage, a requirement for feasibly evaluating cardiac metabolic changes [16].

MATERIALS AND METHODS

Experimental Model

Twelve farm swine (Landrace-Largewhite cross, 36-39 kg) were acclimated for 1 week before any experimental procedure. Thereafter, animals were randomized to the following three groups: I) closed-chest 1.5h left anterior descending (LAD) coronary occlusion with no reperfusion (Group-I; n=3); II) 1.5h LAD occlusion followed by 2.5h reperfusion (Group-II; n=3); III) 1.5h LAD occlusion followed by ischemic post-conditioning (IPost-Co) and then 2.5h reperfusion (Group-III; n=3). The IPost-Co protocol was induced by 6 cycles of 20 seconds of reperfusion and 20 seconds of reocclusion at the onset of reperfusion as previously reported [17].

A sham-operated group of animals (n=4) following the same operating procedure but without ischemia was included.

The study protocol was approved by an institutional animal research committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Myocardial Ischemia/Reperfusion Model and Echocardiography

Twelve hours prior to the experimental induction of acute MI (AMI), a loading dose of clopidogrel was administered to all animals. AMI was experimentally induced as we have previously described [18, 19]. Angiographic images were used to guide angioplasty balloon placement (below the first diagonal branch) and balloon occlusion

was maintained for 1.5h. At the end of the ischemic period animals were either sacrificed without reperfusion (Group I) or the balloon was deflated and the ischemic area allowed to reperfuse (again verified by angioscopy) during 2.5h without (Group II) or with (Group III) previous application of IPost-Co. Heart rate and ECG were monitored throughout the surgical procedure. We used 2D echocardiograms (Phillips iE33) to assess global cardiac function (i.e., left ventricle ejection fraction, LVEF) in all animals before coronary occlusion (at baseline), 1.5h post-occlusion just before reperfusion, and at the end of the reperfusion period (sacrifice).

Protein extraction from Myocardial Tissue

Frozen myocardial tissue samples from the ischemic area were pulverized and resuspended in urea/thiourea buffer (7M Urea, 2M Thiourea, 2%Chaps), incubated 30 minutes in ice and centrifuged at maximum speed for 15 minutes at 4 ºC. Protein concentration in the myocardial extracts was measured with 2D-Quant Kit (GE Healthcare). All processed samples were stored at -80ºC until used.

Proteomic analysis

Protein extracts from the ischemic myocardium were separated by bidimensional gel electrophoresis (2-DE) and protein spots of interest identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF/TOF) [20].

Two-dimensional gel electrophoresis (2-DE):For analytical and preparative gels, respectively, a protein load of 120 µg and 300 µg protein of the urea/thiourea/chaps/DTT soluble extracts were applied to 17-cm dry strips (pH 3-10 linear range, BioRad). Gels were developed by fluorescent staining (Flamingo stain, BioRad). For each independent experiment, 2-DE for protein extracts from each group were processed in parallel to guarantee a maximum of comparability. Each 2-DE run was at least repeated twice. Analysis for differences in protein patterns was performed with the PD-Quest software (BioRad), using a single master that included all gels of each independent experiment. Each spot was assigned a relative value that corresponded to the single spot volume compared to the volume of all spots in the gel, following background extraction and normalization between gels.

Mass spectrometry analysis: Protein spots of interest were excised from 2-DE gels, washed, dehydrated, dried, and enzymatic digested with sequence-grade modified porcine trypsin (Promega), as previously described,[20] and analyzed by matrix –

assisted laser desorption/ionization time-of-flight (MALDI-TOF) using an AutoFlex III Smartbeam MALDI-TOF/TOF (Bruker Daltonics). Samples were applied to Prespotted AnchorChip plates (Bruker Daltonics) surrounding the calibrants provided on the plates. Spectra were acquired with flexControl on reflector mode, (mass range 850 - 4000 m/z, reflector 1: 21.06 kV; reflector 2: 9.77kV; ion source 1 voltage: 19 kV; ion source 2: 16.5kV; detection gain 2.37x) with an average of 3500 added shots at a frequency of 200 Hz. Each sample was processed with flexAnalysis (version 3.0, Bruker Daltonics) considering a signal-to-noise ratio over 3, applying statistical calibration and eliminating background peaks. For identification, peaks between 850 and 1000 m/z were not considered as in general only matrix peaks are visible on this mass range. After processing, spectra were sent to the interface BioTools (version 3.2, Bruker Daltonics) and MASCOT search on Swiss-Prot 57.15 database was done (Mass Tolerance 50 to 100, up to 2 miss cleavage, Global Modification: Carbamidomethyl (C), Variable Modification: Oxidation (M)). Identification was accepted with a mascot score higher than 56.

***In-silico* bioinformatic analysis**

To further explore the physiopathological significance of the differential protein patterns among groups the Ingenuity System Pathway Analysis software (IPA) was used in order to determine the statistically significant canonical pathways in which the identified proteins were involved.

Statistical Analysis

Continuous variables are expressed as median [IQR]. Statistical analysis was performed by non-parametric Kruskal Wallis and Mann-Whitney to assess specific group differences. A value of $p < 0.05$ was considered significant. All statistical analyses were performed with the statistical software package Statview. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

RESULTS

Myocardial proteome in the porcine model

In myocardial tissue extracts 306 spots were consistently detected. Among them, 27% of the identified spots corresponded to 25 non-redundant mitochondrial proteins (Fig. 1 and Supplemental Table 1). Ratios of spot intensities between groups are shown in Supplemental Table 2.

Differential metabolic profile after ischemia and ischemia/reperfusion

When the myocardial proteome of the ischemia group was compared to sham-operated animals (Fig. 1A and 1B) 5 mitochondrial proteins were differentially expressed (Fig. 2A). Among those differential proteins 4 of them showed more than 1.7-fold decreased intensity, being ATP synthase beta subunit 3.6-fold decreased. Only D-beta-hydroxybutyrate dehydrogenase showed a 1.7-fold increase after ischemia.

When myocardium after ischemia/reperfusion was compared to the non-reperfused ischemic myocardium (Fig. 1B and 1C), 11 mitochondrial proteins were differentially expressed (Fig. 2B). Among those proteins 10 showed decreased intensities being D-beta-hydroxybutyrate dehydrogenase and Voltage-dependent anion-selective channel protein 1 not detected after reperfusion. In contrast ATP synthase beta subunit showed a 2.3-fold increase due to reperfusion.

After ischemia/reperfusion a total of 10 mitochondrial proteins showed decreased intensities when compared to sham-operated animals (compare Fig. 1A and C; Fig. 2C).

Comparative analysis between the three groups reported changes in 14 of the 25 identified mitochondrial proteins. Those differential proteins were divided into four groups depending on their behaviour along the ischemia and ischemia/reperfusion process (Table 1): (I) proteins decreased in ischemia and ischemia/reperfusion, (II) proteins decreased in ischemia and normalized in ischemia/reperfusion, (III) proteins that showed an increase in ischemia and decreased levels in ischemia/reperfusion, and (IV) proteins decreased in ischemia/reperfusion.

Ischemic post-conditioning increases mitochondrial proteins and prevents changes due to ischemia and reperfusion

When animals were subjected to IPost-Co and the proteomic profile was compared to the other groups (compare Fig. 1D with 1A, 1B and 1C) there was an important change in the spot intensity of mitochondrial proteins. Among differential proteins 16 showed increased intensities when compared to reperfusion without IPost-Co (Fig. 3A), 15 were increased when compared to ischemia without reperfusion (Fig. 3B).

Among the 14 differential proteins in ischemia and ischemia/reperfusion 57% of the changes were normalized reaching sham values after IPost-Co (Table 2).

***In-silico* bioinformatic analysis**

All protein ratios below and above 1.7-fold change were included in the in-silico analysis. Bioinformatic analysis revealed that the differential proteins were involved in the mitochondrial dysfunction canonical pathway (Fig. 4). When mitochondrial dysfunction related proteins were analyzed in ischemia compared to sham-operated animals differential proteins were mainly localized in complexes I (NADH dehydrogenase), III (cytochrome bc1 complex), and V (ATP synthase) of the respiratory chain of the mitochondria ($p < 0.0001$; Fig. 5A). When the same analysis was performed between ischemia/reperfusion and ischemia alone the same localization of differential proteins was observed ($p < 0.0001$) showing mainly a decrease in the intensity of complexes I and II, and being complex V down-regulated in ATP synthase beta subunit and up- regulated in the alpha subunit (Fig. 5B). In the analysis of IPost-Co animals in comparison to ischemia and ischemia/reperfusion (Fig. 5C and 5D, respectively) an up-regulation of the three complexes was detected ($p < 0.0001$). When compared to sham-operated animals, IPost-Co showed an increase in complexes I and III and a decrease in complex V (Supplemental Figure 1A). Reperfusion decreased complexes I, III, and V under sham levels (Supplemental Figure 1B).

In-silico analysis showed significant changes in the oxidative phosphorilation canonical pathway in the three groups ($p < 0.0001$). Ischemia induced a decrease in NADH and ADP pathways (Fig. 6A). When reperfusion was applied the main change was the down-regulation of the ubiquinol pathway (Fig. 6B). In contrast, in the post-conditioned heart oxidative phosphorilation was induced with significant up-regulation of the NADH, ubiquinol and ADP pathways when compared to ischemia alone (Fig. 6C) and ischemia/reperfusion (Fig. 6D). When compared to sham situation, post-conditioned

hearts showed an up-regulation of the NADH and ubiquinol pathways, and a down-regulation of the ADP pathway (Supplemental Figure 2A). In contrast, reperfusion alone showed decreased levels of the three pathways when compared to sham-operated animals (Supplemental Figure 2B).

DISCUSSION

During different ischemic conditions many biochemical changes happen that initially represent a defensive and protective reaction against ischemic insults [21]. Although the structural and histological changes which occur following MI have been well documented, the study through proteomic approaches of metabolic changes after ischemia affords new insights into potential therapeutic intervention targets. The main finding of this study is that ischemia and reperfusion induce major mitochondrial-related coordinated metabolic changes and that IPost-Co reverts and/or prevents over 57% of those mitochondrial changes that occur after the ischemic event. Here, we have found that ischemia and reperfusion cause a decrease in the level of many mitochondrial proteins that lead to an impairment of the mitochondrial function, while IPost-Co induces an increase in mitochondrial proteins recovering mitochondrial function in the heart. Because of the abundance of mitochondria in the heart, the loss of mitochondrial function has a great impact on the myocardium, as ATP is needed to maintain contractile activity [22]. Recent studies in rat hearts have demonstrated that delayed pre-conditioning by sevoflurane is able to restore the changes in mitochondrial proteins induced by ischemia and reperfusion [23]. Nevertheless, the occurrence of ischemia is difficult to predict, limiting broader use of ischemic pre-conditioning [24]. Instead, IPost-Co is potentially applicable in the clinical scenario [25, 26] and it seems to induce cardioprotection in a similar way to that afforded by ischemic pre-conditioning [7].

The in-silico analysis of the differential proteomic signatures revealed important changes in mitochondrial dysfunction related proteins during ischemia, specifically proteins from complexes I, III, and V in the inner mitochondrial membrane. It has already been demonstrated in previous studies that mitochondria are both targets and sources of injury during cardiac ischemia and reperfusion [12]. Our results are in line with those reported in a rat model of IPost-Co in aortic cross-clamping where an increase in mitochondrial complexes was shown [27]. The primary result of ischemia is

mitochondrial metabolic dysfunction caused by reduced oxygen delivery to the tissue, resulting in a decrease in ATP formation by oxidative phosphorylation [27, 28]. Ischemic mitochondrial damage appears to be a major mechanism of cardiac injury, since reperfusion of myocardium that contain mitochondria with preserved oxidative function markedly decreases myocardial injury [12]. Therefore, the observed changes in IPost-Co would contribute to the preservation of cardiac function as cardioprotection protocols have already shown an up-regulation of mitochondrial function as well as oxidative phosphorylation [29].

The importance of oxidative stress in ischemia/reperfusion injury has been demonstrated in experimental animal models where the overexpression of anti-oxidant proteins reduced apoptosis and improved contractile dysfunction [30-33]. Moreover it has been suggested that redox signalling may be involved in the cardioprotective effect of pre-conditioning due to slight mitochondrial swelling and an increase in respiration [34-37]. Our results demonstrate that IPost-Co induces an up-regulation of oxidative phosphorylation proteins which may lead to an increase in mitochondrial function therefore reducing ischemia/reperfusion injury.

In this proteomic study ischemia induced an up-regulation in complex I. Importantly, there are evidences indicating that mitochondrial respiration chain complex I is one of the most important sources of mitochondrial reactive oxygen species (ROS) [38]. Reperfusion induces an impairment of mitochondrial function, such a transient inhibition of complex I that has already been suggested as an initial attempt to attenuate ROS production [39]. Indeed, the potent mitochondrial antioxidant enzyme manganese superoxide dismutase (Mn-SOD) was up-regulated by both ischemia and reperfusion in order to scavenge the increase in ROS generation [40]. We have found that complex I activity was attenuated when IPost-Co was applied, reducing the impact of ROS generation upon ischemia-related complex I increase. It has been already suggested that ischemic pre-conditioning may attenuate the activity of complex I [12].

Additionally, it has also been previously described that after 30-40 minutes of ischemia complex III impairment leads to oxidative phosphorylation decrease and eventual mitochondrial damage [41]. Interestingly we have found a specific increase in the cytochrome bc-1 complex subunit Rieske and a decrease in the cytochrome bc-1 complex subunit 1 after 90 minutes of ischemia, showing therefore, a differential behaviour of proteins related to the same complex. However, after reperfusion a global decrease of complex III was detected that turn out to a global increase when IPost-Co

was applied. This increase in the activity of complex III-related proteins may have contributed to prevent the accumulation of reducing equivalents likely decreasing ischemia-derived cytotoxic ROS production [42].

As to compex V we have detected decreased levels of proteins after ischemia and reperfusion. Moreover, we have found that IPost-Co up-regulates complex V activity reverting the previously described ischemic damage to the phosphorylation apparatus [43, 44].

Overall, *in silico* analysis has outlined the differential regulation of mitochondrial complexes during both ischemia and reperfusion. These differences in complex activities would lead to mitochondrial uncoupling contributing to ROS accumulation [45, 46]. Importantly, IPost-Co compensates this mitochondrial dysfunction.

To our knowledge this is the first proteomic study reporting on how IPost-Co reverts the coordinated metabolic changes that occur upon ischemia and reperfusion in mitochondrial-related proteins, therefore preserving mitochondrial function and decreasing tissue injury. Even more, we have found that ischemic post-conditioning significantly increases several mitochondrial enzymes involved in oxidative phosphorylation restoring the ischemia-related impairment of the oxidative function. This mitochondrial modulation is therefore, at least in part, an effector mechanism of the post-conditioning-derived cardioprotection.

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LEGENDS

Figure 1. Representative 2-DE image of myocardial tissue from (A) sham operated animal, (B) 1.5h of ischemia, (C) 1.5h of ischemia followed by 2.5h of reperfusion, and (D) ischemic post-conditioning. Spots marked and numbered refer to mitochondrial proteins identified by MALDI-TOF/TOF analysis.

Figure 2. Bar diagrams of the ratio of median spot intensities between (A) ischemia vs. sham, (B) ischemia/reperfusion vs. ischemia, and (C) ischemia/reperfusion vs. sham. Only major changes are represented (1.7-fold increase or decrease).

Figure 3. Bar diagrams of the ratio of median spot intensities between (A) post-conditioning vs. ischemia/reperfusion, and (B) post-conditioning vs. ischemia. Only major changes are represented (1.7-fold increase or decrease).

Figure 4. Canonical pathways involved in mitochondrial dysfunction obtained with the Ingenuity System Pathway Analysis software after the analysis of the identified mitochondrial related proteins. Identified proteins appear in grey.

Figure 5. Canonical pathways involved in mitochondrial dysfunction obtained with the Ingenuity System Pathway Analysis software after the analysis of differential proteins in (A) ischemia compared to sham operated animals; (B) ischemia/reperfusion (I/R) compared with ischemia alone; (C) post-conditioning (IPost-Co) compared with ischemia; and (D) post-conditioning compared with reperfusion without post-conditioning. Proteins in green are down-regulated and in red are up-regulated.

Figure 6. Canonical pathways involved in oxidative phosphorylation obtained with the Ingenuity System Pathway Analysis software after the analysis of differential proteins in (A) ischemia compared to sham operated animals; (B) ischemia/reperfusion (I/R) compared with ischemia alone; (C) post-conditioning (IPost-Co) compared with ischemia; and (D) post-conditioning compared with reperfusion without post-conditioning. Proteins in green are down-regulated and in red are up-regulated.

TABLE 1. Changes in mitochondrial myocardial proteome in ischemia (I) and ischemia/reperfusion (I/R) groups.

Group I	Decreased in I and I/R
4	ATP synthase beta subunit
9	Dihydrolipolyl dehydrogenase
14	Isocitrate dehydrogenase [NAD] subunit alpha
Goup II	Decreased in I and normalized I/R
18	NADH-ubiquinone oxidoreductase chain 2
Group III	Increased in I and decreased in I/R
8	D-beta-hydroxybutyrate dehydrogenase
Group IV	Decreased in I/R
3	3-hydroxyacyl-CoA dehydrogenase type-2
5	ATP synthase subunit alpha
6	Cytochrome b-c1 complex subunit 1
7	Cytochrome b-c1 complex subunit Rieske
10	Electron transfer flavoprotein subunit beta
19	Pyruvate dehydrogenase E1 component subunit alpha
23	Ubiquinone biosynthesis protein COQ9
24	Voltage-dependent anion-selective channel protein 1
25	Voltage-dependent anion-selective channel protein 2

TABLE 2. Changes after IPost-Co in ischemia (I) and ischemia/reperfusion (I/R) differential proteins.

Group I	Decreased in I and I/R	Changes in IPost-Co
4	ATP synthase beta subunit	Normalized
9	Dihydrolipolyl dehydrogenase	Normalized
14	Isocitrate dehydrogenase [NAD] subunit alpha	Normalized
Goup II	Decreased in I and normalized I/R	
18	NADH-ubiquinone oxidoreductase chain 2	Increased
Group III	Increased in I and decreased in I/R	
8	D-beta-hydroxybutyrate dehydrogenase	Increased
Group IV	Decreased in I/R	
3	3-hydroxyacyl-CoA dehydrogenase type-2	Normalized
5	ATP synthase subunit alpha	Normalized
6	Cytochrome b-c1 complex subunit 1	Increased
7	Cytochrome b-c1 complex subunit Rieske	Normalized
10	Electron transfer flavoprotein subunit beta	Increased
19	Pyruvate dehydrogenase E1 component subunit alpha	Normalized
23	Ubiquinone biosynthesis protein COQ9	Increased
24	Voltage-dependent anion-selective channel protein 1	Normalized
25	Voltage-dependent anion-selective channel protein 2	Increased

Figure 1

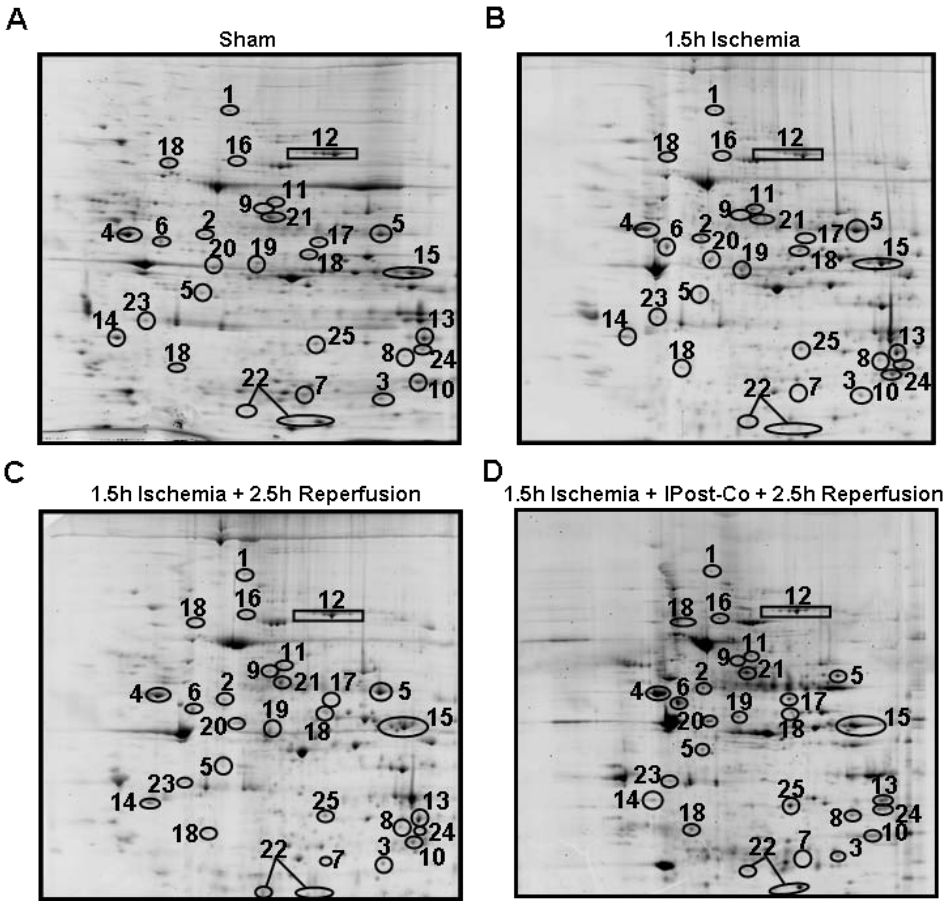

A
Sham
B
1.5h Ischemia
C
1.5h Ischemia + 2.5h Reperfusion
D
1.5h Ischemia + IPost-Co + 2.5h Reperfusion

Figure 2

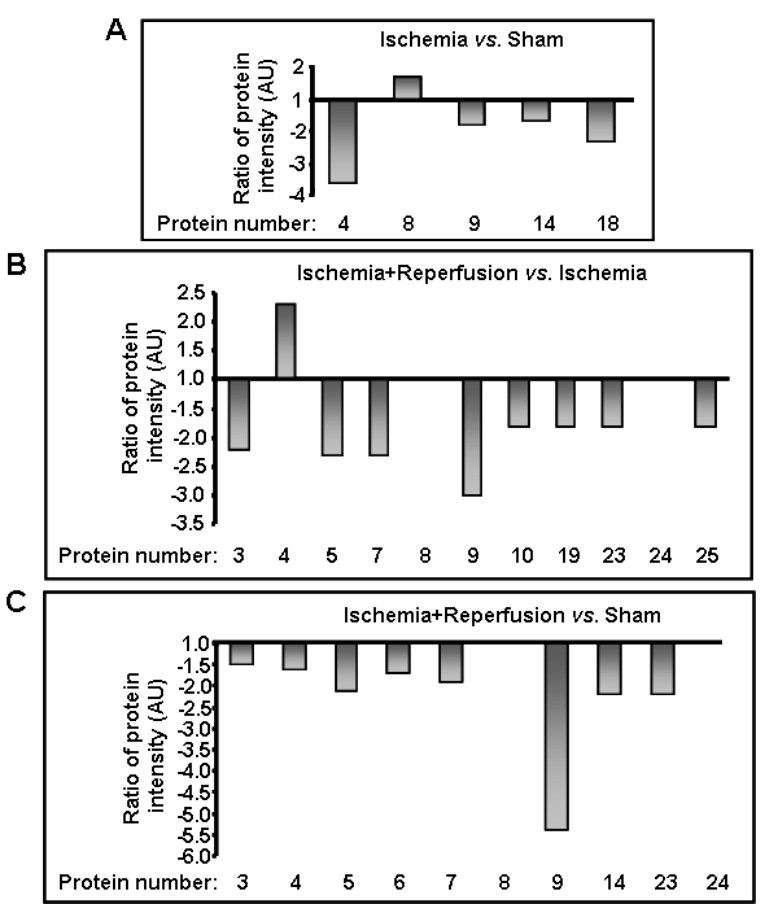

A
Ischemia vs. Sham
Ratio of protein intensity (AU)
2
1
-2
-3
-4
Protein number: 4 8 9 14 18
B
Ischemia+Reperfusion vs. Ischemia
Ratio of protein intensity (AU)
2.5
2.0
1.5
1.0
-1.5
-2.0
-2.5
-3.0
-3.5
Protein number: 3 4 5 7 8 9 10 19 23 24 25
C
Ischemia+Reperfusion vs. Sham
Ratio of protein intensity (AU)
1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
-5.5
-6.0
Protein number: 3 4 5 6 7 8 9 14 23 24

Figure 3

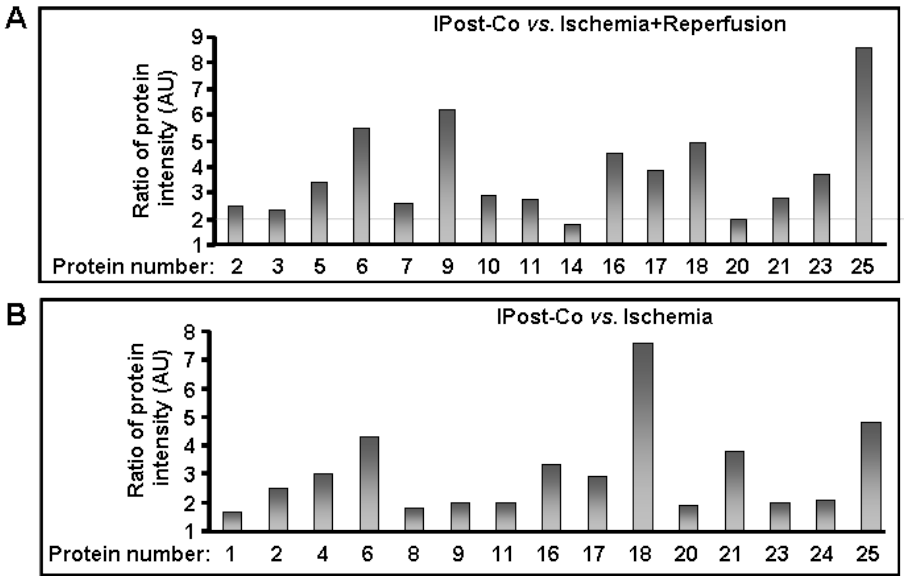

A
IPost-Co vs. Ischemia+Reperfusion
Ratio of protein intensity (AU)
9
8
7
6
5
4
3
2
1
Protein number: 2 3 5 6 7 9 10 11 14 16 17 18 20 21 23 25
B
IPost-Co vs. Ischemia
Ratio of protein intensity (AU)
8
7
6
5
4
3
2
1
Protein number: 1 2 4 6 8 9 11 16 17 18 20 21 23 24 25

Figure 4

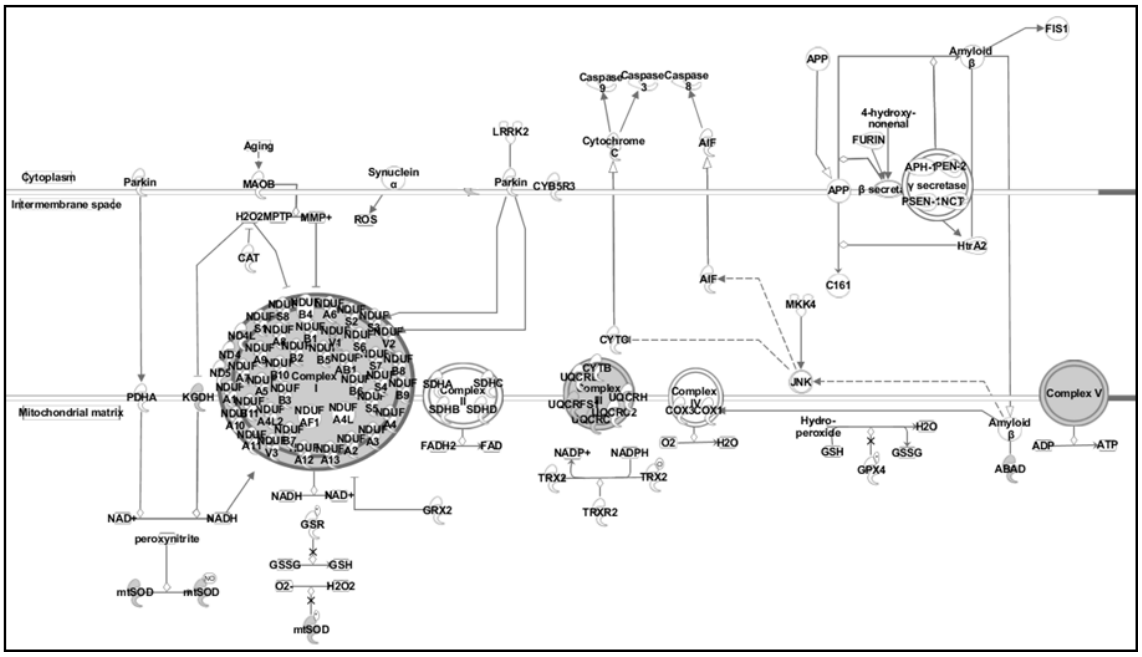

FIS1
Amyloid β
APP
Caspase 9
Caspase 3
Caspase 8
4-hydroxy-nonenal
FURIN
LRRK2
Cytochrome C
AIF
Aging
Cytoplasm
Parkin
MAOB
Synuclein α
Parkin
CYB5R3
APP
β secret.
APH-1 PEN-2 γ secretase PSEN-1 NCT
Intermembrane space
H2O2 MPTP
MPP+
ROS
HtrA2
CAT
AIF
C161
MKK4
CYTC
JNK
PDHA
KGDH
Mitochondrial matrix
SDHA SDHB SDHC SDHD Complex II
CYTB UQCRL UQCRFS1 UQCRH UQCRC2 Complex III
Complex IV COX3 COX1
Complex V
FADH2
FAD
NADP+
NADPH
O2
H2O
Hydro-peroxide
H2O
GSH
GSSG
GPX4
Amyloid β
ABAD
ADP
ATP
TRX2
TRX2
TRXR2
NADH
NAD+
GRX2
NAD+
NADH
GSR
peroxynitrite
GSSG
GSH
mtSOD
mtSOD
O2-
H2O2
mtSOD

Figure 5

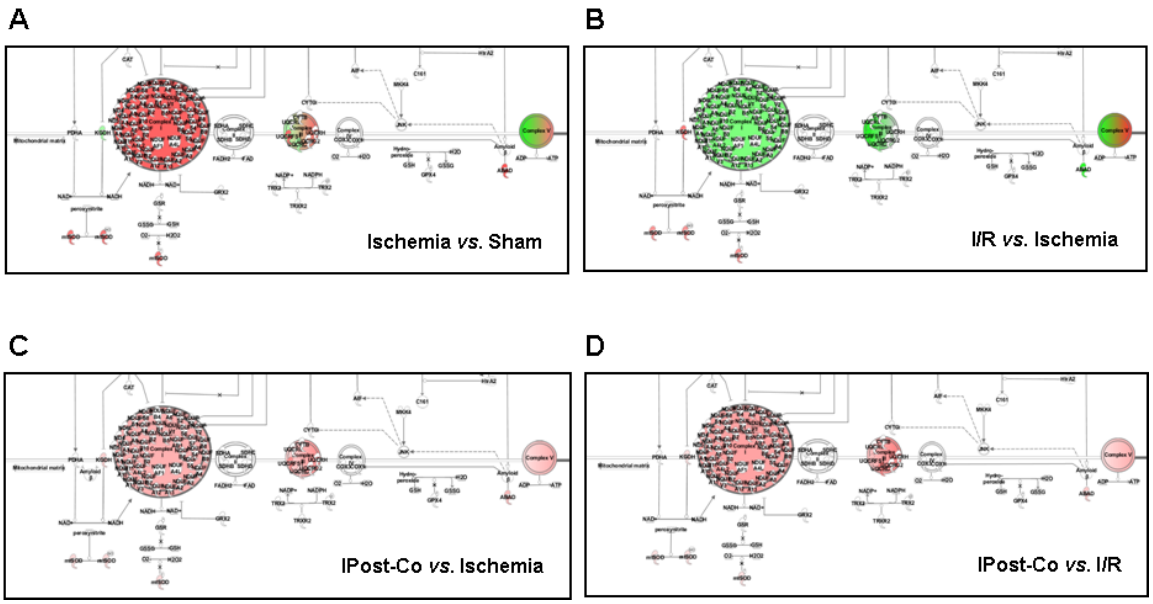
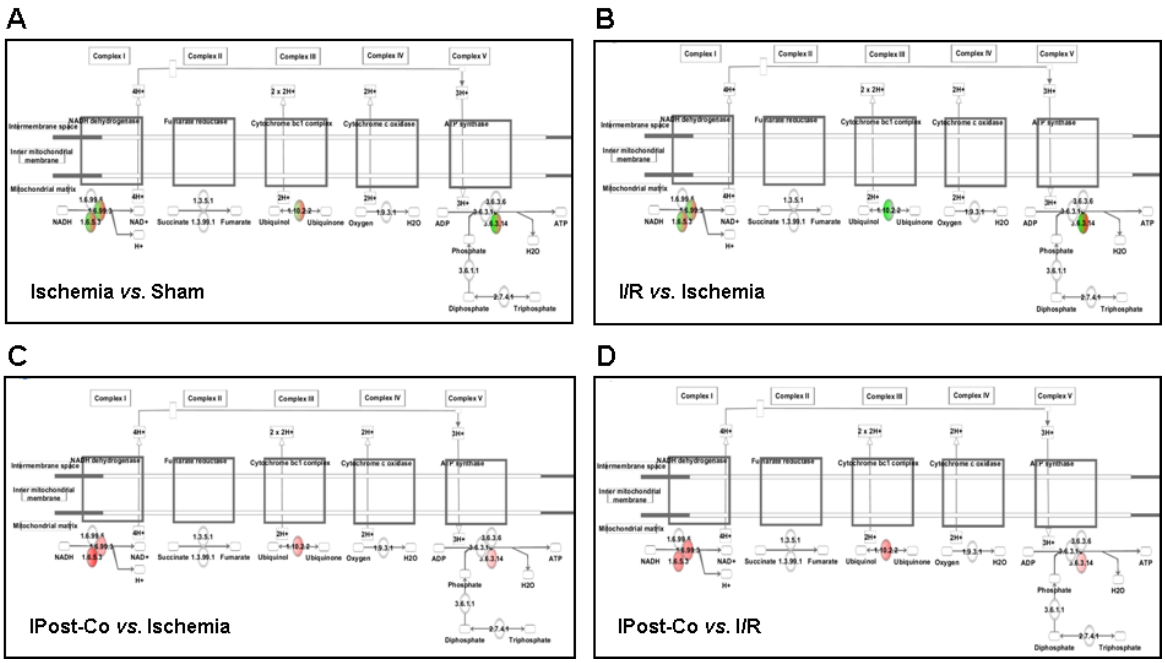

A
Ischemia *vs.* Sham
B
I/R *vs.* Ischemia
C
IPost-Co *vs.* Ischemia
D
IPost-Co *vs.* I/R

Figure 6


A
Ischemia vs. Sham
B
I/R vs. Ischemia
C
IPost-Co vs. Ischemia
D
IPost-Co vs. I/R

SUPPLEMENTAL MATERIAL

Supplemental TABLE 1. Mitochondrial proteins.

Protein Nº	Swiss-Prot Nº	Protname	pI	Mw	Mascot score	Sequence coverage (%)
1	Q148N0 #	2 oxoglutarate dehydrogenase E1 component	6.4	114	77	10.0
2	P36957 *	2 oxoglutarate dehydrogenase E2 component	5.7	52.1	62	11.3
3	Q99714 *	3-hydroxyacyl-CoA dehydrogenase type-2	9.3	16.9	57	20.2
4	P06576 *	ATP synthase beta subunit	4.9	51.6	155	21.7
5	P19483 #	ATP synthase subunit alpha	5.7	29.7	109	17.2
		ATP synthase subunit alpha	9.2	52.4		
6	P31930 *	Cytochrome b-c1 complex subunit 1	5.2	49.9	58	14.6
7	Q9CR68 ||	Cytochrome b-c1 complex subunit Rieske	7.8	17.4	68	14.2
8	P29147 ¶	D-beta-hydroxybutyrate dehydrogenase	9.7	21.4	50	12.0
9	P49819 §	Dihydrolipoyl dehydrogenase	7	63.5	73	20.0
10	Q6UAQ8	Electron transfer flavoprotein subunit beta	8.6	27	93	33.3
11	P55931	Electron transfer flavoprotein-ubiquinone oxidoreductase	7.1	68.6	66	14.0
12	P16276	Heart aconitase	7.9	90.1	105	22.2
		Heart aconitase	8	89.3		
		Heart aconitase	8.2	88.5		
		Heart aconitase	7.7	90.5		
13	P00348	Hydroxyacyl-coenzyme A dehydrogenase	10	23.5	60	15.9
14	Q9D6R2 ||	Isocitrate dehydrogenase [NAD] subunit alpha	4.8	22.8	93	21.6
		Isocitrate dehydrogenase [NAD] subunit alpha	5.9	28.5		
15	P33198	Isocitrate dehydrogenase [NADP]	9.5	41.5	64	29.2
		Isocitrate dehydrogenase [NADP]	9.8	40.1		
16	Q16891 *	Mitochondrial inner membrane protein	6.1	83.7	71	15.0
17	Q0MQI5 ‡	NADH dehydrogenase [ubiquinone] flavoprotein 1	8.5	50.8	102	17.7
18	Q0MQG1 ‡	NADH-ubiquinone oxidoreductase 75 kDa subunit	5.4	19.6	85	7.6
		NADH-ubiquinone oxidoreductase 75 kDa subunit	5.3	82.8		
		NADH-ubiquinone oxidoreductase 75 kDa subunit	8	50.4		
19	P29804	Pyruvate dehydrogenase E1 component subunit alpha	6.9	43.1	116	17.2
		Pyruvate dehydrogenase E1 component subunit alpha	6.9	42.8		
20	Q9Z2I9 ||	Succinyl-CoA ligase [ADP-forming] subunit beta	5.8	42.6	67	16.0
		Succinyl-CoA ligase [ADP-forming] subunit beta	5.7	41.5		
21	Q29551	Succinyl-CoA:3-ketoacid-coenzyme A transferase	7.2	58.5	75	15.0
		Succinyl-CoA:3-ketoacid-coenzyme A transferase	7.4	61.5		
22	P28768	Superoxide dismutase [Mn]	6.8	15.7	70	16.0
		Superoxide dismutase [Mn]	7	15		
		Superoxide dismutase [Mn]	7.1	15		
23	Q2NL34 #	Ubiquinone biosynthesis protein COQ9	5	24.9	58	2.0
24	P21796 *	Voltage-dependent anion-selective channel protein 1	8.6	30.7	134	48.0
25	P68002 #	Voltage-dependent anion-selective channel protein 2	7.5	31.6	63	21.8

Swiss-Prot number: Sus scrofa; * Homo sapiens; # Bos taurus; || Mus musculus; ¶ Rattus norvegicus; § Canis familiaris; ‡ Gorilla gorilla gorilla.

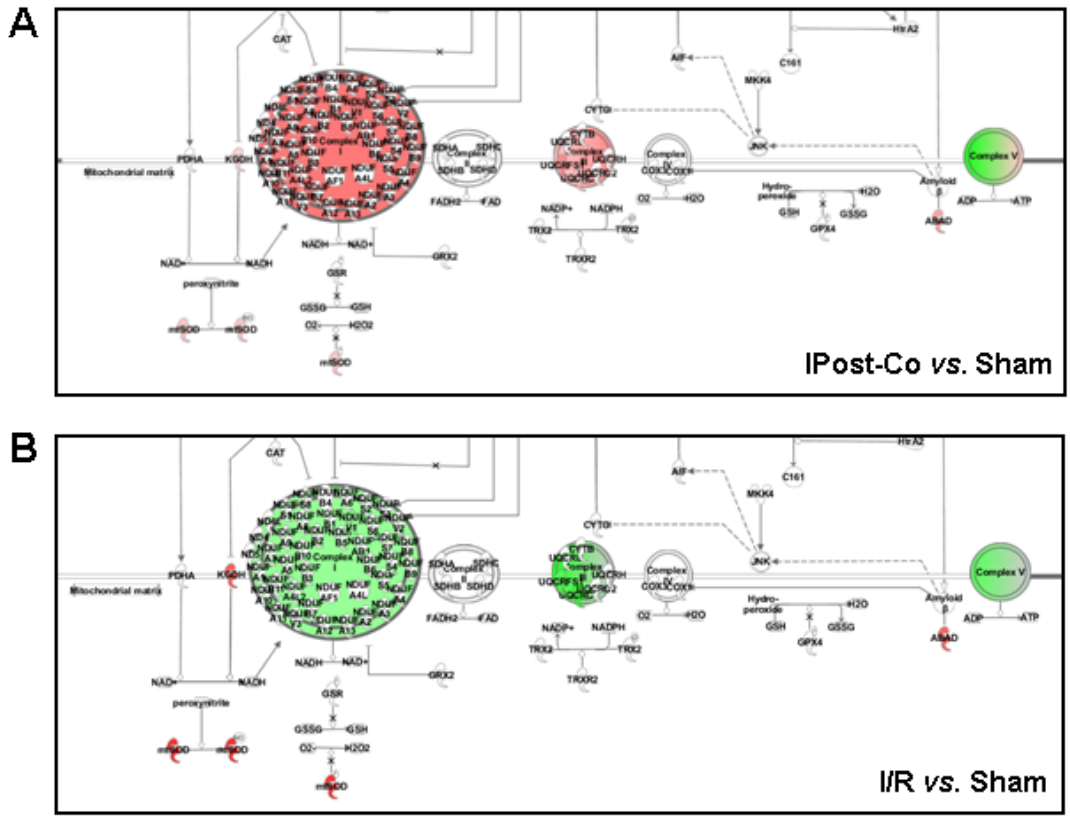
Mascot score: mean value of at least three independent sample identifications.

Supplemental TABLE 2. Ratios of spot intensities between groups.

Protein Nº	Protname	I *vs.* S	I/R *vs.* I	I/R *vs.* S	IPost-Co *vs.* I/R	IPost-Co *vs.* I	IPost-Co *vs.* S
1	2 oxoglutarate dehydrogenase E1 component	0.7	1.5	1.1	1.2	1.7	1.2
2	2 oxoglutarate dehydrogenase E2 component	1.0	1.0	1.0	2.5	2.5	2.6
3	3-hydroxyacyl-CoA dehydrogenase type-2	1.4	0.5	0.6	2.3	1.0	1.5
4	ATP synthase beta subunit	0.3	2.3	0.6	1.3	3.0	0.8
5	ATP synthase subunit alpha	1.1	0.4	0.5	3.4	1.5	1.6
6	Cytochrome b-c1 complex subunit 1	0.8	0.8	0.6	5.4	4.3	3.2
7	Cytochrome b-c1 complex subunit Rieske	1.2	0.4	0.5	2.6	1.1	1.4
8	D-beta-hydroxybutyrate dehydrogenase	1.7	0.0	0.0	ND IR	1.8	3.0
9	Dihydrolipoyl dehydrogenase	0.6	0.3	0.2	6.2	2.0	1.1
10	Electron transfer flavoprotein subunit beta	1.3	0.6	0.7	2.9	1.6	2.0
11	Electron transfer flavoprotein-ubiquinone oxidoreductase	1.1	0.8	0.8	2.7	2.0	2.1
12	Heart aconitase	1.4	1.0	1.3	1.4	1.4	1.9
13	Hydroxyacyl-coenzyme A dehydrogenase	1.0	1.0	1.1	0.8	0.9	0.9
14	Isocitrate dehydrogenase [NAD] subunit alpha	0.6	0.8	0.5	1.8	1.4	0.8
15	Isocitrate dehydrogenase [NADP]	1.3	0.8	1.0	1.6	1.3	1.6
16	Mitochondrial inner membrane protein	0.9	0.7	0.7	4.5	3.3	3.1
17	NADH dehydrogenase [ubiquinone] flavoprotein 1	1.3	0.7	1.0	3.9	2.9	3.8
18	NADH-ubiquinone oxidoreductase 75 kDa subunit	0.4	1.6	0.7	4.9	7.6	3.3
19	Pyruvate dehydrogenase E1 component subunit alpha	1.3	0.6	0.7	1.3	0.8	1.0
20	Succinyl-CoA ligase [ADP-forming] subunit beta	0.7	1.0	0.7	2.0	1.9	1.4
21	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1	0.7	1.3	1.0	2.8	3.8	2.7
22	Superoxide dismutase [Mn]	1.2	1.2	1.3	1.2	1.4	1.6
23	Ubiquinone biosynthesis protein COQ9	0.8	0.6	0.5	3.7	2.0	1.7
24	Voltage-dependent anion-selective channel protein 1	0.8	0.0	0.0	ND IR	2.1	1.6
25	Voltage-dependent anion-selective channel protein 2	1.2	0.6	0.7	8.6	4.8	5.8

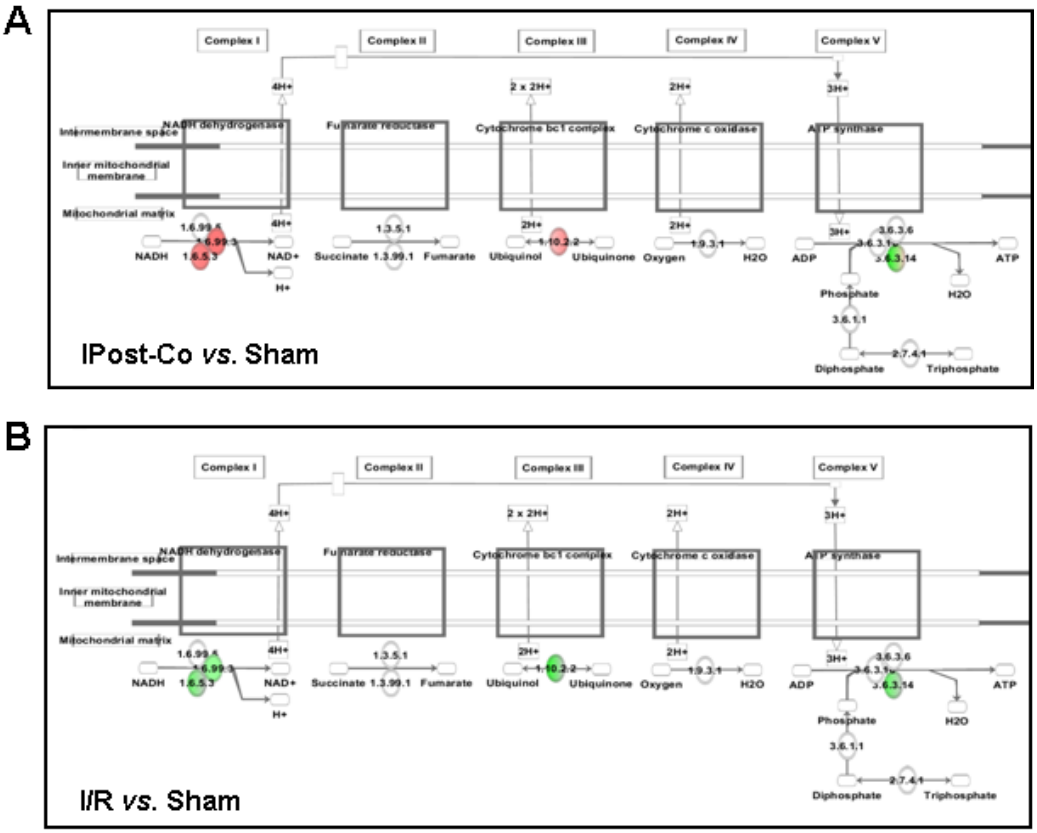
I = ischemia; I/R = ischemia/reperfusion; IPost-Co = ischemic post-conditioning; ND = no detected; S = sham.

Supplemental Figure 1



Supplemental Figure 1. Canonical pathway involved in mitochondrial dysfunction obtained with the Ingenuity System Pathway Analysis software after the analysis of differential proteins in (A) post-conditioning compared to sham operated animals; (B) ischemia/reperfusion (I/R) compared to sham operated animals. Proteins in green are down regulated and in red are up-regulated.

Supplemental Figure 2



Supplemental Figure 2. Canonical pathway involved in oxidative phosphorylation obtained with the Ingenuity System Pathway Analysis software after the analysis of differential proteins in (A) post-conditioning compared to sham operated animals; (B) ischemia/reperfusion (I/R) compared to sham operated animals. Proteins in green are down regulated and in red are up-regulated.

La Dra. Lina Badimón como directora de esta tesis de la doctoranda Judit Cubedo Ràfols certifica que la contribución de la doctoranda a los artículos presentados en esta tesis ha sido la siguiente:

En la publicación **"Proteomic signature of Apolipoprotein J in the early phase of new-onset myocardial infarction"** en la revista ***Journal of Proteome Research*** la realización de los experimentos que configuran las figuras 1 a 6, así como la redacción del manuscrito.

En la publicación **"Differential proteomic distribution of TTR (pre-albumin) forms in serum and HDL of patients with high cardiovascular risk"** en la revista ***Atherosclerosis*** la realización de los experimentos que configuran las figuras 1 a 4 y las figuras adicionales 1 a 4, así como la redacción del manuscrito.

En el manuscrito enviado **"Lipocalin RBP-4 in acute myocardial infarction: effects on vascular function and inflammation"** la realización de los experimentos que configuran las figuras 1 a 6 y la figura adicional 1, así como la redacción del mismo.

En el manuscrito en preparación **"Apolipoprotein A-I glycosylation changes in acute coronary syndromes"** la realización de los experimentos que configuran las figuras 1 a 6 y las figuras adicionales 1 y 2, así como la redacción del mismo.

En el manuscrito en preparación **"Identification of a novel ApoA-I truncated form transported by LDL and increased in diabetics"** la realización de los experimentos que configuran las figuras 1 a 5 y la figura adicional 1, así como la redacción del mismo.

En el manuscrito enviado **"Reperfusion triggered stress protein response in the myocardium is blocked by postconditioning. Systems biology pathway analysis highlights the key role of the canonical aryl-hydrocarbon receptor pathway"** la realización de los experimentos que configuran las figuras 4, 5 y 6 y la figura adicional 1, así como la redacción del mismo.

En el manuscrito en preparación **"Ischemic post-conditioning affords a coordinated change in mitochondrial enzymes impaired by ischemia and reperfusion"** la realización de los experimentos que configuran las figuras 1 a 6 y las figuras adicionales 1 y 2, así como la redacción del mismo.

En la publicación **"Serum proteome in acute myocardial infarction"** en la revista ***Clínica e Investigación en Arteriosclerosis*** la realización de los experimentos que configuran las figuras 1 a 3, así como la redacción del manuscrito.

Además certifica que ninguna de estas publicaciones ha sido utilizada en ninguna otra tesis doctoral.

Firmado,

Dra. Lina Badimón

DISCUSIÓN

Cada vez hay un mayor conocimiento de los mecanismos que contribuyen a la aparición de manifestaciones clínicas de la aterotrombosis tales como el infarto agudo de miocardio, y a su vez hay un incremento de las tecnologías para la identificación de nuevos biomarcadores detectables en sangre. A pesar de ello, desde el punto de vista clínico, sigue habiendo una falta importante de biomarcadores no sólo para la detección temprana y el pronóstico de los eventos aterotrombóticos, sino también para mejorar la estratificación del riesgo y aportar información a los factores de riesgo ya existentes. La mayoría de estudios realizados hasta el momento coinciden en que los biomarcadores disponibles aportan muy poca información adicional incrementando sólo ligeramente la capacidad pronóstica tanto en la prevención primaria como en la secundaria de los factores de riesgo clásicos. Hay, por tanto, una gran necesidad de buscar nuevos biomarcadores mediante el uso de nuevas técnicas analíticas diferentes a las utilizadas habitualmente en la práctica clínica (Blankenberg et al. 2006; Wang et al. 2006; Olsen et al. 2007; Melander et al. 2009; Schnabel et al. 2010). Para tal fin, las técnicas de proteómica representan un avance importante para la búsqueda y caracterización de nuevos biomarcadores. Mediante dichas técnicas se pueden estudiar de forma global los cambios coordinados que se dan tras un infarto agudo de miocardio. Esta "globalidad" de los estudios proteómicos nos acerca a la posibilidad de identificar varios marcadores de la patología estableciendo un multimarcador que aumente la capacidad de predicción del riesgo.

En esta tesis, mediante el uso de técnicas proteómicas, específicamente la electroforesis bidimensional, se han analizado los cambios en el proteoma sérico de pacientes con infarto agudo de nueva presentación dentro de las 6 primeras horas desde el inicio del evento y se ha estudiado la evolución de dichos cambios hasta los 3 días posteriores al ingreso. En las primeras horas posteriores al inicio del AMI se observa un importante cambio en el patrón sérico de proteínas relacionadas con las HDL. Los niveles de HDL están altamente relacionados con el riesgo cardiovascular. De hecho, muchos estudios se han centrado en la posibilidad de utilizar las HDL como diana terapéutica (Choi et al. 2006). A pesar de los esfuerzos, hoy en día aún no está claro si el incrementar farmacológicamente los niveles de colesterol-HDL sería posible y eficaz en humanos. Lo que sí se sabe es que las HDL transportan proteínas implicadas en

funciones tales como la activación del complemento, regulación de la proteólisis y procesos de respuesta de fase aguda (Vaisar et al. 2007), y que la importancia de estas macromoléculas vendría dada más que por sus niveles de colesterol por su composición proteica que determinaría su funcionalidad (Navab et al. 2007). Hay evidencias clínicas y experimentales de que durante los procesos de fase aguda las HDL pierden proteínas con propiedades anti-inflamatorias y citoprotectoras (Van Lenten et al. 2001; Camont et al. 2011).

En esta tesis doctoral, mediante la aplicación de técnicas proteómicas en muestras de suero se han identificado proteínas previamente relacionadas con las HDL, tales como la Apo A-I, A-IV, E, J, transtiretina y paraoxonasa-1 (Rezaee et al. 2006). Es más, el estudio proteómico del suero de pacientes con infarto agudo de miocardio de nueva presentación revela cambios en proteínas relacionadas con las HDL en la fase temprana del evento. Entre éstas proteínas, la ApoJ, también llamada clusterina, muestra un importante cambio en su patrón de distribución en el suero de pacientes AMI. La Apo J está representada por una serie de diferentes isoformas que varían en función del estado fisiopatológico del individuo (Rodriguez-Pineiro et al. 2006), y que se detectan el en proteoma del suero como un conjunto de puntos proteicos que difieren en su masa molecular y su pI. Estas diferentes isoformas se deben a los múltiples sitios de N-glicosilación de la molécula que consisten en azúcares complejos que representan el 20-30% de su peso molecular (Blaschuk et al. 1983; Griswold et al. 1986; Burkey et al. 1991). Este estudio, ha demostrado por primera vez, una predominancia de las isoformas de Apo J de menor peso molecular y un pI más básico. De hecho, el análisis específico de las glicoproteínas del suero revela una importante disminución en las formas glicosiladas de la Apo J dentro de las 6 primeras horas desde el inicio del AMI. Anteriormente, ya se han asociado diferentes isoformas glicosiladas de la Apo J con otras patologías como el cáncer (Pucci et al. 2004) y el Alzheimer (Nilselid et al. 2006). Junto con los cambios en el patrón de distribución proteómico se ha detectado una disminución significativa en los niveles de Apo J en la fase temprana post-AMI, con una posterior recuperación a partir de las 24h desde el ingreso hasta alcanzar los valores normales a las 72-96h. Estas variaciones dependiendo del tiempo transcurrido desde el inicio del evento pueden ser las responsables de la controversia en los diferentes estudios publicados relacionando la Apo J y la enfermedad coronaria (Trougakos et al. 2002; Kujiraoka et al. 2006). A partir de estos resultados no se puede excluir que los

pacientes que han tenido un AMI tuviesen previamente niveles más bajos de Apo J antes del evento y por lo tanto una mayor susceptibilidad. Sin embargo, en el estudio Caerphilly (Mackness et al. 2003) los pacientes con un ACS no mostraron variaciones en los niveles de Apo J previamente al evento. Es más, el hecho de que haya una disminución en la fase temprana del AMI y una posterior normalización de los niveles a las 72-96h parece reflejar una situación transitoria inducida por el AMI. En el momento del ingreso hay una correlación positiva entre los niveles de CRP y de ApoJ lo que podría estar indicando una respuesta sistémica contra la situación pro-inflamatoria derivada de la isquemia. De hecho, hay estudios que atribuyen propiedades anti-inflamatorias a la ApoJ (McLaughlin et al. 2000), que podrían estar mediadas a través de NF-κB (Santilli et al. 2003). Los resultados de esta tesis ponen de manifiesto que la ApoJ está presente en el miocardio de corazón isquémico pero no en tejido normal, lo que podría contribuir al proceso de cicatrización del miocardio. A este respecto, estudios previos ya habían descrito la deposición de Apo J en miocardio infartado (Vakeva et al. 1993). Es más, un estudio reciente basado en un modelo experimental de AMI en rata atribuye a la Apo J un efecto protector sobre el cardiomiocito (Van Dijk et al. 2010). Los cambios detectados en la Apo J apoyan la hipótesis que durante las fases iniciales tras un AMI se dan respuestas sistémicas que tratan de compensar la respuesta inflamatoria del organismo, de hecho, en el modelo experimental porcino de isquemia/reperfusión se ha visto que tras 30min de isquemia ya se dan cambios sistémicos pro-inflamatorios (Vilahur et al. 2009).

Otra de las proteínas con un perfil diferencial en suero de pacientes con AMI es la apolipoproteína A-I. Sin embargo, a diferencia de la Apo J que se modifica en la fase temprana, los cambios en la Apo A-I se manifiestan mayoritariamente entre las 72-96h del ingreso. La Apo A-I es la proteína mayoritaria de las HDL ya que representa el 70% de su contenido proteico total y tiene un papel fundamental en la formación, remodelado y metabolismo de las HDL (Davidson et al. 2007; Tall et al. 2008). La Apo J y la Apo A-I, junto con la paraoxonasa (PON) forman una clase específica de HDL (Jenne et al. 1991) a la que se le ha atribuido un papel protector debido a la reducción de peróxidos lipídicos en membranas y lipoproteínas (Mackness et al. 1995; Mackness et al. 1996). De hecho, se ha descrito un aumento en la acumulación de Apo J, Apo A-I y PON en arterias ateroscleróticas como consecuencia de la captación de HDL por parte de la pared del vaso (Mackness et al. 1997). Por lo que una reducción en los niveles de esta

clase de HDL podría representar una pérdida en la protección frente al estrés oxidativo asociado a la aterosclerosis.

Los cambios observados en la Apo A-I sérica hacen referencia, tanto a los niveles totales como a su patrón proteómico. Mediante 2-DE se ha identificado la Apo A-I como una serie de 5 formas de 28 kDa y un rango de pI entre 5 y 5.75 (formas de 1 a 5). El análisis específico de las glicoproteínas del suero revela que las formas 1, 2, 3 y 5 están glicosiladas y que la forma 4 es la única no glicosilada. A los 3 días tras el AMI los pacientes presentan un patrón de Apo A-I mayoritariamente en su forma glicosilada siendo indetectable la forma no glicosilada (forma 4) en el 62% de los pacientes. La presencia mayoritaria de formas glicosiladas de Apo A-I tras un AMI puede tener un impacto en sus propiedades ya que se ha demostrado que la glicosilación no enzimática con metilglioxal reduce las propiedades anti-inflamatorias de la Apo A-I (Nobecourt et al. 2010). De hecho, se ha visto que la glicosilación de la Apo A-I mediada por fructosa induce la pérdida de propiedades beneficiosas de la Apo A-I y las HDL (Park et al. 2010). Por lo que los cambios observados en la Apo A-I tras el AMI pueden llevar a la pérdida de sus propiedades beneficiosas y a una menor protección de los pacientes.

Los cambios observados en relación a la glicosilación de la Apo A-I llevaron a la realización de estudios complementarios en muestras de pacientes diabéticos. En dichos pacientes hay una correlación entre la intensidad total de las formas correspondientes a la Apo A-I glicosilada con los niveles de hemoglobina glicosilada. Además, se ha detectado un incremento en la forma menos procesada de la Apo A-I dado que la forma más básica representa la isoforma menos madura de la proteína (Jaleel et al. 2010). Junto con las 5 formas de 28kDa se ha detectado una sexta forma de Apo A-I de menor peso molecular (26kDa) y un pI de 5.75, que se ha identificado por primera vez como una forma truncada de Apo A-I a la que le faltan los aa 1-38, que se denomina en esta tesis como Apo A-I-Barcelona (Apo A-I-BCN). Esta forma truncada presenta un incremento del 64% en su intensidad en los pacientes diabéticos. Hay estudios que describen que en pacientes con enfermedad coronaria las metaloproteasas derivadas de los macrófagos proteolizan la Apo A-I asociada a las HDL por los extremos N- y C-terminal (Eberini et al. 2002). La Apo A-I-BCN se ha detectado en la fracción LDL pero no en la HDL. Estudios *in vitro* han demostrado que la pérdida de los residuos 1-43 induce la formación de una estructura terciaria de la Apo A-I de menor estabilidad (Rogers et al. 1997). Concretamente, los 44 aa del extremo N-terminal de la Apo A-I son responsables de la estabilización de la Apo A-I soluble. Recientemente, se ha

demostrado que los residuos 35-49, juegan un papel en la adaptación de la Apo A-I al tamaño de las HDL (Lagerstedt et al. 2011). Es más, formas mutadas de Apo A-I con delecciones en los aa 1-41 y 1-59 presentan alteraciones en la capacidad de unión a lípidos (Fang et al. 2003). Por lo que el aumento de Apo A-I-BCN en los diabéticos puede contribuir a su mayor riesgo cardiovascular mediante el bloqueo de la transición de Apo A-I soluble libre de lípidos a las partículas de HDL (Li et al. 2004). De hecho, los aa del extremo N-terminal son necesarios para la formación de grandes complejos de HDL, y su ausencia conlleva la presencia de partículas de HDL menos estables (Gu et al. 2010). Es más, la presencia de esta forma truncada de Apo A-I en las LDL puede contribuir a la ya descrita presencia de LDL modificadas en los pacientes diabéticos. Diversos estudios han descrito diferentes modificaciones en las LDL de diabéticos (Scheffer et al. 2005) tales como las oxidaciones (Dimitriadis et al. 1995; Yoshida et al. 1997) y glicosilaciones (Moro et al. 1999), que llevan a la formación de partículas LDL más aterogénicas en estos pacientes (Witztum et al. 1982; Regnstrom et al. 1992). Se ha llegado a relacionar el incremento de riesgo cardiovascular en los pacientes diabéticos con la presencia de LDL más pequeñas y densas que son más aterogénicas, y explican, en parte el mayor riesgo de estos pacientes (Vakkilainen et al. 2003) incluso teniendo niveles de colesterol-LDL similares a individuos no diabéticos (Collins et al. 2003).
De la misma forma que ocurre con la Apo J, la Apo A-I interacciona con diferentes proteínas de tal forma que se generan partículas de HDL con diferentes propiedades. Este es el caso de la asociación descrita entre la Apo A-I y la transtiretina (TTR) o prealbúmina, que determina la formación de partículas específicas de HDL a las que se les ha atribuido cambios en su estabilidad (Sousa et al. 2000). Es más, se ha descrito una actividad de la TTR como proteasa que genera moléculas de Apo A-I truncadas por el extremo C-terminal, lo que podría tener un efecto deletéreo en el metabolismo lipídico y además incrementar el potencial amiloidogénico de la Apo A-I (Liz et al. 2004; Liz et al. 2007).

La TTR es una proteína de 14kDa con la capacidad de formar oligómeros que ha sido asociada a procesos de respuesta de fase aguda relacionados con la inflamación (Ingenbleek et al. 2002). En esta tesis se ha demostrado por primera vez una relación inversa entre los niveles de la forma trimérica de la TTR (tTTR) detectada mediante 2-DE y el número de factores de riesgo cardiovascular en los pacientes AMI, siendo la reducción especialmente evidente en los pacientes con diabetes y dislipemia. Además de

esta asociación entre tTTR y la presencia de factores de riesgo cardiovascular, también se ha detectado una disminución en los niveles de tTTR en pacientes con niveles de CRP >3mg/L en el momento del ingreso. De hecho esta asociación inversa entre tTTR y CRP, no se ve cuando se analizan los niveles totales de TTR, subrayando la importancia de la forma específica de tTTR, pero no de los niveles totales, en la respuesta inflamatoria aguda.

La validación de los resultados obtenidos en la 2-DE llevada a cabo mediante ELISA reveló una disminución inicial en los niveles totales de TTR a las 6 primeras horas desde el inicio del AMI con una disminución progresiva hasta alcanzar los niveles más bajos a las 72-96h. La disminución a las 72-96h coincide con la desaparición de la tTTR en la 2-DE y con el punto más alto en los niveles de CRP, por lo que cambios específicos en la forma trimérica de la TTR son representativos del proceso de respuesta inflamatoria. En estudios previos, se ha propuesto la medición conjunta de la CRP y la TTR para el diagnóstico y el seguimiento de infecciones post-operatorias (Ferard et al. 2002).

Debido a la importante disminución en la tTTR en pacientes AMI con diabetes y dislipemia, y a la asociación descrita entre la TTR y la Apo A-I en las HDL, se analizó el efecto de la dislipemia en los niveles de TTR en una población con alto riesgo cardiovascular con hipercolesterolemia familiar heterocigótica (hFH). Los pacientes hFH tienen niveles más bajos de TTR que los individuos controles pero más altos que los pacientes AMI, lo que sugiere que los cambios más marcados en la TTR están asociados a un mayor riesgo y a la precipitación de un evento agudo.

A diferencia de los resultados obtenidos en suero, el análisis proteómico de las HDL reveló sólo la presencia de la forma monomérica de la TTR (mTTR) así como una disminución de esta forma en los pacientes hFH, estando esta disminución asociada a un incremento en el riesgo cardiovascular calculado según las tablas Framingham (FRS). Esta asociación no se detecta cuando se miden los niveles totales de TTR mediante ELISA, mostrando una vez más, que una forma específica de TTR es más sensible a la patología cardiovascular que los niveles totales de la misma. Estos resultados indican que en una población de alto riesgo cardiovascular se dan cambios en la composición de las HDL que pueden contribuir a una reducción en la protección cardiovascular. Estudios previos ya han demostrado la presencia de la TTR en las lipoproteínas (Tanaka et al. 1994) y que ésta representa aproximadamente el 1-2% de la TTR total en el plasma (Sousa et al. 2000). Específicamente, los resultados de esta tesis demuestran que

sólo las HDL3 transportan la mTTR. Se ha propuesto que las propiedades anti-aterogénicas de las HDL3 vienen dadas por la composición de su proteoma que las diferencia de otras sub-clases (Davidson et al. 2009). Por este motivo la distribución diferencial de la mTTR en las diferentes fracciones de HDL podría tener una implicación en las propiedades anti-inflamatorias y anti-oxidantes de las HDL3. Es más, es posible que los bajos niveles de TTR en pacientes hFH no sean debidos a cambios en las LDL sino a una depleción específica de ciertas proteínas de las HDL que podría contribuir a una disminución de sus propiedades protectoras contra la enfermedad cardiovascular.

Uno de los papeles fundamentales de la TTR es el transporte de las hormonas tiroideas, por lo que una disminución en los niveles de TTR provocaría un incremento en los niveles de hormonas tiroideas libres. De hecho, niveles reducidos de TTR se han relacionado a un incremento en los niveles de tiroxina libre (Ingenbleek et al. 1999; Bernstein et al. 2002) lo que ha sido a su vez asociado a una peor supervivencia de los pacientes tras sufrir un AMI (Friberg et al. 2001). Además, cambios en la homeostasis de las hormonas tiroideas han sido relacionados con patologías graves como el infarto de miocardio (Franklyn et al. 1984; Eber et al. 1995).

El otro papel fundamental de la TTR es el transporte de la proteína plasmática de unión a retinol (RBP4) en la sangre, así como la estabilización del complejo retinol-RBP4 (Zanotti et al. 2004). Se ha demostrado que el cociente retinol/RBP4 está asociado con el grosor íntima-media, sugiriéndose que el RBP4 libre de retinol (apo-RBP4) juega un papel en el desarrollo de la aterosclerosis (Bobbert et al. 2010). Por lo que, cambios en los niveles de TTR pueden influenciar los niveles de apo-RBP4 y como consecuencia tener un impacto en el riesgo cardiovascular. De hecho, en esta tesis se muestra tanto mediante técnicas proteómicas como por ELISA, que los pacientes AMI también presentan una disminución significativa en los niveles de RBP4 y que dentro de las seis primeras horas desde el inicio del dolor se ve una correlación positiva entre los niveles de TTR y RBP4.

La función mayoritaria del RBP4 es el transporte del retinol a través de la sangre hacia los tejidos diana (Blaner 1989) por lo que esta disminución en los niveles de RBP4 puede llevar a una disminución en la eficiencia de dicho transporte. El retinol es necesario para mantener la inmunidad y la renovación epitelial por lo que una carencia de retinol en los tejidos puede afectar a la integridad del epitelio e incluso aumentar la

susceptibilidad al estrés oxidativo (Tsavaris et al. 2005). De hecho, se han descrito niveles reducidos de retinol durante las primeras 48h tras un AMI (Scragg et al. 1989) coincidiendo con la bajada de RBP4 detectada en este estudio que se sigue manteniendo hasta las 72-96h tras el ingreso.

La mayor fuente de RBP4 en los humanos es el hígado, aunque también se ha visto secreción por parte de los adipocitos (Yang et al. 2005). De hecho cada vez hay más evidencias de que el RBP4 juega un papel como nexo de unión entre el tejido adiposo y la aterosclerosis. A lo largo de los últimos años, varios estudios han relacionado esta adipoquina con diferentes factores de riesgo cardiovascular como la diabetes tipo 2, obesidad, síndrome metabólico e incluso la edad avanzada (Cho et al. 2006; Graham et al. 2006; Qi et al. 2007; Ingelsson et al. 2009). Es más, la medición de los niveles de RBP4 se ha propuesto como un marcador de respuesta de fase aguda (Baeten et al. 2004; Berton et al. 2010). Recientemente se ha descrito que el RBP4 ejerce un efecto vasodilatador a través del aumento de óxido nítrico (NO), sugiriendo un papel de esta adipoquina en la regulación de la función vascular (Takebayashi et al. 2007). Los resultados de esta tesis demuestran por primera vez una disminución en los niveles de RBP4 en pacientes en la fase temprana post-AMI, revelando que los niveles de RBP4 se ven afectados en una situación patológica que cursa con niveles reducidos de NO. A su vez, el RBP4 induce la expresión de eNOS en células endoteliales y un incremento en la liberación de PGI_2 por parte de las células musculares lisas. Los resultados obtenidos en conjunto aportan relevancia clínica a los efectos vasodilatadores y anti-inflamatorios del RBP4 observados *in vitro* al demostrar cambios agudos en los niveles de RBP4 en condiciones fisiológicas. Por lo que, una reducción en los niveles de RBP4 durante la fase temprana post-AMI puede tener efectos deletéreos debido a la pérdida de sus propiedades vasodilatadoras. Los estudios proteómicos revelan la presencia de dos formas de RBP4 que coinciden con las formas de RBP4 entera y RBP4 truncada en la región C-terminal, descritas en estudios previos (Jaconi et al. 1995), siendo la forma de menor peso molecular es la más sensible a los cambios inducidos por el AMI.

A pesar del desarrollo de una metodología altamente reproducible que permite el estudio de un amplio rango de proteínas del suero, la aplicación de técnicas proteómicas para la búsqueda de biomarcadores en suero presenta inconvenientes para el análisis de moléculas de señalización intracelulares debido a que son moléculas minoritarias que no siempre son liberadas a la circulación. Sin embargo, el estudio de este tipo de moléculas

puede aportar información muy útil sobre el desarrollo de la patología así como de sus posibles dianas terapéuticas. Por ello, los estudios en modelos experimentales pre-clínicos aportan información complementaria a la obtenida a partir de muestras humanas en la búsqueda de biomarcadores. Es por ello que en una segunda fase de esta tesis se ha llevado a cabo el estudio del proteoma del tejido de miocardio en un modelo experimental porcino de isquemia/reperfusión que presenta grandes ventajas para el estudio del AMI. Por un lado, la gran similitud del sistema cardiovascular porcino con el humano permite alcanzar conocimientos que pueden ser llevados a la práctica clínica. Por el otro, se ha utilizado un modelo altamente estandarizado (Vilahur et al. 2009; Vilahur et al. 2009) que presenta la ventaja de seleccionar de forma muy precisa la zona de oclusión coronaria y la posibilidad de controlar la duración de la isquemia y la reperfusión, por lo que se obtienen resultados altamente consistentes y reproducibles que permiten evaluar los cambios metabólicos a nivel cardíaco (Vilahur et al. 2011).

Actualmente, la reperfusión dentro de las primeras horas tras el AMI es la única forma de rescatar el corazón (Huber et al. 2005). A pesar de ello este proceso está asociado al daño celular por la activación de cascadas de señalización deletéreas que pueden acabar provocando la muerte de las células cardiacas y contribuyendo al incremento del tamaño del infarto lo que habitualmente se denomina daño por isquemia/reperfusión (Braunwald et al. 1985).

En este contexto, el pre-condicionamiento isquémico (breves periodos de I/R antes de la isquemia) ha demostrado que es capaz de proteger el miocardio en diversos contextos clínicos de I/R como la cirugía cardíaca, la angina pre-infarto y la angioplastia (Zhao et al. 2003; Tsang et al. 2004; Kin et al. 2008). A pesar de ello, la utilidad clínica del pre-condicionamiento isquémico no es idónea para pacientes con AMI dado que dicha intervención debe llevarse a cabo antes de la isquemia.

Diversos trabajos experimentales y estudios en humanos (Zhao et al. 2003; Staat et al. 2005; Thibault et al. 2008) apoyan que el concepto del post-condicionamiento isquémico (IPost-Co) puede tener un efecto clínico importante que reside en la disminución del tamaño del infarto. Dicha técnica se basa en la aplicación de breves periodos de I/R tras la isquemia y justo antes de iniciar la reperfusión, por lo que puede ser aplicable en la práctica clínica (Staat et al. 2005; Thibault et al. 2008). Dado que no está claro cuál es el mecanismo que interviene en la cardioprotección mediada por IPost-Co, en este trabajo se han aplicado técnicas proteómicas para el estudio de los

cambios que se dan en el tejido cardíaco tras la aplicación del IPost-Co en comparación con los cambios inducidos por la isquemia/reperfusión.

Los resultados de esta segunda fase de la tesis revelan que la isquemia y la I/R inducen importantes cambios en el proteoma cardíaco, entre los que destaca una disminución en las proteínas citoplasmáticas. La alfa actina es la única proteína que aumenta de forma significativa, lo que podría estar indicando un efecto compensatorio para restaurar la pérdida de tejido cardíaco. De hecho, la evaluación clínica de los corazones ha demostrado que el IPost-Co induce una reducción en el tamaño del infarto así como una mejora generalizada de la función cardíaca. Hay estudios que demuestran que el IPost-Co es el primer método que reduce el tamaño del infarto en un 50% en varios modelos *in vivo* y que ejerce cardioprotección en estudios clínicos (Staat et al. 2005; Thibault et al. 2008; Skyschally et al. 2009).

Los resultados obtenidos en esta tesis demuestran que el IPost-Co revierte y/o previene el 43% de estos cambios citoplasmáticos. Es más, el estudio proteómico junto con el análisis *in silico* para la detección de posibles vías metabólicas involucradas ha revelado por primera vez la implicación de la vía de señalización citoplasmática Hsp90/AhR (*heat shock protein 90 kDa/aryl hidrocarbon receptor*) en los cambios inducidos por el IPost-Co.

La Hsp90 participa en los pasos iniciales de la vía de señalización del receptor de hidrocarburo de arilo (AhR). El AhR se encuentra en el citosol asociado a un dímero de Hsp90 (Denis et al. 1988; Perdew 1988; Pollenz et al. 1994). Cuando el AhR se une a su ligando se transloca el núcleo donde dimeriza con el ARNT (Swanson et al. 1993; Pollenz et al. 1994). De hecho, en un modelo de levadura, se ha visto que una bajada en los niveles de Hsp90 afecta a la función del AhR probablemente por una desestabilización de la proteína en ausencia de Hsp90 (Carver et al. 1994). La vía del AhR está involucrada en diferentes cascadas de transducción de señal que pueden llevar a la célula a la proliferación, arresto del ciclo celular o a la apoptosis. De hecho, dentro de los efectores finales de la vía del AhR encontramos moléculas pro-apoptóticas tanto de la vía extrínseca como de la intrínseca. Específicamente, se ha visto que Fas/FasL y Bax son efectores finales de la vía del AhR (Matikainen et al. 2001; Park et al. 2005), por lo que se podría pensar que la reducción de Hsp90 y AhR inducida por el IPost-Co lleva a una reducción en la vía de señalización del AhR que deriva en un bloqueo de los efectores pro-apoptóticos de la vía. Esta hipótesis queda confirmada por el incremento

detectado en la expresión de ARNT inducido por IPost-Co. Es más, el análisis proteómico revela que la I/R induce una disminución en los niveles de β-tubulina y que este efecto es revertido por el IPost-Co. Estos resultados apoyan el papel de la vía de AhR en la protección mediada por IPost-Co ya que se ha sugerido anteriormente que la β-tubulina juega un papel en dicha vía previniendo la translocación al núcleo del ARNT (Zhang et al. 2010). De hecho, en esta tesis se demuestra que el IPost-Co previene tanto la activación del receptor Fas/caspasa-8, como de las vías apoptóticas mitocondriales inducidas por la reperfusión llevando a una reducción del número de células apoptóticas, motivo por el cual no se observan cambios en el marcador de apoptosis bcl-2.

Teniendo en cuenta que la extensión del infarto no sólo se debe a la severidad de la isquemia (Reimer et al. 1977; DeBoer et al. 1983) sino que también a los procesos patológicos que se dan como consecuencia de la reperfusión (Gottlieb et al. 1994; Yaoita et al. 2000; Zhao et al. 2001), y considerando a su vez los resultados que apoyan que la apoptosis es un fenómeno inducido mayoritariamente por la reperfusión (Piper et al. 1998; Vilahur et al. 2011), los mecanismos antiapoptóticos inducidos por el IPost-Co deberían ser considerados un potencial mecanismo para explicar el aumento de miocardio rescatado.

Junto con los cambios observados en las proteínas citoplasmáticas, los estudios proteómicos han revelado que la isquemia y la reperfusión inducen cambios coordinados a nivel de proteínas mitocondriales, y que el IPost-Co revierte y/o previene el 57% de estos cambios mitocondriales que se dan tras el evento isquémico. De hecho, hay datos que evidencian que la mayoría de los cambios citoplasmáticos inducidos por IPost-Co convergen en la modulación mitocondrial de la muerte celular (Zhao et al. 2003; Ovize et al. 2010) lo que lleva al rescate de los cardiomiocitos parcialmente dañados.

Precisamente, en esta tesis se ha visto que la isquemia y la posterior reperfusión causan una disminución en las proteínas mitocondriales, lo que lleva a un deterioro de la función mitocondrial, mientras que el IPost-Co induce un incremento de dichas proteínas recuperando la función mitocondrial del corazón. Debido a la gran abundancia de mitocondrias en el corazón, la pérdida de la función mitocondrial tiene un gran impacto en el miocardio dado que el ATP es necesario para mantener la actividad contráctil (Suleiman et al. 2001). El análisis *in silico* de las proteínas diferenciales revela cambios importantes relacionados con la disfunción mitocondrial durante la

isquemia, específicamente en los complejos I, III y V de la membrana mitocondrial interna. Estudios previos han demostrado que las mitocondrias son tanto diana como fuente del daño inducido durante la I/R cardíaca (Chen et al. 2007).

El principal resultado de la isquemia es la disfunción metabólica mitocondrial provocada por una reducción del aporte de oxígeno al tejido, lo que resulta en una disminución de la formación de ATP en la fosforilación oxidativa (Taegtmeyer et al. 1980; Taegtmeyer et al. 1985). El daño mitocondrial isquémico es una de las causas mayoritarias del daño cardíaco, dado que la reperfusión del miocardio con la función oxidativa mitocondrial intacta disminuye de forma marcada el daño miocárdico (Chen et al. 2007). Los cambios detectados en los complejos mitocondriales durante la isquemia y la reperfusión pueden inducir el desacople de las mitocondrias contribuyendo al incremento en la producción de especies reactivas del oxígeno (ROS) (Sack 2006; Bodyak et al. 2007) lo que puede llevar al consiguiente daño del DNA por oxidación (Rosano et al. 2008). Por este motivo, los cambios mitocondriales observados en el IPost-Co podrían estar atenuando el estrés oxidativo, contribuyendo así a la preservación de la función cardíaca, de hecho, los protocolos de cardioprotección han demostrado inducir un aumento de la función mitocondrial así como de la fosforilación oxidativa (McCully et al. 2009). La importancia del estrés oxidativo en el daño por I/R ha sido demostrada en modelos de experimentación animal donde la sobre-expresión de proteínas anti-oxidantes inducen una disminución de la apoptosis y mejoran la función contráctil (Kang et al. 2003; Shiomi et al. 2004; Nagy et al. 2008; Zhao et al. 2009). Es más, se ha sugerido que la señalización redox puede estar involucrada en el efecto cardioprotector del pre-condicionamiento mediante un incremento en la cadena respiratoria mitocondrial (Pain et al. 2000; Forbes et al. 2001; Juhaszova et al. 2004; Robin et al. 2007). Los resultados obtenidos en este estudio demuestran que el IPost-Co induce un aumento en las proteínas de la fosforilación oxidativa lo que lleva a un aumento de la función mitocondrial reduciendo el daño por I/R. Esta modulación mitocondrial es, al menos en parte, un mecanismo efector de la cardioprotección derivada del IPost-Co.

El estudio específico de las proteínas modificadas durante el IPost-Co puede aportar información sobre posibles marcadores de isquemia y/o dianas terapéuticas. De este modo, la proteína *X1* disminuye de forma importante con la isquemia y recupera los valores normales cuando se aplica el IPost-Co. Estos resultados apuntan tanto a un posible papel como marcador ya que esta proteína es liberada a la sangre, como a un

posible papel terapéutico dado que una aproximación que confiere cardioprotección como es el IPost-Co hace que se normalicen sus valores. Por otro lado, proteínas como la *X2* y la *X3*, aumentan de forma muy importante con el IPost-Co, por lo que pueden ser moléculas efectoras de la protección frente al daño cardíaco y ser, por lo tanto, potenciales dianas terapéuticas ya sea mediante mecanismos que incrementen su expresión o incluso mediante la administración de las mismas.

CONCLUSIONES

A partir de los resultados obtenidos en esta tesis se puede concluir:

I. La apolipoproteína J presenta cambios en su patrón de glicosilación en la fase temprana tras un AMI:

- En las primeras seis horas tras un AMI hay una disminución en los niveles totales de Apo J, acompañada de un aumento específico de las formas menos glicosiladas de la misma. Por lo que el análisis de estos cambios de glicosilación puede llevar a un mejor entendimiento de la función de la Apo J y convertir las formas menos glicosiladas en un marcador de la fase temprana del AMI.
- Tras una isquemia hay una deposición de Apo J en el miocardio, lo que podría contribuir a la cicatrización del mismo tras un AMI.

II. La transtiretina presenta cambios en su patrón de distribución en situaciones de riesgo cardiovascular:

- La forma trimérica de la TTR (tTTR) presente en el suero disminuye tras un AMI de una forma inversa al número de factores de riesgo, siendo estos la dislipemia, la diabetes, la hipertensión y el tabaquismo.
- Los niveles totales de TTR disminuyen en una situación de riesgo cardiovascular como es la hipercolesterolemia familiar heterocigótica (hFH) y de una forma más marcada tras un AMI.
- La forma monomérica de la TTR (mTTR), la única presente en las HDL, está disminuida en pacientes hFH con manifestación clínica previa de patología cardiovascular, viéndose una asociación con el riesgo cardiovascular calculado según la clasificación Framingham.

III. La proteína plasmática de unión a retinol (RBP4) disminuye en la fase temprana tras un AMI lo que puede tener un efecto deletéreo en la función vascular y la situación aguda de inflamación.

- Los pacientes tras una AMI presentan una disminución en los niveles séricos de RBP4.
- La RBP4 puede tener un papel anti-inflamatorio a través de su efecto sobre COX2 a nivel de célula muscular lisa, así como un papel en la función vascular a nivel endotelial a través de eNOS.

IV. La apolipoproteína A-I sufre cambios a nivel molecular tanto en situaciones agudas como de alto riesgo cardiovascular.

- La Apo A-I está tanto N- como O-glicosilada.
- En la fase tardía post-AMI la Apo A-I presenta un mayor grado de glicosilación lo que podrían afectar a su función.
- Hay una distribución diferencial de la Apo A-I en las fracciones lipídicas y solubles del suero.
- Los pacientes diabéticos presentan un incremento importante de una forma truncada de la Apo A-I en su extremo N-terminal (Apo A-I-Barcelona) que también se encuentra en la fracción LDL y que podría afectar al intercambio de las diferentes formas de HDL y contribuir al mayor riesgo cardiovascular de estos pacientes.

V. El post-condicionamiento isquémico (IPost-Co) induce cambios en el proteoma del miocardio que llevan a una protección del mismo en procesos de isquemia/reperfusión.

- El IPost-Co induce una recuperación de las proteínas citoplasmáticas y mitocondriales del miocardio.
- Específicamente, dentro de las vías citoplasmáticas, el IPost-Co induce cambios en la vía del receptor de hidrocarburo de arilo (AhR), en la que participa el Hsp 90, que deriva en una reducción de las vías de apoptosis.
- Los cambios a nivel mitocondrial podrían contribuir a la preservación de la función mitocondrial.
- Las proteínas incrementadas por el IPost-Co son candidatas a moléculas terapéuticas por su posible efecto cardioprotector.

Conclusión global:

La proteómica constituye una herramienta de gran utilidad para el estudio de las enfermedades cardiovasculares tanto en pacientes como en modelos animales que permite tanto conocer los cambios globales que se dan en una situación patológica como el descubrimiento de nuevas vías implicadas en la fisiopatología de las enfermedades cardiovasculares.

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ANEXOS

ANEXO I. Puesta a punto metodología.

"Serum proteome in acute myocardial infarction"

Judit Cubedo * † ¶, Teresa Padró * ¶, Xavier García-Moll ‡ ¶, Xavier Pintó §, Juan Cinca ‡ ¶, Lina Badimon * † || ¶

*Cardiovascular Research Center (CSIC-ICCC), †Ciber ObeNU, ‡Cardiology Service, Hospital Santa Creu i Sant Pau, §University Hospital Bellvitge, ||Autonomous University of Barcelona, and ¶ Biomedical Research Institute Sant Pau (IIB-Sant Pau). Barcelona. Spain

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Serum proteome in acute myocardial infarction

Judit Cubedo[a], **Teresa Padró**[b], **Xavier García-Moll**[c], **Xavier Pintó**[d], **Juan Cinca**[c], **Lina Badimon**[e,*]

[a] *Cardiovascular Research Center (CSIC-ICCC), CIBERobn, Biomedical Research Institute Sant Pau (IIB-Sant Pau), Barcelona, Spain*
[b] *Cardiovascular Research Center (CSIC-ICCC), Biomedical Research Institute Sant Pau (IIB-Sant Pau), Barcelona, Spain*
[c] *Cardiology Service, Hospital Santa Creu i Sant Pau, Biomedical Research Institute Sant Pau (IIB-Sant Pau), Barcelona, Spain*
[d] *University Hospital Bellvitge, L'Hospitalet de Llobregat, Barcelona, Spain*
[e] *Cardiovascular Research Center (CSIC-ICCC), CIBERobn, Autonomous University of Barcelona, and Biomedical Research Institute Sant Pau (IIB-Sant Pau). Barcelona. Spain*

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KEYWORDS
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Abstract
Introduction: Acute myocardial infarction (AMI) is one of the major causes of mortality and morbidity worldwide. Despite the efforts being made, there is a lack of early markers for the prevention, diagnosis and treatment of ischemic syndromes. Proteomic expression profiling technologies are a highly important tool for research into new serum biomarkers for the diagnosis and prognosis of acute coronary syndromes.
Methods: Serum samples were sub-fractionated with different methods for the depletion of high-abundance proteins. The low-abundance fraction was analyzed by two-dimensional electrophoresis (2-DE), followed by protein identification with mass-spectrometry (MALDI-TOF). The proteomic profiles of serum samples from AMI patients and controls were analyzed and compared.
Results: Through depletion of six high-abundance proteins in 2-DE analysis of serum samples, 569 spots were detected, of which 131 spots were only detected in the AMI group and 27 were only detected in controls. The comparative analysis between AMI-patients and controls revealed a group of differential protein spots involved in seven different biological functions. The main changes were found in proteins involved in the immune system and lipid metabolism.
Conclusions: In this study, by using a 2-DE differential approach, we developed a highly reproducible methodology for the analysis of coordinated changes in serum proteome patterns that occur within the first 6 hours after the onset of an AMI.

* Autor para correspondencia.
E-mail address: lbadimon@csic-iccc.org (L. Badimon).

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PALABRAS CLAVE
Infarto de miocardio;
Suero;
Proteómica

Resumen
Introducción: El infarto agudo de miocardio (IAM) es una de las mayores causas de mortalidad y morbilidad en el mundo. A pesar de todos los esfuerzos hay una falta de marcadores para la prevención, el diagnóstico y el tratamiento de la fase temprana de los síndromes isquémicos. Las técnicas proteómicas son una herramienta muy importante para la búsqueda de nuevos biomarcadores para el diagnóstico y el pronóstico de los síndromes coronarios agudos.
Métodos: Las muestras de suero se sub-fraccionaron mediante diferentes métodos para eliminar las proteínas mayoritarias. La fracción de proteínas de menor abundancia se analizó mediante electroforesis bidimensional (2-DE), seguida de la identificación de proteínas mediante espectrometría de masas (MALDI-TOF). Se analizó y comparó el patrón proteómico de los pacientes IAM y el grupo control.
Resultados: Mediante la eliminación de las seis proteínas mayoritarias en el análisis por 2-DE se detectaron un total de 569 spots, de los cuales 131 sólo se detectaron en el grupo infarto y 27 sólo en el grupo control. El análisis comparativo entre los pacientes IAM y los controles reveló un grupo de spots proteicos con un patrón de distribución diferencial entre ambos grupos. Estos spots diferenciales pertenecen a siete grupos diferentes en función de su papel biológico, de los cuales los cambios más importantes se ven en las proteínas involucradas en el sistema inmune y en el metabolismo lipídico.
Conclusiones: En el presente estudio, mediante el uso de técnicas de 2-DE hemos desarrollado una metodología altamente reproducible para el análisis de los cambios coordinados que se dan durante las seis primeras horas desde el inicio de un evento.

Introduction

Ischemic atherothrombotic syndromes induce structural and functional modifications that are reflected in serum levels of proteins and other biomarkers. However, biomarkers characterization is nowadays incomplete and their prognostic value is not clear. Increased concentrations of inflammatory biomarkers such as C reactive protein (CRP), serum amyloid A, myeloperoxidase and interleukin-6 (IL-6) are detectable in a substantial proportion of patients with acute coronary syndromes. Several biomarkers of myocardial ischemia are under investigation, ischemia-modified albumin (IMA) is among the most thoroughly studied of these markers.[1] There are also biomarkers for the detection of cardiac injury, from those ones the protein of choice is troponin.[2]

Some of those biomarkers lack of consensus because, up to now, it is not clear whether their measurement would be useful in the diagnosis and prognosis of ischemic atherothrombotic syndromes.

Despite the success of cardiac troponins as markers of myocardial injury there is still a need for the development of early markers for prevention, diagnosis and treatment of ischemic syndromes.

Proteomics is one of the most important strategies for the study of complex protein associations. By using proteomic techniques we can determine modifications in protein structure, expression levels, and post-translational modifications that may be associated to the presence of disease. Indeed, proteins are excellent targets for disease diagnostic and prognostic markers and to develop therapeutic strategies. Proteomic techniques are a good tool for the analysis of the protein content in a determined sample.[3]

Serum has the advantage of being one of the most accessible sources for biomarker discovery, but also has the disadvantage of its great complexity as it has a lot of different proteins in a very dynamic range of concentrations. Near 85% of serum proteome is represented by a reduced group of proteins that are known as the high abundant fraction and usually have no interest in proteomic analysis as they are very well characterized. Potential serum biomarkers are in very low abundance and so they are masked by high abundant proteins such as albumin and IgGs.

The aim of this study is to characterize the serum proteomic profile in healthy individuals and acute myocardial infarction patients by using two different subfractionation methods.

Materials and methods

Study population

The study population comprised 27 new-onset AMI patient (20 men and 7 women; mean age: 61 ± 2 years) who were admitted with chest pain and suspected of ACS at the Emergency Room of Santa Creu i Sant Pau Hospital.

At the emergency department, routine diagnostic procedures were applied to establish the onset of symptoms as accurately as possible (i.e. description of chest pain, pulmonary edema, severe dyspnea, and syncope). In addition to the general patient history, clinical examination, 12-lead ECG, and laboratory tests were also run to characterize AMI-patients. All AMI-patients showed (1) typical chest pain lasting more than 30 minutes; (2) ST segment elevation >0.2 mV in at least 2 contiguous leads; (3) admission to the hospital within the first 6 hours after chest pain onset; (4) normal serum CK and CKMB levels at admission; (5) negative troponin T at admission (excluding subacute myocardial infarction); (6) sinus rhythm. Exclusion criteria were a previous documented or suspected myocardial infarction and antithrombotic treatment because of the AMI onset before arriving to the emergency room and time of

Table 1 Background description of AMI-patients and healthy donors.

	AMI patients	Healty donors	*P* value
N	27	60	-
Age (mean ± SEM)	61 ± 2	61 ± 1	NS
Females/Males	7/20	15/45	-
Total cholesterol (mg/dL)	204 ± 7.9	216.4 ± 5.4	NS
HDL cholesterol (mg/dL)	44.3 ± 2.1	48.8 ± 1.8	NS
LDL cholesterol (mg/dL)	130.5 ± 6.9	142.3 ± 4.8	NS
Triglycerides	169.6 ± 12.3	129.2 ± 11.5	0.02
Background medicaton, n (%)			
ASA	9.8	-	-
ACEI	17.1	-	-
Statins	17.1	-	-
A2RA	4.9	-	-
β blocers	7.3	-	-
Ca-Antagonists	9.8	-	-
NTG	2.4	-	-
OAD	17.1	-	-
CRP levels (mg/L)[a]	7.6 ± 9.9	-	-
TnTmax (μg/L)[b]	7.3 ± 5.9	-	-
Necrosis %[c]	13.5 ± 9.8	-	-

ASA: acetylsalicylic acid; ACEI: angiotensin-converting enzyme inhibitors; A2RA: angiotensin 2 receptor antagonists; NTG: nitroglycerine; OAD: oral antidiabetic drugs.
[a] CRP levels: C-reactive protein values at admission.
[b] TnTmax: Defined as the highest serum T-troponin levels after the event.
[c] Necrosis (%): Percentage of necrotic mass of the myocardium (calculated as percentage of the total cardiac mass measured by cardiovascular magnetic resonance).

blood collection. Delayed contrast-enhanced (CE) cardiovascular magnetic resonance studies were performed within the first week after acute ST-segment elevation AMI in all patients to evaluate the MI location and the necrotic lesion. The control group included sixty healthy individuals (45 men and 15 women; mean age: 61 ± 1 years) who attended to a routine health check. Background description of AMI-patients and control individuals are listed in Table 1.

The Ethics Committee of the Santa Creu i Sant Pau Hospital approved the project and the studies were conducted according to the principles of Helsinki's Declaration. All participants gave written informed consent to take part in the study.

Blood collection and sample preparation

- *Blood samples obtention.* Venous blood samples of AMI-patients, before starting any medication, and control individuals were collected to prepare serum that was aliquoted and stored at −80 °C.
- *Serum subfractionation.* For proteomic studies, serum samples were sonicated (six cycles of 15 seconds each) in ice[4] and filtrated (0.22 μm) by centrifugation to avoid the presence of impurities. Highly abundant proteins were depleted using two different depletion methods one that uses an antibody affinity resin to selectively remove albumin and IgGs (Albumin and IgG Removal Kit from GE Healthcare) and another that removes the six most abundant serum proteins using a specific affinity cartridge with binding capacity for albumin, IgGs, IgAs, transferrin, α1 antytripsin and haptoglobin (Multiple Affinity Removal Spin Cartridge, Agilent Technologies) as reported by the providers. Serum depleted samples (called the total serum fraction) were concentrated and desalted by centrifugation with 5 kDa cut-off filter devices and sample buffer was exchanged to a urea containing buffer (8 M Urea, 2% Chaps). Protein concentration in the serum extracts was measured with 2D-Quant Kit (GE Healthcare). All processed samples were stored at −80 °C until used.

Biochemical analysis

C-reactive protein (CRP), lipids (Total-, HDL-, LDL-cholesterol, and triglycerides), kidney parameters (urea and creatinine), liver parameters (bilirubin, GOT and GPT) and T-troponin levels were measured by standard laboratory methods.

Proteomic analysis

The proteomic analysis of serum samples was performed by two-dimensional electrophoresis (Figure 1A) followed by mass spectrometry identification (Figure 1B).

- *Two-dimensional gel electrophoresis (2-DE).* For analytical and preparative gels, respectively, a protein load of 120 μg and 300 μg protein were dissolved in the rehydration buffer (8 M urea, 2% chaps, 0.2% ampholytes, 1.6% DTT and 0.002% bromophenol blue) soluble extracts was applied by cup-loading to 18-cm dry strips (pH 4-7 linear

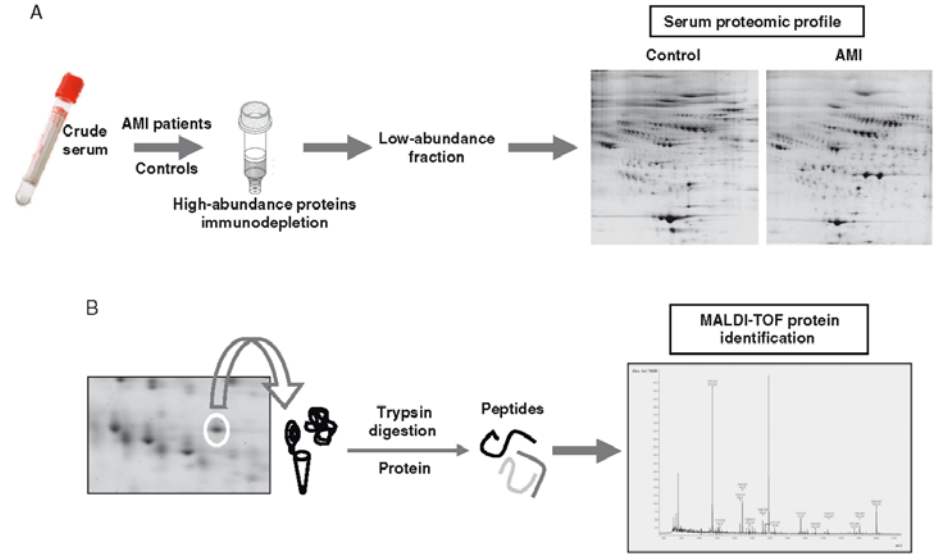

Figure 1 (A) Experimetal workflow of serum sample sub-fractionation and the analysis by 2-DE of the low abundance fraction in acute myocardial infarction patients and the control group. (B) Working scheme of 2-DE spots excision, trypsin digestion and peptide mass fingerprint analysis by mass spectrometry (MALDI-TOF) for protein identification.

range, GE Healthcare). Isoelectrofocusing was performed in a Protean IEF Cell (BioRad) at the following conditions: (1) linear step at 250 V for 45 min; (2) linear step at 500 V for 1 h; (3) linear step at 1000 V for 1 h; (4) linear step at 4000 V for 1 h; (5) linear step at 10,000 V for 1 h; (6) linear step at 10,000 V until reaching 70,000 V. After the first dimension strips were equilibrated 15 min in an equilibration buffer (6 M urea, 2% SDS, 50 mM Tris-HCL pH 8.8 and 30% glycerol) containing 1% DTT and 15 min in the same buffer with 2.5% iodoacetamide.

The second dimension was resolved in 10% SDS-PAGE in an Ettan Dalt six electrophoresis unit (GE Healthcare) at 5 w/gel during the first 30 minutes and afterwards at 17 w/gel. Gels were developed by fluorescent staining (analytical gels) or comassie blue (preparative gels). For each independent experiment, 2-DE for protein extracts from controls and patients were processed in parallel to guarantee a maximum of comparability. Each 2-DE run was at least repeated twice. Analysis for differences in protein patterns was performed with the PD-Quest 8.0 (BioRad), using a single master that included all gels of each independent experiment. Each spot was assigned a relative value that corresponded to the single spot volume compared to the volume of all spots in the gel, following background extraction and normalization between gels.

- *Mass spectrometry analysis*. Protein spots of interest were excised from 2-DE gels, washed (25 mM Ambic), dehydrated (25 mM Ambic/50% ACN followed by 100% ACN), dried, and enzymatically digested with one gel volume of sequence-grade modified porcine trypsin (Promega). Peptides from in-gel-trypsin digestion were desalted and concentrated by ZipTipU-C18 (Millipore), mixed 1:1 with 5 mg/mL α-cyano-4-hydroxy-cinnamic, and spotted on a stainless steel mass spectrometry slide. Protein identification was performed by peptide-mass fingerprinting using an Ettan MALDI-TOF Pro (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer, GE-Healthcare) operating in delayed extraction/reflector mode. MALDI-generated mass spectra were internally calibrated using trypsin autolysis products, Ang III (angiotensin III), and ACTH (adrenocorticotropic hormone) peaks. The peptide masses were searched against the National Center for Biotechnology Information nonredundant mammalian database using ProFound™ and confirmed using a Mascot 2.3 search from Matrixscience, selecting the SwissProt database. For the present study, protein identification was based on the measurement with a Mascot score higher than 55 and a minimum coverage of 20%. Minimal expectation for valid identification was <0.005 and $P<0.05$.

Statistical analysis

Data are expressed as mean and standard error of the mean ± SEM unless stated. N indicates the number of subjects tested. Statistical analysis for differences between control and AMI groups were performed by the ANOVA single factor or including covariates, Fisher PSLD as post-hoc analysis, and the Student's t-test or the non-parametric Mann-Whitney test, as indicated. Correlations between variables were

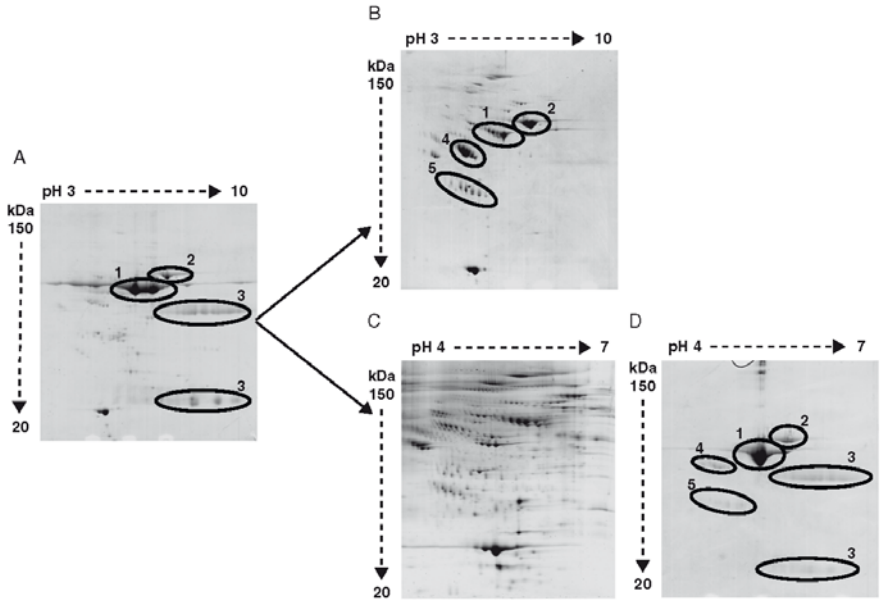

Figure 2 Representative image of 2-DE of (A) crude serum, (B) serum depleted of albumin and IgGs, (C) serum depleted of six high-abundant proteins (albumin, IgGs, IgAs, transferrin, haptoglobin and α1-antitrypsin), and (D) the retained fraction containing those six high-abundant proteins. In crude serum only albumin (1), transferrin (2) and IgGs heavy and light chain (3) are detected. After albumin and IgGs depletion α1-antitrypsin (4) and haptoglobin (5) are apparent. After six high abundant protein depletion the amount of detected proteins is increased. The retained fraction containing the six high-abundant proteins show a very similar profile of that obtained with crude serum.

determined single and multiple regression models. A P value $\leq$0.05 was considered significant.

Results

Clinical characteristics of the study population

There was no significant difference in age, sex and cholesterol levels (Total-, HDL- and LDL-cholesterol) between AMI-patients and controls. AMI-patients showed significant higher levels of triglycerides (P=0.02), urea (P=0.03), creatinine (P<0.001), and also significant higher levels of hepatic parameters as bilirubin (P<0.001), GOT (P<0.001), and GPT (P<0.001) when compared to controls. No T-troponin levels were detected at arrival to the hospital.

The high abundance fraction interferes in the detection of serum proteome

As shown in Figure 2 the same serum samples were analyzed using two different depletion methods in parallel and compared to crude serum.

Bidimensional electrophoresis of crude serum only displayed two big spots corresponding to albumin and transferrin and two areas of blurred spots identified as heavy and light chains of IgGs respectively (Figure 2A).

After removing albumin and IgGs other proteins like transferrin, α1-antytripsin and haptoglobin turned out to be the most detected proteins and prevent other less abundant proteins from being detected (Figure 2B). The identification by MALDI-TOF analysis of protein spots obtained after removing albumin and IgGs revealed 25 non-redundant proteins (data not shown). The immunodepletion of albumin, IgGs, IgAs, transferrin, haptoglobin and α1 antytripsin allowed the further identification of 90 non-redundant low abundance proteins not detected with the first method (Figure 2C). A total of 569 spots were detected in serum samples. In the AMI group a total of 542 spots were detected. The control group depicted 438 total spots, from which 411 spots were also detected in the AMI group. There were 131 spots only present in the AMI group and 27 spots only detected in the control group. The detected serum spots were mainly in the pI range between 4 and 7, and in a molecular weight range between 150 and 20 kDa. The identified proteins are involved in different processes listed in Table 2. The proteomic profile of the retained fraction containing those six high-abundant proteins was very similar to the crude serum profile (compare Figure 2A with 2D). The identification by mass spectrometry of the detected spots in the retained

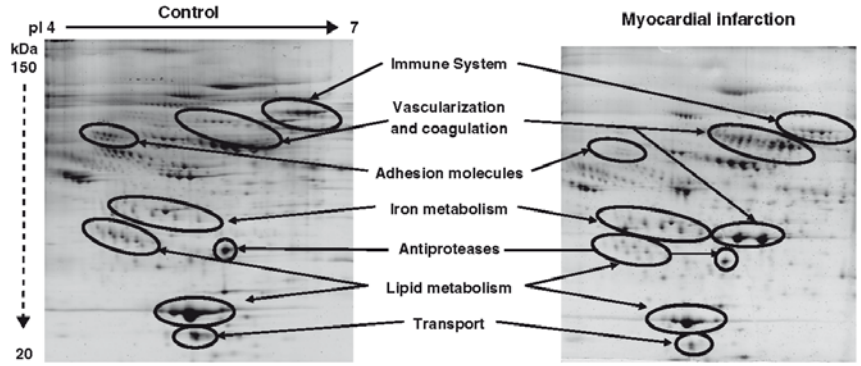

Figure 3 Representative 2-DE image of control and myocardial infarction serum samples. There are several spot proteins with differential expression profile between AMI patient and control individuals. These differential proteins are involved in seven different biological functions: immune system, vascularisation and coagulation, adhesion molecules, iron metabolism, antiproteases, lipid metabolism, and transport proteins.

Table 2 Groups of proteins identified in serum proteome.

	Biological funcation	N of protiens
1	Immune system	28
2	Vascular system and coagulation	24
3	Lipid metabolism	18
4	Iron metabolism	9
5	Transport/Binding	9
6	Antiprotease activity	7
7	Citoskeleton reorganisation	5
8	Acute phase response	4
9	Adhesion molecules	3
10	Signaling	3
11	Interaction with DNA and/or histones	3
12	Antioxidant molecules	2

fraction only revealed the presence of high-abundant proteins.

Proteomic profile of serum samples in AMI patients

Serum samples (after removal of the six most abundant proteins) from 27 AMI-patients were analyzed individually and samples of healthy donors were analyzed in 6 pools of 10 individuals each in order to obtain a more representative pattern of a healthy population. All samples were run in duplicates by 2-DE and proteins were identified by MALDI-TOF mass spectrometry.

Among the 569 detected serum spots, 270 showed a statistically significant differential expression profile between AMI patients and control individuals, being 127 of them identified as 28 non-redundant proteins involved in seven different biological functions (Figure 3).

From the identified spots with a differential expression profile, 23 showed at least a two-fold increase in their intensity, 16 depicted at least a two-fold decrease, and 45 showed changes in their distribution profile in AMI patients because changes in molecular weight and/or pI, presumably due to post-translational modifications (Table 3).

Discussion

There is an increasing need for the identification of new biomarkers in order to obtain tools for an early detection and prognosis of atherothrombotic events such as acute myocardial infarction. To this aim serum proteomics reveals as a leading technology for the research and characterization of new biomarkers. Accordingly, in this study we have used proteomic analysis to identify new changes in serum proteome

Table 3 Groups of proteins with differential profile in AMI patients compared to healthy donors.

Group	N of protiens	Increase	Decrease	Distribution
Immune system	7	3	6	8
Vascular system and coagulation	5	5	1	20
Lipid metabolism	6	2	1	7
Iron metabolism	3	11	2	-
Transport/Binding	3	2	1	2
Antiproteae activity	3	-	-	8
Adhesion molecules	1	-	5	-

in association to the clinical manifestation of cardiovascular disease such as AMI.

Serum is a very rich source for biomarker discovery but because of its great complexity it must be sub-fractionated prior to proteomic analysis. Therefore to analyze serum samples by bidimensional electrophoresis a previous step to remove the high abundance proteins is essential. Years ago the depletion of human albumin was performed via Cibacron Blue columns.[5] In case of IgGs, resins of immobilized protein A or G have been widely used.[6] But these methods lacked of specificity and reproducibility. Nowadays there are several methods based on columns containing a mixture of antibodies for the depletion of various high abundance proteins at the same time.

In this work the selected depletion method was the one removing six high abundance proteins (albumin, transferrin, IgGs, IgAs, haptoglobin and α1 antitrypsin). By using this immunodepletion method we have developed a highly reproducible technique for the identification and analysis of a wide range of low abundance proteins from serum proteome. The use of these antibody-based columns has reduced the nonspecific binding during depletion, in fact by 2-DE analysis we did not detect nonspecific proteins in the retained fraction. Unfortunately, we can not exclude that some low-abundance proteins, not detectable by 2-DE analysis were lost during the depletion protocol. Nevertheless, our methodological approach represents an improvement in protein detection compared to previous studies.[7]

Early systemic changes after the onset of an AMI have not been fully characterized and therefore there is a dearth of biomarkers that could indicate early ischemic damage. The analytical power of modern proteomic technologies could help to identify these systemic changes and therefore facilitate our understanding of the pathophysiology of cardiovascular diseases.[8] In the present study, by applying proteomic technologies, we have detected significant changes in groups of proteins involved in several different biological functions such as: immune system, vascular system and coagulation, lipid metabolism, iron metabolism, transport/binding, antiprotease activity, and adhesion molecules.

Among the proteins showing a differential profile between AMI-patients and control individuals there are seven immune system related proteins. There are several evidences of the relevance of immune response processes and related molecules in atherothrombosis.[9,10]

In this study we have found that acute myocardial infarction is related to a differential proteomic profile in proteins involved in lipid metabolism. Some authors[11] suggest, for example, that the relevance of HDL in cardiovascular diseases is not through its levels (HDL-cholesterol levels) in plasma but through its composition, structure and function. Therefore, the more in depth study of proteins involved in lipid metabolism such as lipoproteins may lead to a better understanding of their role in atherothrombotic diseases.

In AMI-patients differential proteins did not only showed increased or decreased intensity values in comparison to healthy donors, but also depicted changes in their distribution pattern presumably due to post-translational modifications or the presence of different protein isoforms. These protein modifications, such as phosphorylations and glycosylations, play an important role in the molecular pathology of a disease.[12] The specific study of serum phospho[13] and glycoproteomes[14] could represent and important source for biomarker discovery. The most important post-translational modification in serum proteome is glycosylation. In fact there are several studies relating differential glycosylation states of serum proteins in relation to specific diseases.[15–17]

In this study, by using a 2-DE differential approach we have developed a highly reproducible methodology for the analysis of coordinated changes of serum proteome that exist within the first six hours after the onset of an acute myocardial infarction. Further studies are ongoing in our group in order to determine the specific changes of the proteins involved in those biological functions that have shown to be differentially represented in the proteomic profile of patients in the early phase after an AMI.

Disclosures

No conflicts to disclose.

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ANEXO II. Cambios mitocondriales en el post-condicionamiento isquémico: Candidatas a moléculas cardioprotectoras.

Patente proteínas X1, X2, X3 en curso: Moléculas protectoras frente al estrés oxidativo y daño en el órgano diana.

Mediante la aplicación de técnicas proteómicas para el análisis de muestras de miocardio procedentes de un modelo experimental pre-clínico de isquemia/reperfusión se han observado cambios significativo de las proteínas X1, X2 y X3 tras la aplicación de una estrategia cardioprotectora de uso clínico como es el post-condicionamiento isquémico aplicado durante la revascularización del vaso.

Concretamente la proteína X1 además de su interés como molécula protectora se ha visto que puede considerarse un marcador de daño por tisular. Los niveles de X1 en el miocardio disminuyen significativamente con la isquemia y siguen bajos tras la posterior reperfusión. Paralelamente, los niveles en suero aumentan. Cuando se aplica el post-condicionamiento isquémico los niveles de X1 en el miocardio se recuperan hasta alcanzar los niveles fisiológicos **(Figura 1)**, por lo que la X1 estaría actuando como molécula efectora de la protección frente al daño tisular inducido por estrés oxidativo.

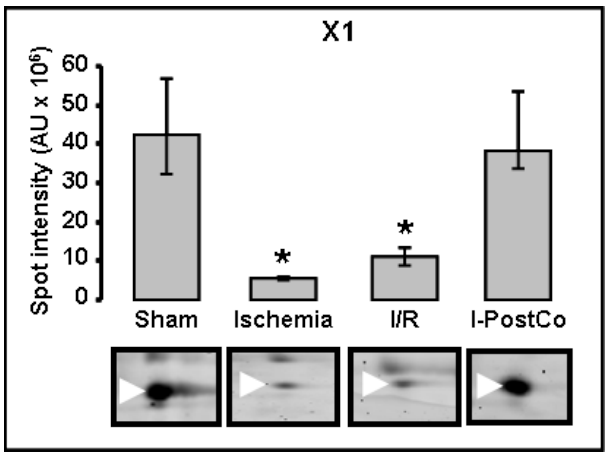

Figura 1: Cambios en la proteína X1 en la 2-DE de tejido miocardio.

En el caso de la proteína X2, en el tejido de miocardio se detectan dos isoformas una de 20kDa y otra de 17kDa. La forma de 17kDa aumenta de forma muy marcada cuando se aplica el post-condicionamiento isquémico **(Figura 2)**. Por lo que la forma específica X2-17 estaría actuando como molécula efectora de la protección frente al daño tisular inducido por estrés oxidativo.

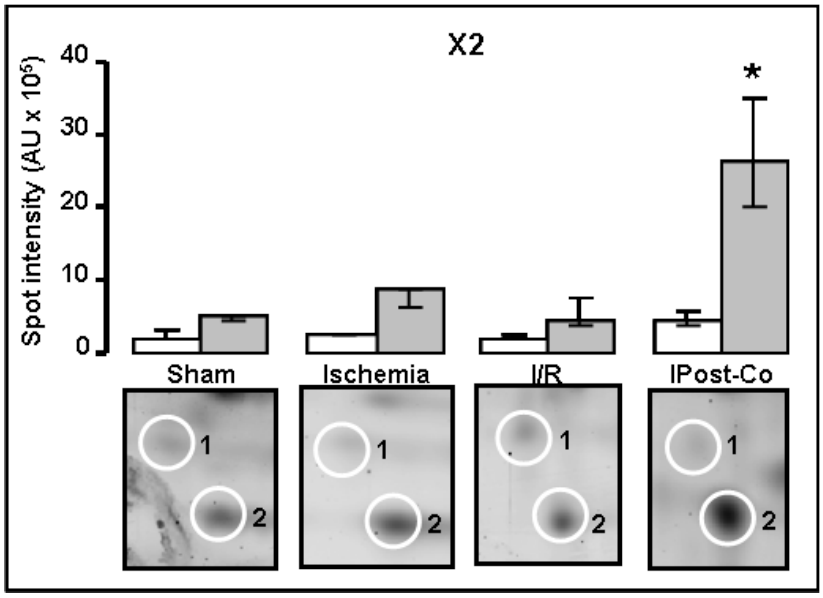

Figura 2: Cambios en la proteína X2 en la 2-DE de tejido miocardio.

La proteína X3, tiene el mismo patrón que la forma X2-17, aumenta de forma muy marcada cuando se aplica el post-condicionamiento isquémico **(Figura 3)**, actuando también como molécula efectora de la protección frente al daño tisular inducido por estrés oxidativo.

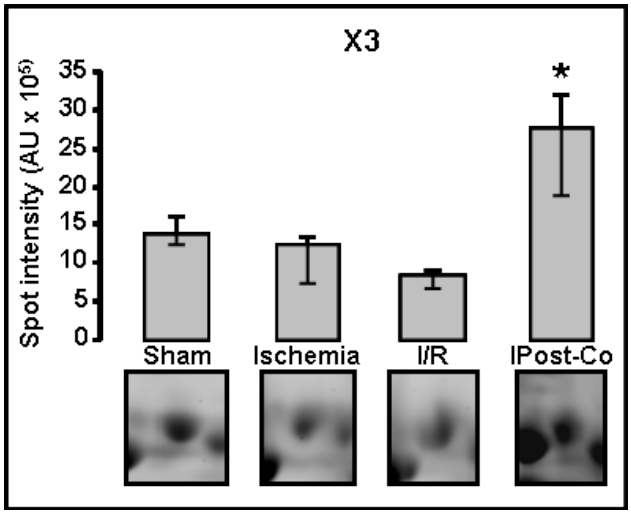

Figura 3: Cambios en la proteína X3 en la 2-DE de tejido miocardio.

La patente pretende proteger:

1. Uso de al menos una de las proteínas del conjunto, X1, X2 y X3, como moléculas protectoras frente al estrés oxidativo.
2. Uso de al menos una de las proteínas del conjunto, X1, X2 y X3, como moléculas protectoras del daño en el órgano diana sobre el cual el estrés oxidativo tiene efecto.
3. Uso terapéutico de las proteínas X1, X2 y X3 solas o en combinación para la protección frente al daño tisular.
4. Uso específico de la proteína X1 como marcador de daño tisular.