SUPPLEMENTAL MATERIAL

De Hoog VC. Serum extracellular vesicle protein levels are associated with acute coronary syndrome

Supplemental 1: Identification and selection of extracellular vesicle protein biomarkers

FAME-ER cohort

FAME-ER (Fatty acid-binding protein in Acute Myocardial infarction Exclusion in the Emergency Room) is a prospective single-center cohort study among ACS-suspected patients presenting to the emergency department of a regional hospital (Meander Medical Center, Amersfoort, The Netherlands).[1]

This cohort consists of 541 patients, enrolled between 2007 and 2009. Frozen serum samples were available from 471 patients. All adult (>18yr) patients presenting to the emergency department suspected of having an ACS were eligible for inclusion, except patients with an ST-elevation myocardial infarction (STEMI). Venous blood samples were drawn at arrival before diagnosis and serum samples were frozen for further analysis. Final diagnosis was made retrospectively by consensus of 2 independent cardiologists, with all patient information and digital files at their disposal, including serial troponin measurements, serial ECG, coronary angiography, echocardiography, cardiac exercise tests and information from hospital discharge letters, according to the recent guidelines of the ESC/ACCF/AHA/WHF[2]. In case of non matching diagnoses the event was reviewed by an expert panel of three cardiologists who tried to achieve diagnostic consensus. If opinions diverged the majority opinion prevailed.

The study complies with the Declaration of Helsinki, was approved by the regional medical ethical committee and written informed consent was collected from all patients.

Athero-Express cohort

Athero-Express is a longitudinal vascular biobank study which includes biomaterials from patients undergoing carotid endarterectomy (CEA) and femoral endarterectomy in two Dutch hospitals (University Medical Center, Utrecht and St. Antonius Hospital, Nieuwegein). The primary objective of the study is to investigate the relation between single plaque characteristics at baseline and clinical outcome during follow-up. The study design has been described previously[3]. The study has been approved by the institutional review boards of both hospitals and written informed consent was obtained from all patients. All patients underwent clinical follow-up 1 year after

the surgical intervention and filled out postal questionnaires one, two and three years after the surgical intervention.

The primary outcome was a composite encompassing all vascular events and interventions: vascular death, non-fatal myocardial infarction, non-fatal stroke, and vascular interventions that were not foreseen at the time of inclusion.

Secondary outcome was any major vascular event: vascular death, non-fatal myocardial infarction, non-fatal stroke and non-fatal aneurysm rupture. Definitions and assessment procedures of the outcome events were described previously[3].

Quantitative proteome analysis and selection of serum extracellular vesicle proteins associated with ACS

Forty ml serum of the 30 confirmed ACS patients was pooled and split in two samples of 20 ml for ultracentrifugation. The same was done for the 30 age- and gender matched confirmed non-ACS patients. After a short centrifugation step (1850 g, 10 min RT), the four samples were centrifuged for 30 min at 30.000 g at 4 °C followed by centrifugation of the supernatant at 100.000 g for 60 min at 4 °C. The pellet was resuspended in PBS and again centrifuged at 100.000 g for 60 min at 4 °C. The pellet was then dissolved in 4% SDS and used for proteomics analysis.

The relative protein expression in the four samples was quantified by iTRAQ-based shotgun proteomics with a LC-MS/MS MuDPIT approach. For the 4-plex iTRAQ experiment, 200µg peptides from each sample/condition was labeled with iTRAQ reagent. iTRAQ labelled peptides were fractionated using ERLIC with a PolyWAX LP weak anion-exchange column (4.6 × 200 mm, 5 µm, 300 Å, PolyLC, Columbia, MD, USA) on a Shimadzu Prominence UFLC system using solvent A (85% acetonitrile (ACN), 0.1% acetic acid), and solvent B (30% ACN, 2% formic acid). Thirty fractions were collected with a 70 minute gradient of 100% buffer A for 10 minutes, 0%–10% buffer B for 20 minutes, 10%–100% buffer B for 30 minutes, and 10 minutes at 100% buffer B at a flow rate of 0.5 mL/minute. The collected fractions were dried with a vacuum centrifuge. All fractions were analyzed using a QStar Elite mass spectrometer coupled with an online TempoTM nano-MDLC system (Applied Biosystems). Identification and quantitation of proteins was performed using ProteinPilot software (Applied Biosystems).

First selection was based on proteins with identical duplos (ACS/non-ACS both lower then 0.8 or both between 0.8and 1.2 or both above 1.2). Second selection was based on proteins with lower (ACS/non-ACS <0.8) or higher (ACS/non-ACS >1.2) expression in group 1 vs. group 2. This revealed a list of 127 proteins.
This list of 127 proteins was uploaded and analyzed in Ingenuity Pathway Analysis software (www.ingenuity.com). 35 proteins of these initial 127 proteins were not listed in the Ingenuity database, identifying 92 different types of proteins, including transmembrane receptors, transporters and transcription regulators and proteins that are not present in serum.
On these 92 proteins, a Core analysis was performed revealing 16 networks showing the indirect and direct relationships between the proteins. Based upon the Ingenuity knowledge database, a network shows the indirect and direct relationships between proteins, genes, metabolites and protein complexes. For Network 7-16, each Network contained 1 protein of the 92 proteins. Network 6 contained 9 proteins of the 92 proteins; Network 4&5, 10 proteins and Network 3, 11 proteins.
Network 1 (score 36, Organismal injuries & disorders, Connective tissue disorders, Immunological disease) and 2 (score 32, Cellular Movement, Hematological System development & function, Immune Cell trafficking) contained respectively 19 & 16 (35) proteins out of the 92 proteins and were merged.

From the Athero-Express discovery proteomics comparing patients that had a secondary cardiovascular event during follow-up vs Athero-Express patients that had no cardiovascular event during follow-up, serum extracellular vesicle proteins that were related to secondary cardiovascular events during follow up were added to the merged Networks 1 & 2 when they had a direct relationship with one of the 35 selected proteins. This identified Cystatin C as a potential serum extracellular vesicle protein related to ACS and was added to the 35 proteins bringing the total number to 36 potential serum extracellular vesicle proteins related to ACS.
From these 36 proteins, 3 proteins were selected for which a capture antibody, biotinylated detection antibody and antigen was available. After spike-recovery and cross-reactivity tests it was determined that this could be measured within 1 multiplex panel. The 3 proteins were: C5a, pIgR and Cystatin C.

References to Supplemental 1

1. Oerlemans MI, Mosterd A, Dekker MS, de Vrey EA, van Mil A, Pasterkamp G, Doevendans PA, Hoes AW, Sluijter JP. Early assessment of acute coronary syndromes in the emergency department: the potential diagnostic value of circulating microRNAs. *EMBO Mol Med* 2012. Epub ahead of print.

2. Thygesen K, Alpert JS, White HD, Biomarker Group, Jaffe AS, Apple FS, Galvani M, Katus HA, Newby LK, Ravkilde J, ECG Group, Chaitman B, Clemmensen PM, Dellborg M, Hod H, Porela P, Imaging Group, Underwood R, Bax JJ, Beller GA, Bonow R, Van Der Wall EE, Intervention Group, Bassand JP, Wijns W, Ferguson TB, Steg PG, Uretsky BF, Williams DO, Clinical Investigation Group, Armstrong PW, Antman EM, Fox KA, Hamm CW, Ohman EM, Simoons ML, Global Perspective Group, Poole-Wilson PA, Gurfinkel EP, Lopez-Sendon JL, Pais P, Mendis S, Zhu JR, Implementation group, Wallentin LC, Fernandez-Aviles FF, Fox KM, Parkhomenko AN, Priori SG, Tendera M, Voipio-Pulkki LM, Universal definition of myocardial infarction: Kristian Thygesen, Joseph S. Alpert and Harvey D. White on behalf of the Joint ESC/ACCF/AHA/WHF Task Force for the Redefinition of Myocardial Infarction. *Eur Heart J* 2007;**28**:2525–2538.

3. Verhoeven BA, Velema E, Schoneveld AH, de Vries JP, de Bruin P, Seldenrijk CA, de Kleijn DP, Busser E, van der Graaf Y, Moll F, Pasterkamp G. Athero-express: differential atherosclerotic plaque expression of mRNA and protein in relation to cardiovascular events and patient characteristics. Rationale and design. *Eur J Epidemiol* 2004;**19**:1127-33.

Supplemental 2: Serum extracellular vesicle electron microscopy methods

Serum extracellular vesicles were isolated from serum with ExoQuick™ as described before ('Methods'). The pellet was resuspended in 500 µl of PBS and stored at 4°C until use. Prior to incubation the vesicles were diluted 1:20 in PBS. A grid (100 M) was placed on a drop of the diluted vesicle suspension for 10 minutes at room temperature (RT). The grid was subsequently placed in a drop of 1% glutaraldehyde in PBS for 5 minutes at RT and then rinsed 10 times for 1 minute with dH2O.

The grid was stained on a drop of uranyl acetate (pH 7.0) for 10 minutes at RT and then rinsed once with dH2O. After this, the grid was rinsed with two drops of methyl-cellulose uranyl (pH 4.0) followed by an incubation of 10 minutes on ice with methyl-cellulose uranyl (pH 4.0). In the following step, the grid was looped out of the methyl-cellulose uranyl and the methyl-cellulose was reduced by placing the grid on filter paper to get an even film. After drying for 10 minutes at RT, the grid was carefully removed from the loop and analyzed with an EM microscope (Jeol Jem-1010, Japan).

Supplemental 3: Specification of the non-ACS control group

Table S1. Specification of the non-ACS control group (n = 331)

Diagnosis	N (%)
Cardiovascular - other	**75 (23)**
Stable angina	51 (15)
Arrhythmia	16 (5)
Heart failure	7 (2)
Pericarditis	1 (0)
Pulmonary	**1 (0)**
Pneumonia	1 (0)
Gastrointestinal	**28 (8)**
Oesphagitis / GERD	18 (5)
Oesophageal spasm	3 (1)
Cholelithiasis	1 (0)
Cholecystitis / cholangitis	5 (2)
Other	1 (0)
Musculoskeletal	**8 (2)**
Myalgia	7 (2)
Rib fracture	1 (0)
Psychiatric	**7 (2)**
Anxiety disorder / hyperventilation syndrome	6 (2)
Depression	1 (0)
Other	**87 (26)**
Atypical chest pain	75 (23)
Other	12 (4)
Unknown	**125 (38)**

Supplemental 4: Troponin I measurement and ROC curves of Troponin I and pIgR, Cystatin C and C5a in patients with ACS and controls

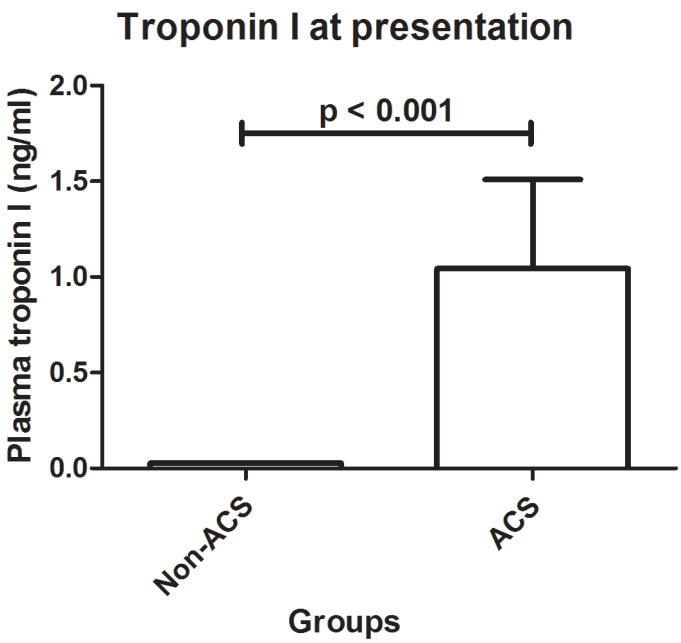

Figure S1. Mean Troponin I levels (µg/L). Non-ACS 0.03 ± 0.02 (n = 331) vs. ACS 1.04 ± 5.52 (n = 140), p < 0.001 (Mann-Whitney U test)

Table S2. ROC curve AUC's of Troponin I and markers in combination with risk factors

A. All patients (n = 471)

Marker	**AUC**	**95% CI**	**Sign**
Troponin I	0.839	0.796 – 0.883	<0.001
RF	0.715	0.665 – 0.765	<0.001
RF with			
Troponin I	0.875	0.841 – 0.910	<0.001
pIgR	0.718	0.668 – 0.768	<0.001
Cystatin C	0.726	0.677 – 0.774	<0.001
C5a	0.718	0.669 – 0.767	<0.001
Prot Conc	0.718	0.669 – 0.768	<0.001
RF with Troponin I and			
pIgR	0.875	0.839 – 0.910	<0.001
Cystatin C	0.875	0.840 – 0.910	<0.001
C5a	0.874	0.839 – 0.909	<0.001
Prot Conc	0.874	0.839 – 0.909	<0.001

Prot Conc: total serum extracellular vesicle protein concentration, in mg/ml; Sign: significance (p-value); AUC: area under the curve; RF: risk factors: age, gender, previous MI, hypertension, hypercholesterolemia, DM, smoking (current and former).

B. Men only (n = 263)

Marker	AUC	95% CI	Sign
Troponin I	0.859	0.811 – 0.907	<0.001
RF	0.696	0.631 – 0.761	<0.001
RF with			
Troponin I	0.878	0.837 – 0.919	<0.001
pIgR	0.716	0.652 – 0.779	<0.001
Cystatin C	0.721	0.659 – 0.783	<0.001
C5a	0.708	0.644 – 0.773	<0.001
Prot Conc	0.719	0.656 – 0.782	<0.001
RF with Troponin I and			
pIgR	0.878	0.836 – 0.920	<0.001
Cystatin C	0.878	0.837 – 0.920	<0.001
C5a	0.877	0.835 – 0.919	<0.001
Prot Conc	0.878	0.836 – 0.920	<0.001

Prot Conc: total serum extracellular vesicle protein concentration, in mg/ml; Sign: significance (p-value); AUC: area under the curve; RF: risk factors: age, previous MI, hypertension, hypercholesterolemia, DM, smoking (current and former).

Supplemental 5: pIgR, Cystatin C and C5a localization in serum extracellular vesicles

Serum extracellular vesicles were isolated by ultracentrifugation from serum as described under 'Supplemental 1'. The pellet was resuspended in 20 µl PBS and subsequently mixed with 2.5 M sucrose. Gradient sucrose ultracentrifugation was done using a gradient from 0.4 to 2 M and centrifugated over night at 4°C at 39.000 rpm in a SW40 rotor using a Beckman Le-80K ultracentrifuge at low acceleration and low brake.
After centrifugation, 12 fractions were harvested and the density was measured, extracellular vesicles were collected using a SW60 rotor at 100.000g for 1 hour at 4°C. The pellet was resuspended in SDS ample buffer and used for Western Blot analysis.
Western Blot analysis was performed on 4-12% gradient Bis-Trisgel (NuPage, Invitrogen), or on 12% Bis-Trisgel (NuPage, Invitrogen) for C5a. After blotting, the blot was incubated with the following primary antibodies: CD9 (Santa Cruz Biotechnology sc-53679, 100 µg/ml): dilution 1:200; pIgR (Lifespan Biosciences LS-A9790, 1.0 mg/ml): dilution 1:500; Cystatin C (R&D Systems MAB11962, 500 µg/ml): dilution 1:200; C5a (R&D Systems MAB2037, 500 µg/ml): dilution 1:500.
For the secondary antibody a Goat anti-mouse HRP (DAKO P0447) (1000 µg/ml) was used in a dilution of 1:2000 for CD9, Cystatin C and C5a and a Goat anti-rabbit HRP (DAKO P0448) (250 µg/ml) was used for pIgR. Visualization was done with an ECL kit (Sigma) and image capture and analysis was done on a Biorad Chemidoc XRSplus.

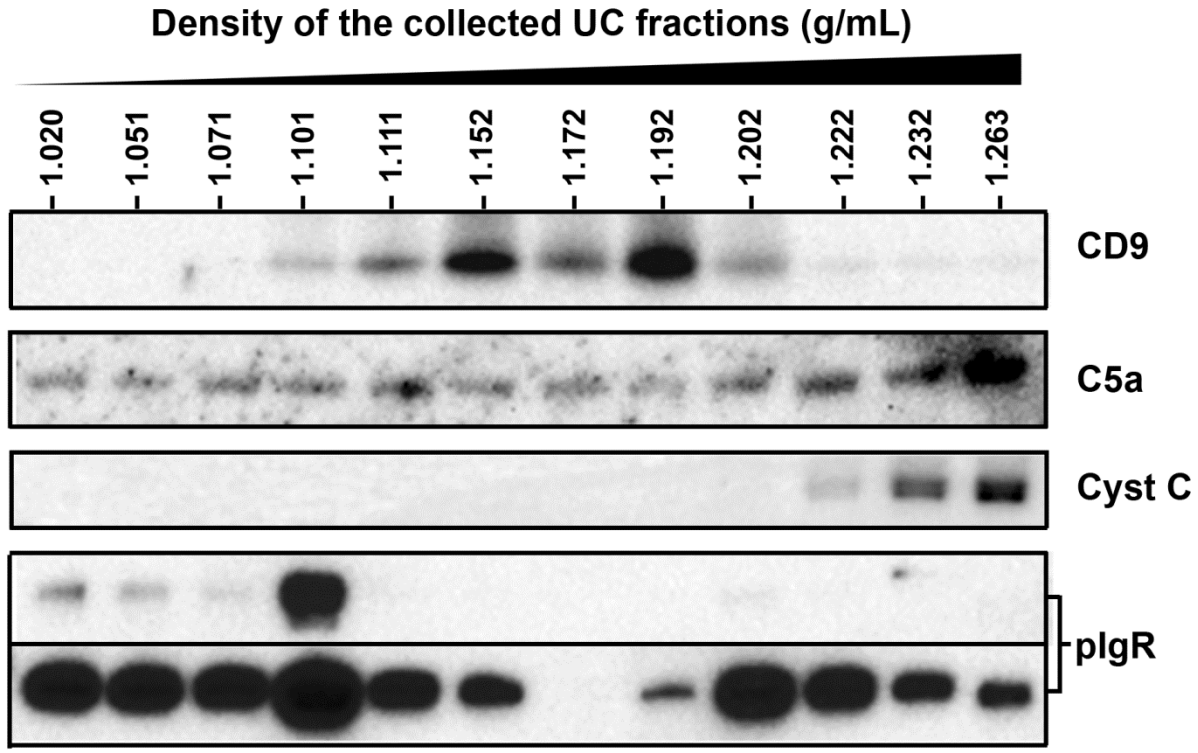

Figure S2. Serum extracellular vesicles obtained from the floatation experiment were analyzed with Western Blot as described in the 'Supplemental material 5'. CD9 was used as the marker for exosomes. C5a, Cystatin C and pIgR were all present in extracellular vesicles, floating at different densities. Of note, two bands were observed for pIgR.

Supplemental 6: Freeze-thaw cycles and Luminex measurement

Freeze-thaw cycles were performed on two different serum samples from healthy volunteers. Fresh serum was used at room temperature and an aliquot was taken (Cycle 0) for Exoquick serum extracellular vesicle isolation. The remaining serum was frozen on dry ice and thawed after which a next aliquot for Exoquick isolation was taken (Cycle 1). This was repeated up to 4 cycles. The Exoquick samples were subsequently used for vesicle isolation after which Luminex analysis for pIgR, Cystatin C and C5a was performed on the pellet lysate. All proteins remained stable during four freeze-thaw cycles within the same serum sample *(figure S3)*.

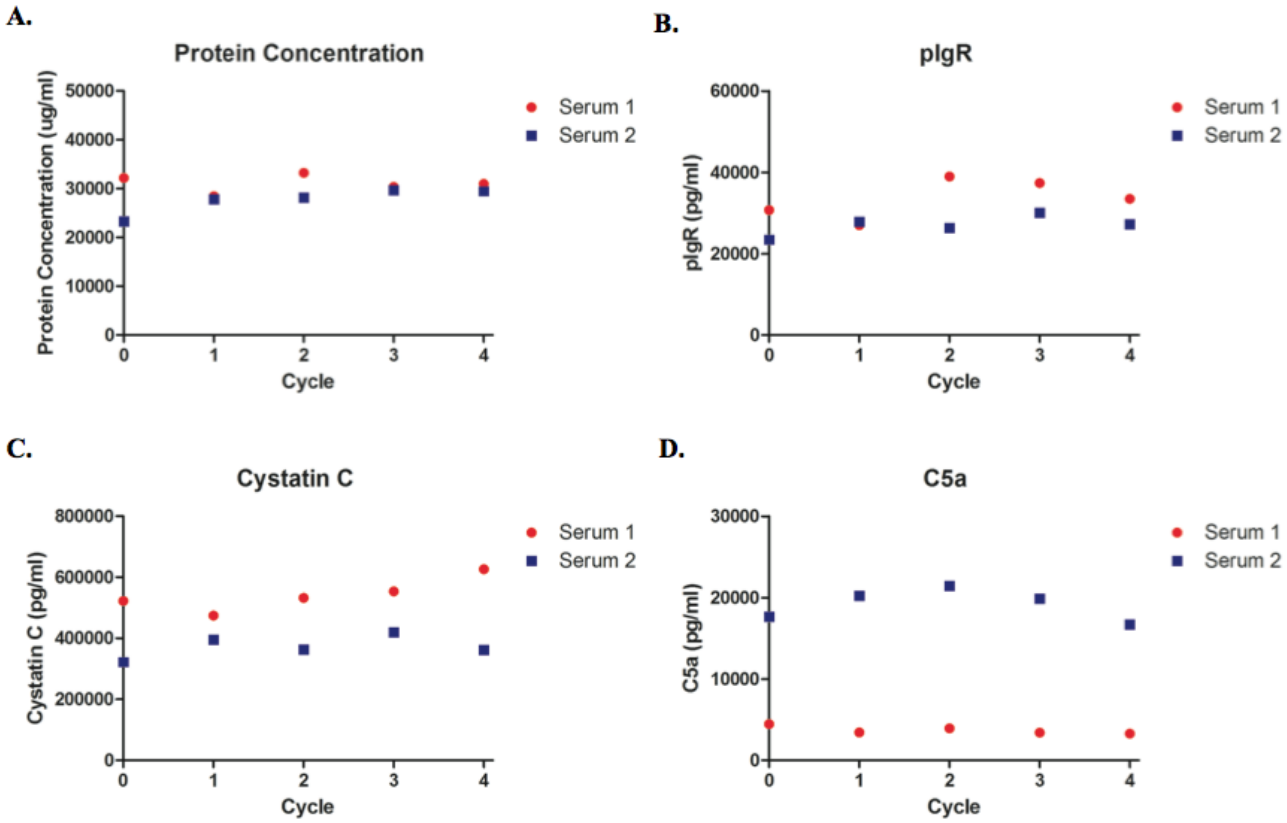

Figure S3. Extracellular vesicle protein concentrations after several freeze-thaw cycles. Concentration of pIgR, Cystatin C, C5a and total protein concentration in serum extracellular vesicles after Exoquick isolation, measured in 2 serum samples of healthy volunteers. Cycle 0: fresh serum (not frozen); Cycle 1: after 1 freeze-thaw cycle; Cycle 2: after 2 freeze-thaw cycles; etcetera. Panel A: total serum vesicle protein concentration (µg/ml); Panel B: pIgR concentration (pg/ml); Panel C: Cystatin C concentration (pg/ml); Panel D: C5a concentration (pg/ml).