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Identifying exosomal biomarkers to predict cardiac events

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Abstract

Every year, millions of people in the world die from cardiovascular diseases. Most of these victims show no symptoms of disease for decades as the disease silently progresses, but may suddenly become afflicted with a myocardial infarction or stroke. At present there is a regrettable lack of important disease markers that could help identify patients at risk with regard to secondary cardiovascular events. Biomarkers for this would of course be extremely valuable. If such markers could distinguish between patients with different responses to treatment and overall outcome, that would be of major added value as this potentially could pave the way for differentiated preventive measures. In this study we show that specific proteins isolated from plasma-derived exosomes are indeed associated with an increased risk for secondary cardiac events.

Keywords

Exosomes, biobank, filtration, ultracentrifugation, ExoQuick, CD9, CD81.

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Identification of exosomal biomarkers to predict cardiac events

Hanna Inganäs

Populärvetenskaplig sammanfattning

Varje år dör miljoner av människor i världen på grund av hjärt och kärlsjukdomar. Enbart i USA så motsvarar detta en död var trettioåttonde sekund. De flesta människor vet inte om att de kan drabbas förrän det är för sent, eftersom sjukdomen sakta smyger fram under flera år. I CAD, den vanligaste typen av hjärt- kärlsjukdom i den västerländska världen, ansamlas plack i artärerna. Dessa plack, eller kalkförhårdnader, kan slitas loss och förhindra blodets fria flöde. De kan på detta sätt blockera kärl i hjärtat där de kan ge upphov till en hjärtattack eller stroke.

Forskarna vill på ett tidigt stadium kunna förutse om en patient befinner sig i riskzonen eller ej och för detta krävs nya metoder. Ett sätt att gå till väga på är att mäta biomarkörer i blod. En biomarkör kan vara en specifik mätbar substans som utsöndras vid sjukdom och kan användas som ett mått på hur sjukdomen fortskrider.

Under de senaste åren så har mycket forskning pågått för att hitta biomarkörer för hjärt och kärlsjukdomar. Vid laboratoriet för experimentell kardiologi på universitetssjukhuset i Utrecht, Holland så pågår forskning just för att hitta biomarkörer för stroke. Man har bland annat lyckats påvisa specifika biomarkörer funna i plack, liksom i exosomer, dvs. små vesiklar i blod innehållande proteiner som kan associeras med stroke. Denna studie syftar till att ta fram en bättre och snabbare metod för att isolera exosomer från blod och jämföra uttryck i markörnivåer hos patienter som har haft en primär och en sekundär infarkt.

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Concepts & abbreviations

Atherosclerotic plaque	Accumulation of debris such as calcium in the arteries
Biobank	A storage of biological samples
Biomarker	A substance used as a biological indicator
CAD	Coronary Artery Disease
CD9	Protein encoded by the CD9 gene
CD81	Protein encoded by the CD81 gene
Coronary Artery	Blood vessel in the myocardium
CVD	Cardio Vascular Disease
Endocytosis	A process in which molecules are engulfed by cells
Exocytosis	A process in which a cell directs the contents of secretory vesicles out of the cell
ExoQuick	Commercial kit for isolating exosomes
Golden standard	Best performed method available for a specific situation
HSP	Heat Shock Protein
LDS	Lithium Dodecyl Sulphate
Luminex	A technology from Luminex Corporation in which multiple analytes can be measured simultaneously.
MHC class I	One of two Major Histocompatibility complex molecules
MOPS	3-(N-morpholino) propanesulfonic acid
MVB's	Multivesicular bodies
Myocardium	The muscular layer of the heart
Myocardial infarction	Ischemia of the heart; heart attack
Proteomics	Study of proteins expressed by the genome
SBI	System Biosciences
Stroke	A rapid developing loss of brain function due to disturbances in the blood supply to the brain

1. Introduction

1.1 Introduction

Every year, millions of people in the world die from cardiovascular diseases (CVDs). In the USA alone, this is 2,300 Americans a day or one cardiovascular death per 38 seconds. Cardiovascular disease is a silent killer. Most individuals show no evidence of disease for decades as the disease advances and are suddenly confronted with a myocardial infarction or stroke. CVD is the underlying cause for about 56% of all deaths in the USA (2006) and is also in all other Westernized countries the major and leading killer compared to other diseases.[1]

In the western world, coronary artery disease (CAD) is the most common CVD. In CAD, athermanous plaques accumulate within the walls of the coronary arteries. After years of accumulation these plaques can rupture, leading to a subsequent thrombotic event that can close off the coronary artery leading to a heart attack[2]. Treatment is to open the artery as quickly as possible to reperfuse the heart muscle again.

The patient group suffering from manifestations of CVD is rapidly increasing due to the fast growing elderly population, the lower mortality following myocardial infarction and stroke and the increase in risk factors like diabetes and kidney failure[3]. There is a specific lack of important disease markers that can help identify patients at risk of developing secondary cardiovascular events. Biomarkers that could distinguish between patients suffering from the same disease, but with different response to treatment and overall outcome, would be of major added value as this high-risk patient group could be targeted for aggressive preventive measures.

The ideal approach in the search of biomarkers is an unbiased approach. Novel molecular techniques such as proteomics opened new possibilities for this purpose.

Recently in the Experimental Cardiology laboratory, this technique was used successfully to discover biomarkers for cardiovascular disease in atherosclerotic plaques. As the ideal biomarker can be measured in the human plasma, which is easily obtainable, a proteomic analysis was performed on human plasma samples. The procedure was hampered by the presence of (redundant) high-abundant plasma proteins such as albumin and immunoglobulin's, which complicated the detection of other potentially interesting proteins.

1.2 Exosomes

When stimulating the biosynthetic pathway proteins can be secreted from eukaryotic cells either by a regulated release- or by constitutive exocytosis of secretory or stored granules. Scientists have also shown that an alternative pathway also can be used to secrete vesicles; the endocytic pathway. This happens when cellular compartments named multivesicular bodies (MVBs) fuse with the membrane of a cell to release its vesicles.[4]

The endocytic pathway was described several decades ago in differentiating red blood cells but only lately has the pathway been established in a larger group of different cell types. It results in the release of certain membrane- and cytosolic proteins into the extracellular milieu as a way of cell-to-cell communication. The MVBs secrete vesicles, called exosomes, whose sizes range between 50 and 100 nm, see figure 1. The spherical membrane-enclosed vesicles, which possibly could be formed by inward budding, consist of a bi-lipid membrane that contains hydrophobic soluble components. The hollow space within the vesicle is topologically equivalent to the cytoplasm and can carry significant amounts of mRNA, microRNA and protein. Proteins and lipids are selectively recruited by the MVBs using the membrane. Through specific stimuli the MVBs can either fuse with lysosomes to degrade the content or secrete the exosomal vesicles through fusion with the plasma membrane.[4, 5]

The secretion of exosomes were first discovered in the 80s when Stalh and Jonestone were culturing reticulocytes. They could see how exosomes were released from intracellular vesicles to the extracellular medium as large multivesicular endosomes fused with the plasma membrane. Using an ultracentrifuge Stahl and Jonestone manage to isolate and purify these secreted vesicles, and named them exosomes. It took many years until the exosome was excepted among scientists, because at the time when they were discovered people thought endosomes only functioned as a transitional compartment holding trash waiting to be degraded by lysosomes. In 1996 the functionality of exosomes became clear when internal vesicles of multivesicular endosomes by Epstein-Barr virus transformed B-cells were described. Finally the exosomes were excepted.[5]

In the last decades exosomes from several cell types have been studied and described; reticulocytes, B- and T-lymphocytes, dendritic cells, mast cells, platelets, macrophages and alveolar lung cells. In early studies the secretion of exosomes were studied in cell types in vitro, but later also in body fluids. Scientist have now found exosomes in amniotic fluid, blood, breast milk and urine to give some examples. Because of the small size exosomes are undetectable by regular confocal microscopy and only visible by electron microscopy[6]. Exosomes have several functionalities. They function as intracellular communicators, transfer RNA and proteins and they also play a part in the immune responses.[4,5]

Many groups have performed proteomic analysis on vesicles derived either from cell lines or body fluids containing exosomes such as serum, urine and ascites fluid[11]. These analysis show common characteristics among mammalian exosomes for instance size, density and overall protein composition. Most of the exosomes contain MHC class I molecules and heat-shock protein, Hsp70 and Hsp90. Nearly all vesicles contain the cytoplasmic proteins; tubulin, actin and actin-binding proteins as well as proteins for signal transduction. The most commonly tetraspanins found on the membranes of exosomes are CD9, CD63, CD81 and CD82. Depending on where the exosomes are secreted from, they express proteins that can be traced back to a specific host cell. For this reason there may be valuable information regarding ongoing (patho) physiologic processes in the human body and including information on future cardiovascular events.[7]

Currently a lot of research is being performed on exosomes to develop new diagnostic- and therapeutic applications. Most of this research is being performed in the fields of oncology and immunology and not so much in the field of cardiovascular disease[8]. Recent proteomic studies in the Experimental Cardiology Laboratory at the University Medical Center Utrecht have indicated that proteins present in exosomes derived from pooled plasma are associated with secondary cardiac events. If this could be verified in an antibody based assay on individual plasma samples then measurement and characterization of exosome proteins could potentially be used as a blood based test for future secondary cardiac events.

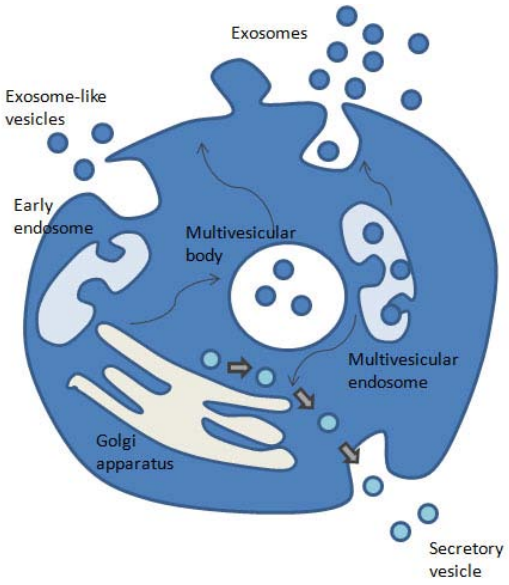

Figure 1 Schematic figure over a cell secreting exosomes and other exosome-like vesicles. The multivesicular endosomes and a multivesicular body (MVB) can be seen. Intracellular trafficking takes place both between the cell membrane and the sub cellular compartments.

1.3 The Biobank

In 2002 the Experimental Cardiology Laboratory at the University Medical Center Utrecht and the Vascular Surgery departments in the St. Athonius Hospital (Nieuwegein) and UMC Utrecht started to collect samples to form a biobank containing atherosclerotic plaques and blood for a study called Athero-Express[9]. At this time no one had collected data from patients undergoing surgery of carotid arteries, femoral arteries or abdominal aneurysms in connection with long term follow up studies[10].

Patients who had experienced a myocardial infarction at one of the two participating hospitals were asked to participate in this study. By filling out extensive questionnaires and signing a written consent the patients were included in the biobank study. Atherosclerotic plaques and blood were then collected during surgery, dissected and frozen. Parts of the plaques were used for protein, RNA and DNA isolation, but precious material as tissues was also stored for further analysis.[10]

What makes this biobank unique is that all patients have a follow up at one-, two and three years after surgery. Due to this follow-up, plaque and blood can be used to find biomarkers associated with secondary events that occur in approximately 25% of the biobank patients. Today more than 2000 patients have been included in the biobank, making it at present the largest of its kind worldwide[10]. For about 60% of the patient both serum and heparin plasma are available[8].

1.4 The Aim of this study

In this study we will investigate if characterization of plasma exosome proteins is feasible and contains predictive information for secondary cardiac events.

The golden standard method for isolating exosomes from serum or plasma is ultracentrifugation, which is both expensive and time consuming. In this study, we investigate new methods of isolation, normalization and measurement of exosome proteins identified by earlier proteomic studies. Later one of the methods will be selected and used to isolate exosomes and verify whether differences in marker expression can be found within a cohort of 60 carotid atherectectomy serum samples from which 30 patients had a secondary cardiac event and 30 were free of secondary events during 3 year follow-up.

1.5 Specific research questions

Several research questions need to be addressed as the investigation proceeds, such as:

- Today ultracentrifugation is used as a golden standard when isolating exosomes, though it is expensive and time consuming. Can filtration or ExoQuick, a new method to isolate exosomes, be alternative methods of use instead?
- What should be used for normalization when performing the verification study?
- Markers will be measured in the Luminex system in two patient groups; patients with a secondary cardiac event (event) and patients not suffering from a secondary cardiac event (control). First of all, is it possibly to detect the chosen markers and second, will there be any differences between the two groups?

2. Materials & Methods

2.1 Plasma and Serum

While setting up the exosome isolation protocol heparin whole blood was taken from healthy donors and centrifuged at 1850 x g for 10 minutes to separate the blood plasma from the blood cells. As time proceeded also serum and plasma collected from patients in the Athero-Express biobank was used.

2.2 Isolation methods

2.2.1 Ultracentrifugation

To be able to carry out research in biochemistry, molecular biology and medicine etc. isolation of cells and sub cellular molecules are of high importance. Centrifugation is a widely used research technique using the gravity or g-force to separate particles from their surrounding media in a batch or a continuous-flow mode. Depending on how long time the liquid suspension is spun, the particles or cells will separate and settle at the bottom of the container due to the g-force.[12]

During centrifugation of a cell homogenate, the large particles will sediment faster compared to the small ones. Centrifugation at 1000 x g for 10 minutes will form a pellet with unbroken cells and heavy nuclei at the bottom of the centrifuge tube. Transferring the supernatant into a new tube and exposing it for further centrifugation will separate the particles of intermediate size and so on. Very small particles will not separate at unless they are exposed to a high centrifugal force. [12]

Since exosomes are very small, only 50-100 nm, an ultracentrifuge is needed to separate them from the plasma or serum. The ultracentrifuge is optimized for spinning the samples at a very high speed and can accelerate up to 1,000,000 x g. Exosomes need to be spun at 100,000 x g for at least one hour[3]. Using an ultracentrifuge has up to now been the only way to isolate exosomes, however the method is very time consuming and fairly complicated. Not all laboratories have access to an ultracentrifuge, so other reproducible methods would be preferred.

Plasma was thawed and centrifuged at 1850 x g for 10 minutes to get rid of debris from the freeze thawing process. 1 ml of plasma was then put into a 30 ml centrifugation tub, added up with PBS and spun at 10,000 x g for 30 minutes to get rid of big cell debris. After the spinning most of the mixed plasma/PBS was taken out into a new clean tube, only leaving the pellet of debris left. The remaining part of the new tube was filled up with new PBS and spun at 100,000 x g for 1h. After centrifuging the supernatant was removed leaving the pellet and a bit of liquid containing the exosomes. This liquid was transferred into a new smaller ultracentrifugation tube, filled up with new PBS and centrifuged at 100,000 x g for another

hour. Using a smaller tube made the pellet more visible to the eye when removing the PBS after the last hour of spinning. The supernatant was removed and the pellet containing the exosomes was directly lysed with 100 µl of Roche lysis buffer, since continuous work would be done on the intracellular proteins. The samples were stored in -20°C.

2.2.2 Filtration

Two different sizes of membrane filters were used; with 300 kD and 1000 kD cut off, but the procedure was same.

Similar to the ultracentrifugation method, the plasma was centrifuged at 1850 x g for 10 minutes to get rid of the debris from freeze thawing. A series of 2, 1.5, 1.0, 0.5, 0.25 and 0.1 ml plasma was then aliquoted, filled up to 15 ml with PBS respectively and mixed. Every mix was then filtrated through sterile 0.45 µm syringe filter followed by 0.22 µm filter and the flow through was collected. The flow through was further added to a 300 kD/1000 kD membrane filter and centrifuged at 3000 x g for 90 minutes at room temperature. The membrane was then washed with the filtrated residue before the residue was taken off the filter into clean tubes. The PBS/plasma mix that had gone through the filter was discarded. The tubes were again filled up to 15 ml with PBS and the mix added to the same filter as previous. The membrane was once again spun at 3000 x g for 90 minutes at room temperature. The residue was thereafter collected and 200 µl of the residue was mixed with an equal amount of lysis buffer and stored in the -20°C freezer.

2.2.3 ExoQuick™

While we were busy testing the membrane filters a new product from System Biosciences, SBI, called ExoQuick™ was released on the market. It is a clear liquid, which you add to the serum or plasma sample. Accordingly to SBI's datasheet[13] ExoQuick™ exosome precipitation reagent should be more effective than any other methods available for isolating exosomes, so we decided to test it ourselves since no other information was given by the producer.

Accordingly to the manufacturer's protocol serum was centrifuged at 3000 x g for 15 minutes to remove cells and cell debris. The supernatant was them taken over to a new tube and filtrated through a 45 µm filter. To 250 µl of plasma 63 µl of ExoQuick™ exosome precipitation solution was added and stored in the fridge over night. The biofluid was then centrifuged at 1500 x g for 30 minutes at 4°C. The supernatant was aspirated and the tube spun down again for another 5 minutes at the same speed. 500 µl of PBS was added to the tube and vortexed for 10 seconds to wash the pellet. The biofluid was then centrifuged again at 1500 x g for 30 minutes at 4°C.The last bit of fluid was removed, the pellet resuspended in 100 µl lysis buffer and stored in the -20°C freezer.

2.3 Verification methods

2.3.1 Protein measurement

The protein concentration was measured using a BSA kit from Thermo Scientific Pierce. A standard curve was made from the stock solution that came with the kit and diluted in Roche lysis buffer. The standard curves final concentration was; 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml. 25 µl of each sample/standard was added to a well in a 96-well plate. 200 µl of working reagent was thereafter added to each well. The plate was covered and incubated at 37 °C for 2 hours and thereafter was the optical density measured at 540 nm.

2.3.2 SDS PAGE followed by Coomassie staining

To see the amount of contamination from other proteins coomassie stainings were performed using Invitrogens NuPAGE® Novex® Bis-Tris Mini Gels.

Samples were thawed and vortexed well. 6.5 µl of sample was mixed in an eppendorf tube with 2.5 µl of NuPAGE Lithium Dodecyl Sulphate (LDS) Sample Buffer (4X) and 1 µl of NuPAGE Reducing Agent (10X). The samples were heated up at 70°C for 10 minutes. While waiting for the samples the running buffer containing 25 ml 20X NuPAGE MOPS (also called 3-(N-morpholino)propanesulfonic acid) SDS Running Buffer and 475 ml deionized water were prepared and added to the XCell SureLock TM Mini-Cell, the container holding the gel. The wells were washed with the running buffer using a syringe and the samples were added together with the ladder, SeeBlue®Plus2 Prestained Standard (1x). 500 µl of NuPAGE® antioxidant was added to the chamber before the gel was run at 200 V for about 50 minutes.

After running the gel was taken out of its casing and placed in a tray containing SimplyBlue™ SafeStain. The tray was heated up at 480 Watt for 30 seconds and after left on a shake table for 10 minutes. The SimplyBlue™ SafeStain was then poured of and deionized water was added to distain the gel for a few hours. A picture could then be taken.

2.3.3 Western blotting

To be able to separate and detect specific proteins in a sample the analytical technique Western blotting is often used. The technique which was invented in the 1979 and separates denatured protein samples by the length of the peptide. The proteins are then transferred to a nitrocellulose membrane, where the proteins are probed and detected by antibodies. [13] To verify that we actually had isolated exosomes western blotting for the markers CD9 and CD81[7], which are known exosome markers, were performed.

The same procedure as for the coomassie staining (see above) took place. After the gel was finished running it was taken out of the casing and the stacking gel was removed. It was then assembled in the following order to start blotting; plastic support, sponge, whatman paper,

gel, nitrocellulose membrane, whatman paper, sponge, plastic support. The gel cassette was then put into a holder and blotted at 250mA for 90 minutes. After blotting the membrane was incubated with 5% milk in PBS-0.1%Tween for 1 hour, followed by 1 hour of incubation with the primary antibody (CD9 or CD81)[7]. The membrane was washed 3x5 minutes with PBS-0.1%Tween and incubated with the secondary antibody; goat anti rabbit for 1 hour. After incubation the membrane was washes 2x5 minutes with PBS-0.1%Tween and left to sit in PBS to the picture was to be taken using Molecular Imager ChemiDoc™ XRS+ with the Image Lab™ software. Before taking the picture of the blot an ECL substrate was mixed, 1:2, and added to the blot.

2.3.4 Luminex

The Luminex System, made by Luminex Corporation, is an analyzer system which makes it possible to measure biomarkers in plasma. Based on the principles of flow cytometry the system makes it possible to measure up to 100 analytes simultaneously in a single microplate well using a very small sample volume.[14, 15]

The system can be divided into three different parts; the xMAP microsperes, the cytometry-based instrument or Luminex analyzer as it is also called and the assay designed around the microspheres. One hundred 5.6 micron-sized polystyrene microspheres serve as the solid surface on which the assay is built on and are individually dyed with different intensities of red and infrared fluorophores. Each bead is filled with a different ratio of the two fluorophores. This gives the bead a unique spectral signature which the xMAP detection system can identify, see figure 2. Different microspheres can then be combined within the assay which makes it possible to multiplex 100 different tests in a single reaction. [14, 15]

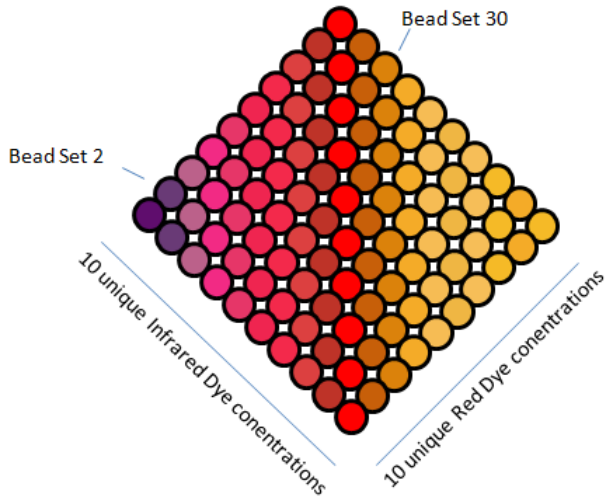

Figure 2 Representation of the one hundred unique microspheres individually dyed with red and infrared fluorophores serving as the surface where the Luminex assay takes place.

In the Luminex analyzer two lasers are combined with a fluidic system and a real-time signal processor. Beads pass by the reader in the fluidic system and are aligned into a single file

within the detection chamber. The reader can then detect the beads, one by one, and interrogate for bead color and assay signal strength (PE florescence intensity). [14, 15]

The system uses one green and one red laser to distinguish between the beads. The green laser is used to excite the PE dye at 532 nm and the solid state red laser is used to excite the color inside of the beads and establish their color or region. The red laser is also used for doublet discrimination by light scatter. Four detectors are then used to measure the florescence of the assay, determine which of the beads are passing (1-100) and discriminate between single and aggregated beads, see figure 3. [14, 15]

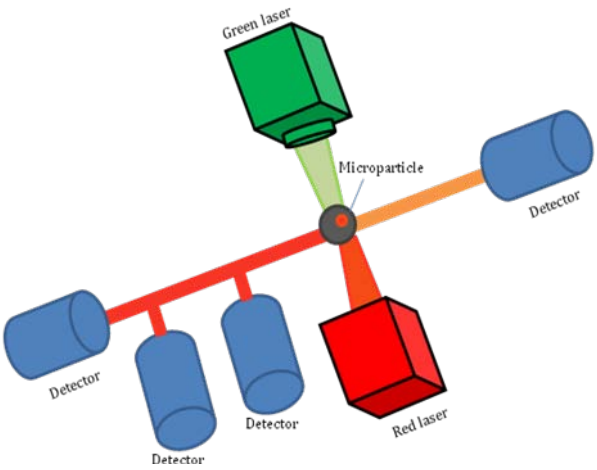

Figure 3 A graphic representation of the Luminex analyzer. Four detectors and two different colored lasers can be seen. The lasers excites the two dyes used and the detectors measure the florescence of the passing bead.

Because of the surface chemistry on the microspheres it is possible to couple capture reagents such as antibodies, peptides or receptors to them. [14, 15]

2.4 Verification study

The goal of the project was to find new plasma-based biomarkers that are predictive for a secondary coronary event using the Luminex bead system. 60 samples were selected from the Athero Express biobank to show the proof of concept for the study. 30 of these samples were individual serum samples that during follow up had suffered a secondary coronary event. The other 30 samples served as controls, since they did not have any secondary event during follow-up. The controls were age and sex matched with the events.

Exosomes from 1 ml of serum was isolated using the ultracentrifuge, lysed in Roche lysis buffer and stored in -20°C till further use.

While sample preparation was taken place another technician was busy setting up single-plex assays in the Luminex system, where one marker of interest per test was measured. In total 28 markers were approved since they fulfilled the two criteria's for quantitative protein analysis; good linear response of the calibrators and a dynamic range of 2 to 3 logs. The single-plex assays where then combined into multi-plex assays with the same quality criteria's as before. Four different multiplex assays (panel 1-4) were needed to combine the single-plex assays see fig. 4. Note that the names of the markers are confidential and sham names have been given.

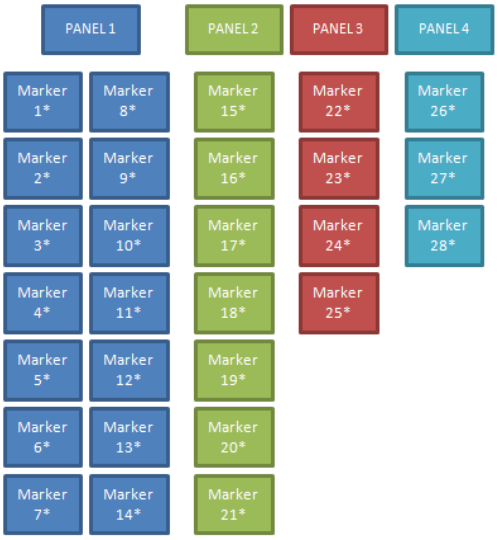

Figure 4 The chosen Luminex panels. Twenty eight single-plex assays have been combined and put together into four multi-plex assay panels. Sham names have been given to the different markers.

3. Results

3.1 Previous Proteomics Run & Patient Selection

To identify exosome biomarkers for cardiovascular disease a quantitative proteomics run was performed in 2009 on a selection of stored Heparin Plasma samples from the Athero-Express biobank. The selection of samples was based on one type of cardiovascular event, a hard clinical endpoint, which was believed to facilitate the detection of proteins between events and controls. For this, only coronary events were chosen, since these are related to coronary arteries directly in contrast to peripheral events that derive from different arteries. 52 patients were selected based on these criteria's (events group) and gender and age matched with 52 patients serving as controls (control group): patients who did not suffer from any cardiovascular including coronary events in a follow-up time longer then the matched event. Next to gender and age, the events and controls were matched when possible for diabetes status and statin use (a drug that lowers the plasma cholesterol amounts) [16]. The samples within the event group were pooled to one sample and the same was done with the control group samples. From these two pooled samples, the exosomes were isolated by ultracentrifugation.[8]

The proteomics run revealed a list of 116 proteins that differed between the event and control group. 102 of these proteins could be found in the Ingenuity database and when analyzed 60% of these showed to be located in cytoplasm, nucleus and plasma membrane. None of these proteins are normally present in plasma proving that the ultracentrifuged pellet actually contained exosomes.[8]

3.2 Ultracentrifugation

Ultracentrifugation was used a lot in our studies, both as a golden standard, but also to produce samples. Spinning usually resulted in an invisible pellet, with a protein concentration of about 200 µg/ml.

Fig. 5 A shows three different samples of; 1 ml, 2 ml and 3 ml plasma made from one patient pool. The protein concentrations for the same samples can be seen in Table 1. The amount of exosomes seem to increase with an increasing amount of plasma, as can be seen in the western blot for CD9, while the protein concentrations seem to fluctuate more.

Table 1 Protein concentrations of ultracentrifuged plasma samples. The concentrations are measured for 5 samples containing 1, 2 or 3 ml plasma. See figure 5A and 5B for CD9 expression.

Sample ID (as seen in fig. 5A)	1 ml	2 ml	3 ml	1 ml healthy	1 ml patient
Volume plasma (ml)	1	2	3	1	1
Measured protein concentration (µg/ml)	188.77	317.49	379.33	375,06	313,58

In figure 5B a triplicate of 1 ml plasma samples, made from a patient pool and a healthy donor pool are visualized. More intense bands are seen in the healthy donor samples, indicating higher amounts of CD9.

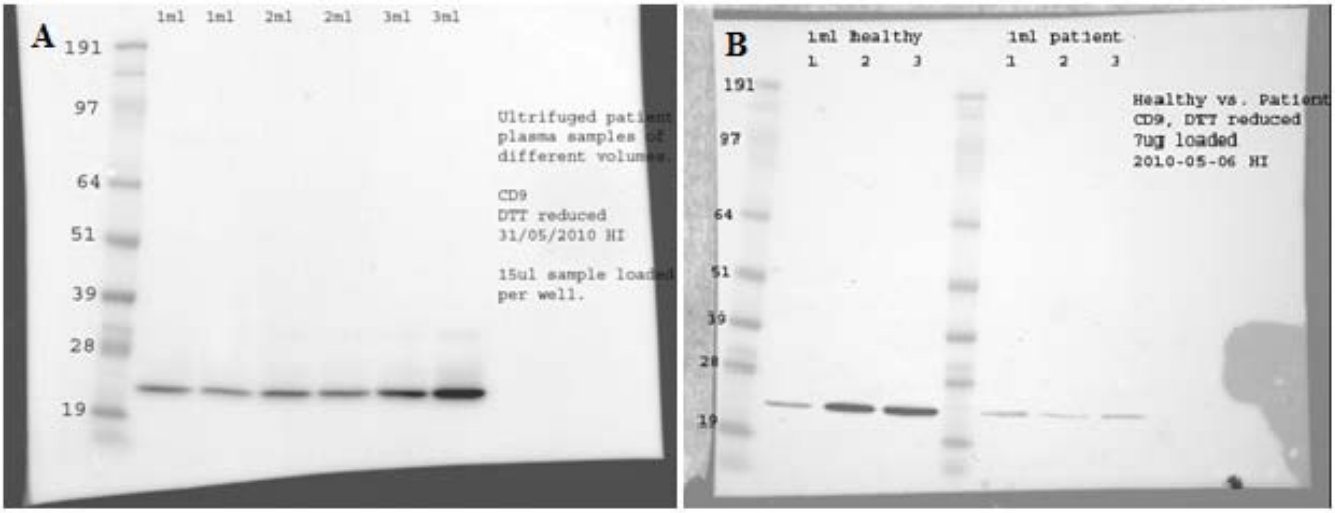

Figure 5 Western blots for CD9 **A.** Western blot for CD9 of 3 different ultracentrifuged patient plasma samples made in duplex. The samples contain 1, 2 and 3 ml plasma respectively. **B.** Western blot for CD9 comparing healthy donor and patient plasma with each other. The samples are blotted in triplicate. The patient samples show less intense bands compared to the healthy donor.

3.3 Filtration

Coomassie staining in a series of samples filtrated through a 300kD membrane (Fig. 6A) shows that all samples contain high amounts of different plasma proteins. The 0.1 ml sample contained the least amount of proteins compared to the other samples, but as the volume of plasma increased, so did the amount of proteins as can be seen in the figure. What was also noted was that the residue that could be collected after filtration had approximately the same volume as the plasma added to the filter in the first place. Looking at the samples filtrated through a 1000kD filter only very small amounts of proteins were detected. This was the same for the whole series of samples.

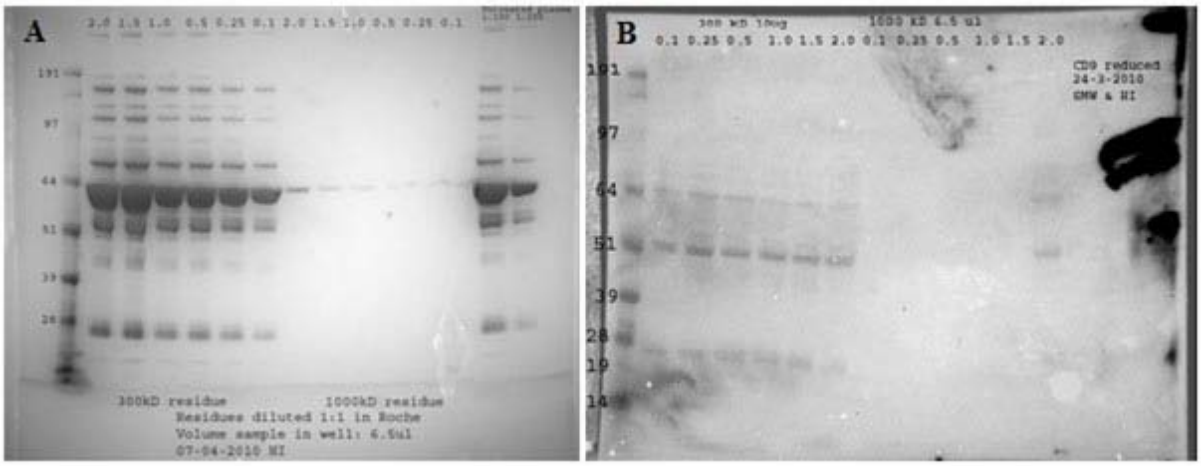

Figure 6 Coomassie stainings of filtrated samples. **A.** Coomassie staining of plasma samples; 2.0, 1.5, 1.0, 0.5, 0.25 and 0.1 ml, filtrated through 300kD membrane filters (left) and 1000kD membrane filters (right). Two untreated plasma samples can also be seen to the very right in a dilution of 1:100 and 1:200. **B.** Western blot for the same set of samples as in 5A. The left sets of samples are filtrated through 300kD membrane filters, while the right set of samples are filtrated through 1000kD filters. CD9 is approximately 25kD.

Performing a Western blot on the same set of samples (fig. 6B) to detect CD9 showed that for all samples filtrated through the 300kD membrane, CD9 was detected. The CD9 band detected by the antibody is expected to be at a height of ~25kD, but also other bands, more intense, were seen. The CD9 Western blot for the samples filtrated through the 1000kD filter only showed a very weak signal for the 2.0 ml sample. No CD9 was detectable in the other samples.

3.4 ExoQuick

We first tested if the ExoQuick solution made by SBI indeed precipitates exosomes and could be used as a substitute for ultracentrifugation. For this, several ExoQuick treated samples from patients and healthy donor's serum and plasma have been tested using CD9 western blot (fig. 7) and compared with ultracentrifugation. Even though the ExoQuick samples are 1:50 times diluted, the CD9 levels are still higher compared to an ultracentrifuge sample in 1:1 dilution.

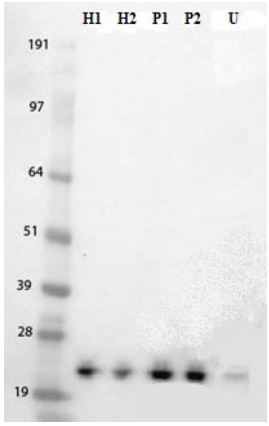

Figure 7 Samples made using ExoQuick and visualized using a CD9 western blot. Two healthy donors and two patient samples can be seen. An ultracentrifuged sample was also put on the gel to visualize the difference between the two methods when exosomes are being isolated.

3.5 Normalization

To be able to quantify exosomal biomarkers you need to normalize for the number of isolated exosomes and/or protein. The amounts of housekeeping protein most correlate to the amount of plasma used per patient. This can be done by measuring a protein known to be found in all exosomes like CD9, CD81, beta tubulin etc. So far none of these proteins work in the Luminex system, so normalization using these proteins was done with the marker CD9 on Western blot instead.

To be sure that Western blot was quantitative for CD9 an experiment was carried out where 20, 6 and 3 ml of plasma were spun in the ultracentrifuge. Depending on what volume of plasma had been spun the pellets were lysed in 200, 60 and 30 µl of lysis buffer respectively, which would correspond to a concentration of approximately 1 mg/ml. Western blots for CD9 were carried out for the different samples in which we expected all bands to give the same signal. As can be seen in fig. 8 the amount of exosomes that were isolated did not vary between the samples, which proved the CD9 Western blot to be a good standard for exosome isolation.

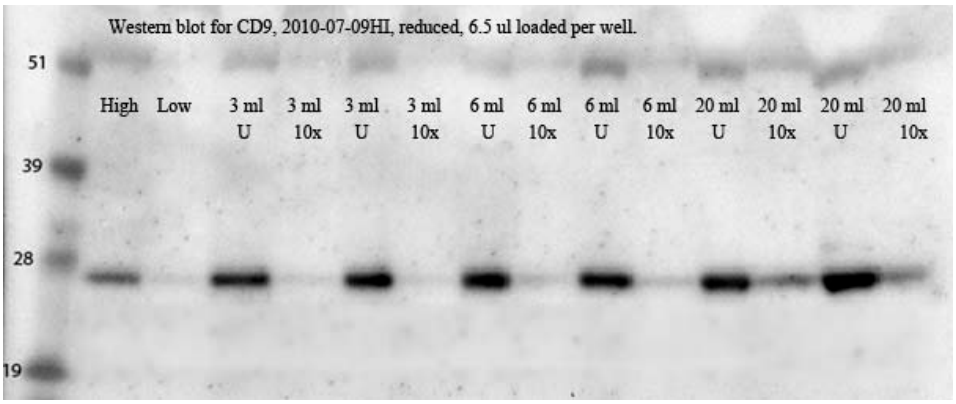

Figure 8 Western blot for CD9 performed on 3, 6 and 20 ml ultracentrifuged samples. The samples were after centrifugation lysed in 30, 60 and 200 µl lysis buffer respectively. 6.5 µl per sample was loaded on the gel, which corresponds to a concentration of approximately 1 mg/ml. U in the figure stands for undiluted and 10x for 10 times diluted.

3.6 Verification study

To find new plasma-based biomarkers that are predictive of a secondary coronary event a verification study was set up measuring markers of interest in 60 individual serum samples. 30 of these samples came from patients that had suffered a secondary coronary event during follow up (events) and 30 of the samples came from patients who had not suffered one any secondary event (controls).

Using the Mann Whitney test six different markers were found to differ between the control and event group. Marker 16, 19, 20, 26, 27 were significantly higher in the event group, while marker 28 was significantly elevated in the control group, see figure 8. Marker 6 and 13 showed a trend ($p=0.15<0.20$) towards being higher in the event group. See table 2 for minimum, maximum, mean, median and p-values. All markers that are not included in table 2 were either not measurable or out of the range of the standard curve.

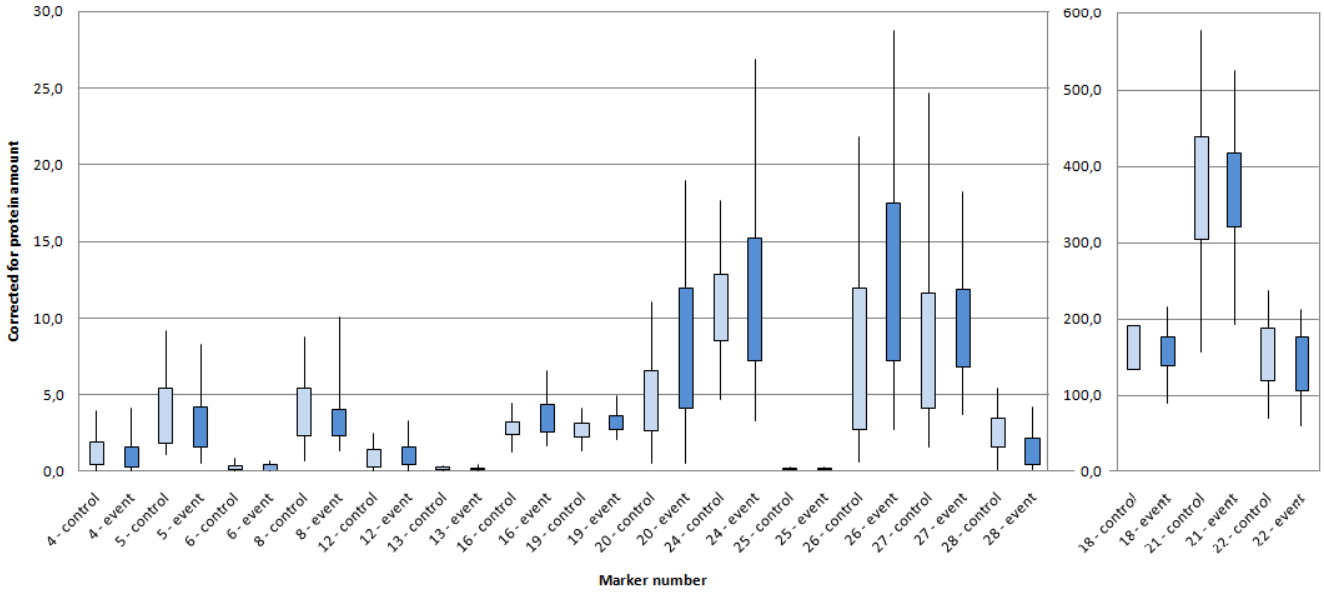

Figure 9 Combined box plot of 28 different serum markers measured in the Luminex system. 30 patients experiencing a secondary cardiac event (event) have been compared with 30 patients who only experienced a primary cardiac event (control). Light blue colors indicate control samples, while dark blue are events. Marker 1, 2, 3, 7, 9, 10, 11, 14, 15, 17 and 23 were not measurable in any of the samples. Note that marker 18, 21 and 22 contain higher protein amounts.

Table 2 Calculated values for the different serum markers in the 30 vs. 30 study.

Marker #	N	Min.	Max.	Mean	Std. dev.	Median [IQR] Controls	Median [IQR] Events	P	Estimated odds ratio [95% CI] and p-value
4	59	0.0	9.67	1.35	1.61	0.8	0.7	0.617	1.1 [0.8 – 1.5] p=0.568
5	59	0.0	22.0	3.9	3.7	3.2	2.6	0.355	1034 [0.896 – 1.194] p=0.643
6	59	0.0	1.44	0.32	0.30	0.19	0.24	0.339	1.6 [0.290 – 9.1] p=0.581
8	59	0.69	14.6	4.5	2.8	4.2	3.6	0.976	1077 [0.895 – 1.297] p=0.430
12	59	0.0	3.32	0.82	0.83	0.43	0.64	0.378	1418 [0.746 – 2.97] p=0.286
13	59	0.06	1.06	0.24	0.17	0.21	0.16	0.095**	0.843 [0.041 – 17.488] P=0.912
16	59	1.26	9.70	3.4	1.5	2.9	3.7	0.025*	1.7 [1.1 – 2.8] p=0.025
18	59	88.6	306.7	163.4	44.3	151.1	154.6	0.891	1 [0.990 – 1.013] p=0.814
19	59	1.37	6.53	3.07	0.98	2.7	3.3	0.001*	4 [1.6 – 9.9] p=0.003
20	59	0.00	18.97	6.4	4.8	4.0	7.0	0.018*	1.2 [1.0 – 1.3] p=0.016
21	59	157.07	640.75	3744162	10180821	349.8	376.5	0.617	1001 [0.996 – 1.006] p=0.799
22	59	58.6	420.9	164.1	81.6	156.3	147.9	0.868	1 [0.994 – 1.007] p=0.872
24	59	3.31	40.47	11.2	5.9	9.6	9.3	0.891	1 [0.948 – 1.144] p=0.401
25	59	0.09	0.39	0.18	0.06	0.18	0.16	0.596	0.150 [0.000 – 1093.0] p=0.676
26	59	0.0	38.1	11.1	8.5	8.4	11.6	0.057**	1065 [0.995 – 1.140] p=0.068
27	59	1.6	31.4	9.0	5.6	6.6	7.9	0.069**	1056 [0.958 – 1.165] p=0.273
28	58	0.0	41.4	2.5	5.6	2	0.3	0.014*	0.785 [0.589 – 1.044] 0.096

Outcome of data analysis when measuring 28 different plasma markers of interest using the Luminex system. All data are corrected for protein concentration. * P<0.05 either from Mann Whitney or the logistic regression **P>=0.05 and <0.10 from Mann Whitney or logistic regression. The number of participants, maximum- and minimum value, mean, median and standard deviation as the significance are given for the different markers.

4. Discussion

The search for new biomarkers in the field of secondary prevention is rather unexplored. The group of patients who have already suffered their first cardiovascular event is rapidly increasing due to the lower mortality rate following myocardial infarction and stroke, the increase in risk factors such as obesity, diabetes and kidney failure, and the fast growing elderly population[1]. There is a specific lack of important disease markers that can help identify patients at risk of developing secondary cardiovascular events. Biomarkers that can distinguish which patients are at the highest risk of secondary event would be of major added value as this patient group could be targeted for aggressive preventive measures.

Earlier studies have indicated that proteins present in exosomes, derived from pooled patient plasma, are associated with secondary cardiac events[8]. Since blood is easily accessible and drawing blood a standard procedure in hospitals this could potentially be used as a blood based test for future secondary cardiac events.

4.1 Ultracentrifugation

Since the 1980's it has been known that exosomes can be isolated from plasma using ultracentrifugation[5], though the procedure is both expensive and very time consuming. We have seen that it also is very difficult to isolate exosomes and get the same results time after time, see table 1. This difference in protein concentration for instance is most likely due to the handling of the sample. The exosomal pellet is barely visible during isolation and even when handling with the greatest care what is expected to be the same differ.

In figure 5A we have shown that when spinning a larger volume of plasma the isolation of exosomes increase, which can be seen as more intense bands on the CD9 western blot. Also the protein amount rises as expected. This is of course of high relevance, since spinning more plasma would indeed lead to a more visible pellet and perhaps therefore more precise care for it. Spinning even larger volumes would of course be very handy, however in practice it will not help, since plasma from patients can only be obtained in small volumes.

Figure 5B show the result from a CD9 western blot performed on six different ultracentrifuge samples, originating from a healthy and a patient pool, all containing 1 ml of plasma. Even though you would expect the intensity of the samples to stay the same within the groups, since they are coming from the same tubes and the same amount of protein was loaded on the gel, the intensity of the bands differ among each other. In other words Western blot is not the most reliable method to use when it comes to detecting markers of interest in a sample. Even though the same sample is measured on one gel the result might not always give the same results due to the way of handling and external influences the blot. What is also interesting is that the expression of CD9 expression is higher in the healthy group compared to the control group. This has been observed several times and could be explained by CD9 having a protective role in myocardial infarctions. To be able to tell for sure further experiments needs

to be performed and verified in the Luminex system. Unfortunately there is no CD9 marker for the Luminex system commercially available yet, so it will have to wait.

4.2 Filtration

Our studies show that neither 300kD nor 1000kD membrane filters can be used as an alternative instead of using ultracentrifugation when isolating exosomes. As can be seen in figure 6A and 6B the plasma that was filtrated through a 300 kD filter resulted in a lot of plasma protein contamination even though different plasma volumes were used. Using the 1000kD filter, however, no exosomes could be detected. This is probably due to clogging up the pores of the 300kD filter whereas the pores of the 1000kD filter are probably too big and all proteins including exosomes were going through the filter.

We collected and examined the flow-throughs from the different samples that had been run over the two different sizes of filters and as expected the flow-through from the 300kD filter showed barely any proteins while there was a lot of protein in the samples filtrated with the 1000kD membrane. Testing if the filters could show any differences in exosome content between plasma taken from patients and from donors gave no results. Experiments using several filters after one another with a hope to get rid of the bigger bulky proteins did also not help. As a last attempt we tried coating the membrane with Glycine to decrease the stickiness of the filter, but this did not help either. We conclude that neither of the filters could be used for isolating exosomes, since enrichment of exosomes in comparison with the plasma protein concentration was low and plasma protein contamination would complicate the detection of other potentially interesting proteins in a later stage.

4.3 ExoQuick

Using pooled plasma from both healthy donors and patients show that by using ExoQuick exosomes could be isolated in an easy way, with a high yield and quality compared to ultracentrifugation. Only 250 µl of plasma or serum is needed for one reaction[11] and even when diluting the sample 50 times after isolation exosomes are found in higher concentration compared to the ones isolated by ultracentrifugation, as can be seen in figure 7. A lot of samples can be made at the same time that speeds up the process of testing a lot of different samples. It is also possible to run the samples that are precipitated with the ExoQuick solution in the Luminex system but it needs further optimization.

To be able to run the isolated exosomes in the Luminex we have seen that the pellet needs to be washed once in PBS in order to give measurable values. This also decreases the stickiness of the pellet when dissolving it again in lysis buffer, which could be a problem according to the manufacturer[11].

Further optimization is needed before we can use ExoQuick in a standardized protocol. Nevertheless, this method has a very good potential to isolate exosomes and is likely to be used instead of ultracentrifugation.

4.4 Normalization

As in any study results needs to be normalized before any conclusion can be drawn from the experiments. Optimal would be to normalize the data using a housekeeping protein when measuring samples on the Luminex system. So far no housekeeping exosome protein is identified that works in the Luminex system. Because of this, we detected the housekeeping protein CD9 on western blot next to measuring protein concentration.

4.5 Verification study

In this study we have showed that it is possible to detect differences between patients who have suffered a secondary coronary event compared to patients who have not, when measuring different markers on serum in individual samples. From the twenty eight markers that were measured, marker 16, 19, 20, 26, 27 showed to be significantly up regulated in the event group, while marker 28 was significantly elevated in the control group, see figure 8. Marker 6 and marker 13 showed a trend being higher in events. This confirms our hypothesis that exosomes contain measurable information that could predict a future secondary cardiac event. For 11 markers no data was generated because the measured values were out of range. The dilutions of the samples need to be adjusted so that they are within the range of the standard curve so they can be remeasured. Hopefully this can lead to the discovery of additional markers showing differences between the event and control group.

The CD9 western blot one of the methods to normalize the Luminex data, assuming that CD9 would reflect the protein content of exosomes. Due to technical problems when performing the western blot; small sample volumes, low protein concentrations and a poorly functional blotting system, the western blot did not work. Fall back was to normalize using the protein concentration instead. This is after all thought to be a more reliable method compared to western blotting.

The verification study was carried out in 60 individual plasma samples and further validation studies needs to be performed in larger validation cohorts to verify these results. A follow up study is likely to be a nested case cohort study, in which a random sub-set of patients is drawn from a larger CAD cohort. Due to the random selection from the whole cohort, the selected patient group will consists of mostly control patients and only a low number of cases. Subsequently, additional cases from the larger cohort can be added to the cases group.

5. Conclusions

This study shows the proof of concept that there is information in blood exosome protein concentration, isolated by ultracentrifugation and measured by Luminex, which is associated with future secondary cardiac events.

To isolate the exosomes filtration is not a method of choice, since the filter clog up very easily, which leaves high concentrations of contaminating plasma proteins such as albumin and immunoglobulin's in the sample.

A better and more efficient way of isolating exosomes from serum and plasma is to use SBI's new product: ExoQuick^{TM}. ExoQuick makes it possible to use small amount of serum or plasma and result in a higher yield and better quality of the samples compared to ultracentrifugation. ExoQuick can therefore be used as an alternative to ultracentrifugation. Normalization for exosomes can be done using CD9 Western blotting and to correct for protein amount.

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