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Comparative Proteomic Analysis of Extracellular Vesicles Isolated by Acoustic Trapping or Differential Centrifugation

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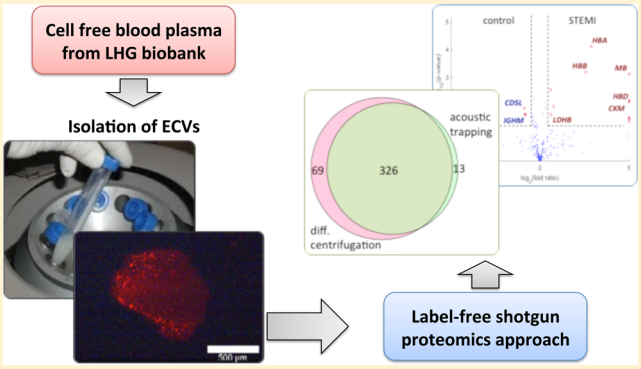
Supporting Information

ABSTRACT: Extracellular vesicles (ECVs), including microparticles and exosomes, are submicrometer membrane vesicles released by diverse cell types upon activation or stress. Circulating ECVs are potential reservoirs of disease biomarkers, and the complexity of these vesicles is significantly lower compared to their source, blood plasma, which makes ECV-based biomarker studies more promising. Proteomic profiling of ECVs is important not only to discover new diagnostic or prognostic markers but also to understand their roles in biological function. In the current study, we investigated the protein composition of plasma-derived ECVs isolated by acoustic seed trapping. Additionally, the protein composition of ECVs isolated with acoustic trapping was compared to that isolated with a conventional differential centrifugation protocol. Finally, the proteome of ECVs originating from ST-elevation myocardial infarction patients was compared with that of healthy controls using label-free LC−MS quantification. The acoustic trapping platform allows rapid and automated preparation of ECVs from small sample volumes, which are therefore well-suited for biobank repositories. We found that the protein composition of trapped ECVs is very similar to that isolated by the conventional differential centrifugation method.



Extracellular vesicles (ECVs), including microparticles (MPs) and exosomes, are submicrometer membrane vesicles released by diverse cell types upon activation or stress. On the basis of the growing number of publications during the last two decades, the investigation of ECVs is increasingly gaining attention in the life sciences.

Circulating ECVs are potential reservoirs of disease biomarkers, and the complexity of these vesicles is significantly lower compared to their source, blood plasma, which makes ECV-based biomarker studies a promising prospect.[1−3] Proteomic profiling of ECVs is important not only to discover new diagnostic or prognostic markers but also to investigate their roles in diverse biological processes.

Elevated levels of ECVs have been associated with various forms of cardiovascular disease.[4,5] One example is the submicrometer vesicles shed by platelets upon activation, which have been shown to be both procoagulant[6] and proinflammatory[7] and reflect the level of general platelet activation in patients with myocardial infarction.[8] Another example is endothelial cell-derived ECVs, which are released from the vasculature as a response to inflammation or injury and have, for example, shown potential as biomarkers for atherosclerosis.[9]

High-resolution liquid chromatography coupled to high-resolution mass spectrometry together with data processing software and search algorithms has become a powerful toolbox for in-depth proteomics profiling of biological specimens.[10] The common shotgun proteomics analyses of isolated plasma ECVs, depending on the applied extraction and LC−MS analysis methods, allow the identification of hundreds to a few thousand proteins.[11−16] Previous proteomics studies used various instrumentation and separation techniques prior to MS analysis, and besides the general plasma ECV population, platelet and endothelial cell-derived subfractions have also been investigated

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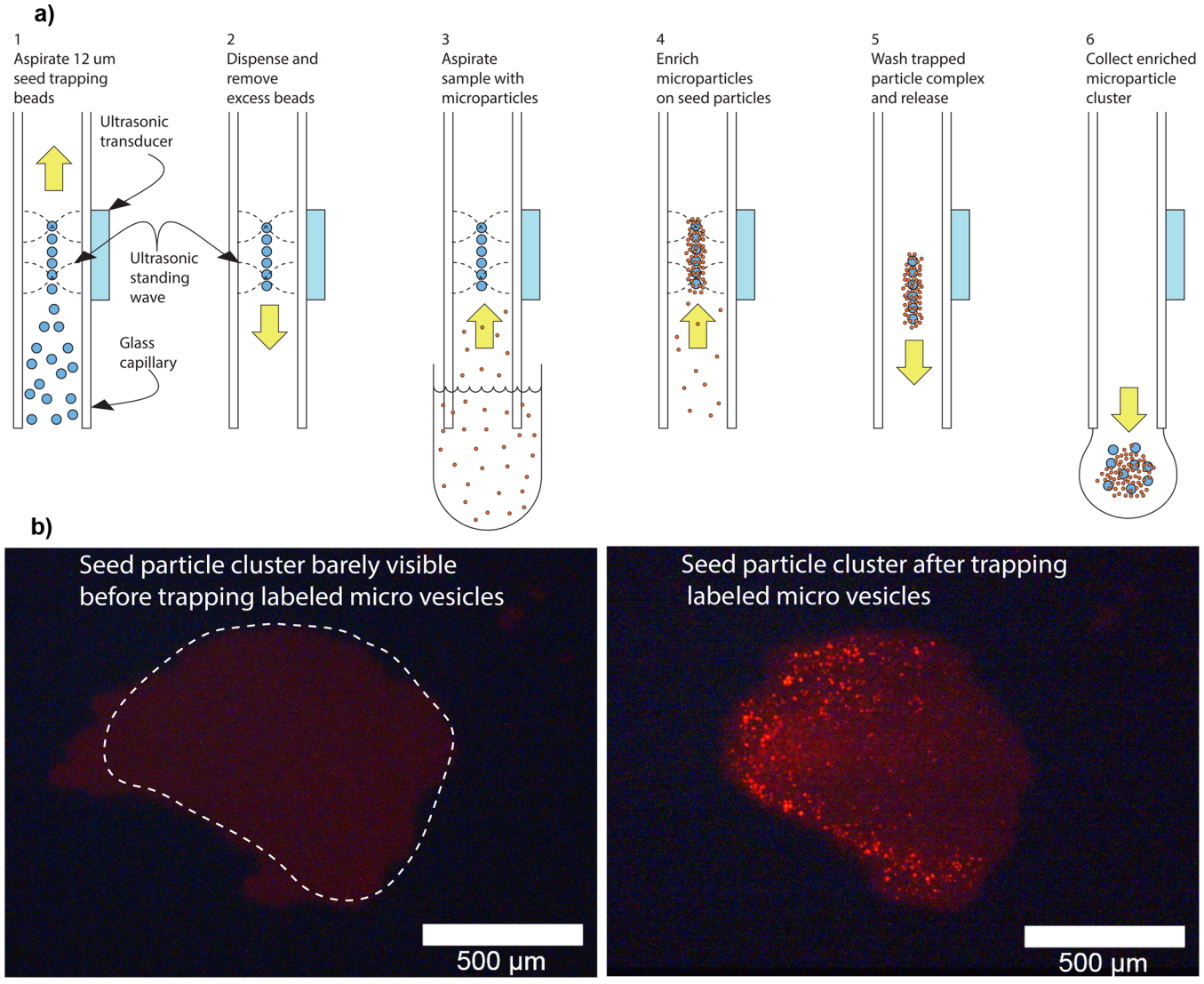

Figure 1. (a) Schematic of the capillary-based acoustic seed trapping enrichment and purification of circulating extracellular vesicles (ECVs). (1) First, 12 μm polystyrene seed particles are aspirated and retained in the acoustic trap while (2) excess particles are removed. (3) Sample containing ECVs is aspirated across the trapping zone, and (4) the ECVs are acoustically enriched on the seed particle cluster. (5) The enriched ECV/seed particle cluster is washed from plasma components and (6) released into 30 μL of PBS buffer. (b) Fluorescent image showing the seed cluster (consisting of 12 μm polystyrene particles) prior to and after trapping of ECVs stained with CD42-PE.

and compared.[11,12,17,18] LC−MS/MS analysis following SDS-PAGE separation has been performed for proteomic profiling of platelet MPs[12] and also for their comparative analysis with plasma MPs using both label-free and ICAT labeling.[14] 2D gel electrophoresis with MS/MS was used in a few studies to identify distinct protein features between MP, plasma, and platelet samples;[19] MPs originated from platelets activated with different stimulus[20] or different disease groups.[21] These studies led to the identification of hundreds of proteins altogether, involved mostly in metabolic processes, signal transduction, and communication. Furthermore, different protein signatures were identified in the various comparative studies. However, the overlap in the reported proteomics studies is quite small, which may be explained by the various ECV isolation protocols applied in the different studies and potentially with a varying degree of contamination from the highly abundant plasma proteins.

There is reason to believe that many of the methods and protocols used to isolate plasma ECVs, such as ultracentrifugation, impair the integrity of the vesicles and affect the outcome of various analyses.[22−24] We recently presented a noncontact microfluidic technique for trapping ECVs from plasma using acoustic standing waves.[25] This technique evades the need for excessive g-forces and large sample volumes associated with standard ultracentrifugation.

In the current study, we investigated the protein composition of plasma-derived ECVs isolated by either a novel microfluidic technology called acoustic seed trapping or conventional differential centrifugation. The motivation for comparing the acoustic trapping isolation method relative to that of differential centrifugation-based preparation is the ease of use, rapid and automated preparation, and most importantly the ability to address small sample volumes, which enables access to biobank repositories and hence longitudinal studies in established biobank cohorts. The proteome of ECVs originating from ST-elevation myocardial infarction (STEMI) patients was compared with healthy controls using label-free LC−MS quantification.

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MATERIALS AND METHODS

Materials. Formic acid (reagent grade ≥95%), acetone (Chromasolv for HPLC), ammonium bicarbonate (AMBIC), dithiothreitol (DTT), iodoacetamide (IAA), and RIPA buffer were purchased from Sigma-Aldrich (Steinheim, Germany); acetonitrile and water (LiChrosolv Hypergrade for LC-MS) were from Merck (Darmstadt, Germany). Sequence-grade trypsin and ProteaseMAX Surfactant were purchased from Promega (Madison, WI).

Clinical Materials. Human plasma samples were obtained from the LUNDHEARTGENE Biobank, which includes peripheral venous samples from patients admitted to the coronary care unit. Blood samples were collected 5−6 h after coronary reperfusion. All study enrollments followed the recommendations of the Declaration of Helsinki. Oral and written information was given to the patients, and confirmed consent was received in writing before inclusion.

Plasma Extracellular Vesicle Extraction. The acoustic trapping platform has previously been described in refs 26 and 27 and was recently reported for ECV trapping by Evander et al.[25] Briefly, a rectangular borosilicate capillary (2 × 0.2 mm^2 I.D.) was used as a fluidic channel, and a 4 MHz PZT transducer was attached through a thin layer of glycerol to the outside of the capillary. The transducer was used to generate a local $\lambda/2$ acoustic standing wave that creates a large pressure and velocity amplitude gradient where cells or particles are trapped noncontact in the capillary by acoustic forces. These, primary acoustic forces will have very little effect on small objects, e.g., ECVs. However, by first capturing large "seed particles" (12 μm polystyrene particles), a particle−particle force is created that will attract smaller particles to the vicinity of the seed particles. Acoustic focusing and trapping of cells over extended time periods have previously been shown not to influence cell viability or function.[28,29] Considering that ECVs have the same type of phospholipid bilayer membranes as cells, it can be anticipated that acoustic trapping does not influence membrane integrity of ECVs.

The entire enrichment sequence is illustrated in Figure 1. ECVs were trapped in an automated setup combining an acoustic trapping unit with a robotic 96-well plate (AcouTrap, AcouSort AB, Lund, Sweden). A sample volume corresponding to 30 μL of plasma (diluted 1:1 with PBS) was aspirated at 30 μL/min into the already trapped seed particle cluster. The trapped vesicles were then washed, and finally, the ultrasound was deactivated to release the cluster in 30 μL of PBS with 0.35% BSA. For comparison, a well-established differential centrifugation protocol was used.[30] The protocol consists of two centrifugation steps, 1,600*g* for 15 min followed by pelleting of ECVs at 20,000*g* for 1 h.

Protein Extraction and Digestion. RIPA buffer (100 μL) was added to the ECV samples; they were vortexed and incubated at 4 °C for 5 min. The lysates were then centrifuged at 8,000*g* for 10 min at 4 °C to pellet the debris, and the supernatants were transferred to new tubes. Protein concentration was determined by using the bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Micro BCA kit, Pierce/Thermo Scientific, Rockford, IL). Then, 200 μL of ice-cold acetone was added to an aliquot (50 μL) of ECV lysate containing 30 μg of total protein, vortexed, and incubated for 1 h at −20 °C; it was then centrifuged at 16,000*g* for 10 min at 4 °C. The supernatant was carefully removed, and the pellet was washed twice with 200 μL of cold acetone and then air-dried for 5 min at room temperature.

The protein pellet was solubilized in 20 μL of 0.2% ProteaseMAX solution for 10 min and then diluted with 75 μL of 50 mM ammonium-bicarbonate buffer pH 7.8 (AMBIC); the proteins were reduced with dithiothreitol by the addition of 1 μL of 0.5 M DTT (incubation at 56 °C for 30 min) and alkylated with iodoacetamide by the addition of 3 μL of 0.5 M IAA (incubation at room temperature for 20 min in the dark). The samples were digested by the addition of 1 μL of 1% ProteaseMAX solution and 1 μL of 1 μg/uL of sequence grade trypsin for 16 h at 37 °C. The proteolysis was terminated by the addition of 20% formic acid to a final concentration of 2%. Samples were dried using a speedvac concentrator and resuspended in 30 uL of 0.1% formic acid before the nanoLC-ESI−MS/MS analysis.

NanoLC−MS/MS Analysis. The LC−MS/MS analysis was performed on an Orbitrap Fusion mass spectrometer equipped with an Easy n-LC 1000 pump (Thermo Scientific, Waltham, MA). Two microliters of samples were injected onto an Acclaim PepMap 100 precolumn (75 μm x 2 cm, C18, 3 μm, 100 Å, Thermo Scientific, Waltham, MA), and following online desalting and concentration, the tryptic peptides were separated on an EASY-Spray column (25 cm × 75 μm ID, PepMap RSLC C18, 2 μm, 100 Å, Thermo Scientific, Waltham, MA). Separations were performed in a 90 min nonlinear gradient using 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B at a flow rate of 300 nL/min. Peptides were eluted with a linear gradient of 5 to 22% B in 65 min, followed by 22 to 32% B in 9 min and 32 to 98% B in 8 min, and finished by holding at 98% B for another 8 min. The samples were injected in random order.

Data-dependent acquisition (DDA) was used running the Orbitrap Fusion Instrument Control Software v1.2. Full MS scans were acquired in the Orbitrap mass analyzer over the m/z range of 400−1500 with resolution of 120,000 (at m/z 200), target AGC value of 4e5, and maximum injection time of 50 ms. Universal method was used, where precursors with charge state ≥2 were selected for a maximum 3 s cycle (3 s Top Speed mode). Precursor ions were filtered using a 45 s dynamic exclusion window and an intensity threshold of 5000. CID fragmentation was used with normalized collision energy of 35%, and tandem mass spectra were acquired in the Iontrap mass analyzer with rapid scan rate and using quad isolation with 1.6 m/z window. AGC target and maximum injection time were set to 1e2 and 300 ms, respectively, and all available parallelizable time was enabled.

Data Analysis. MS/MS spectra were searched using SEQUEST HT search engine integrated into Proteome Discoverer software v1.4 (Thermo Scientific, Waltham, MA). UniProt Human database was used (June 2015 release including 20164 sequences) with tryptic specificity allowing for up to two missed cleavage sites. Ten parts per million precursor tolerance and 0.6 Da fragment tolerance were used. Oxidation (M) was treated as variable, and carbamidomethylation (C) was treated as a fixed modification. Search results were filtered by using 1% FDR.

Label-free quantification (LFQ) was performed with MaxQuant software v1.5.2.8.[31] Andromeda search engine integrated into MaxQuant software was used for MS/MS spectra search. UniProt Human database was used (June 2015 release including 20164 sequences) with tryptic specificity allowing for up to two missed cleavages. Ten parts per million

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precursor tolerance and 0.5 Da fragment tolerance were used. Oxidation (M) and N-terminal acetylation were treated as variable, and carbamidomethylation (C) was treated as a fixed modification. Search results were filtered by using 1% FDR, and at least two peptides (unique + razor) were recommended for each protein.

Protein annotation and enrichment analysis was performed using Panther Classification System (http://pantherdb.org).[32,33] The DAVID system was also used for assessment of statistical significance of the lists of detected proteins.[34] Further statistical analysis was done using Matlab v7.11 (Mathworks, Natick, MA). Two-sample *t* test was performed to determine the significant differences in protein LFQ intensity data considering only those proteins that were quantified in at least 3 samples in at least one group. A p-value < 0.05 was considered significant. For analysis of differentially regulated proteins, QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) was used to generate relationship networks and perform functional analyses.

3. RESULTS AND DISCUSSION

We performed the proteomics analysis of plasma-derived ECVs from 20 subjects, of which 10 were ST-elevation myocardial infarction (STEMI) patients and 10 were age- and gender-matched controls. ECVs were extracted from cell-free plasma with either acoustic trapping or differential centrifugation.

Protein Identification and Annotation. In total, 1014 protein groups (1325 merged proteins) were identified with high confidence using SEQUEST HT. Of these, 408 protein groups (550 merged proteins) were identified with at least 2 peptides (Supplementary Table 1). To define the "core" protein composition of our plasma-derived ECVs, we further tightened the filtering parameters considering only those proteins that were found in at least half of the samples in each group. We identified 251 and 280 protein groups in centrifuged control and STEMI samples, and 242 and 250 protein groups were identified in trapped control and patient samples, respectively. Found in all groups were 75% (226) of these proteins (Figure 2). The core proteins are listed in Supplementary Table 2.

Functional classification of the ECV proteins was also performed by assigning biological processes, molecular functions, and cellular localization to the protein identifications (Figure 3). Within the category of biological processes, the largest number of proteins are involved in metabolic (GO:0008152) and cellular processes (GO:0009987) (Figure 3A). Fewer but still a considerable number of proteins are linked to processes such as biological regulation (GO:0065007), immune system processes (GO:0002376), localization (GO:0051179), developmental processes (GO:00032502), and response to stimulus (GO:0050896). Proteins involved in biological adhesion (GO:0022610), cellular component organization or biogenesis (GO:0071840), locomotion (GO:0040011), reproduction (GO:0000003), apoptotic (GO:0006915), and multicellular organismal (GO:00032501) processes were also identified in the ECV proteome. On the basis of the molecular function classification, large portions of the identified proteins have catalytic (27%), enzyme regulator (13%), receptor (13%), structural molecule (13%), or transporter (7%) activity or are involved in binding (mostly protein, calcium ion, calcium-dependent phospholipid, and nucleic acid binding) (25%)

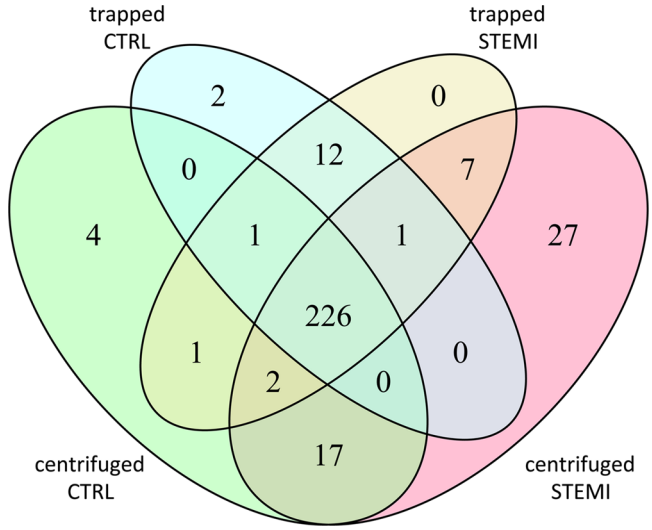

Figure 2. Venn diagram of the "core" proteins detected in the four different ECV sample groups. Core proteins were detected in at least 5 samples (in 50% of the samples) from a particular group.

(Figure 3B). Proteins with nucleic acid binding transcription factor, protein binding transcription factor, antioxidant, and translation regulator activity were also found but to a lower extent (Figure 3B). More than two-thirds of the identified proteins were derived from extracellular regions, cell parts, and organelles (Figure 3C), and the rest of the proteins originated from extracellular matrix, macromolecular complexes, membranes, cell junctions, or synapses.

The functional classification of the different protein lists (total vs "core" protein lists) with regards to biological processes and molecular functions shows a very similar picture, i.e., the distribution of the different subgroups is very much alike (Figure 3A and B, Supplementary Figure 1). In contrast, the cellular localization of the "core" proteins differs from the localization of all identified proteins. Nearly half of the "core" proteins are linked to the extracellular region (46%), whereas the corresponding number is only 29% with respect to the full protein list (Figure 3C, Supplementary Figure 1).

By comparing our protein list with lists generated by other groups earlier, very few common proteins could be found. The greatest overlap (35%) was obtained in comparison with the data reported by Geiger's group in 2015.[15] Out of the reported 1325 proteins, 864 have not been identified previously as plasma-derived ECV proteins. The most plausible explanation of the very small overlap between the different studies is the lack of a general sample preparation protocol.[23,30] A large number of protocols exist in the literature with slightly different centrifugation and blood handling conditions, and as has been demonstrated previously,[13,23] these parameters can have a significant effect on the analysis results.

Previous publications showed that most of the ECV proteins are involved in metabolic and cellular processes (~40% altogether), which agree well with our findings. Concerning the molecular functions, proteins with catalytic activity and proteins involved in binding constitute the largest subgroups in all ECV studies.[11,12,14,15] In our data set, proteins with receptor and enzyme regulator activity are slightly overrepresented, whereas proteins with structural molecule activity are underrepresented in comparison with former publications (Figure 3B, Supplementary Figure 1). By comparing our full protein list

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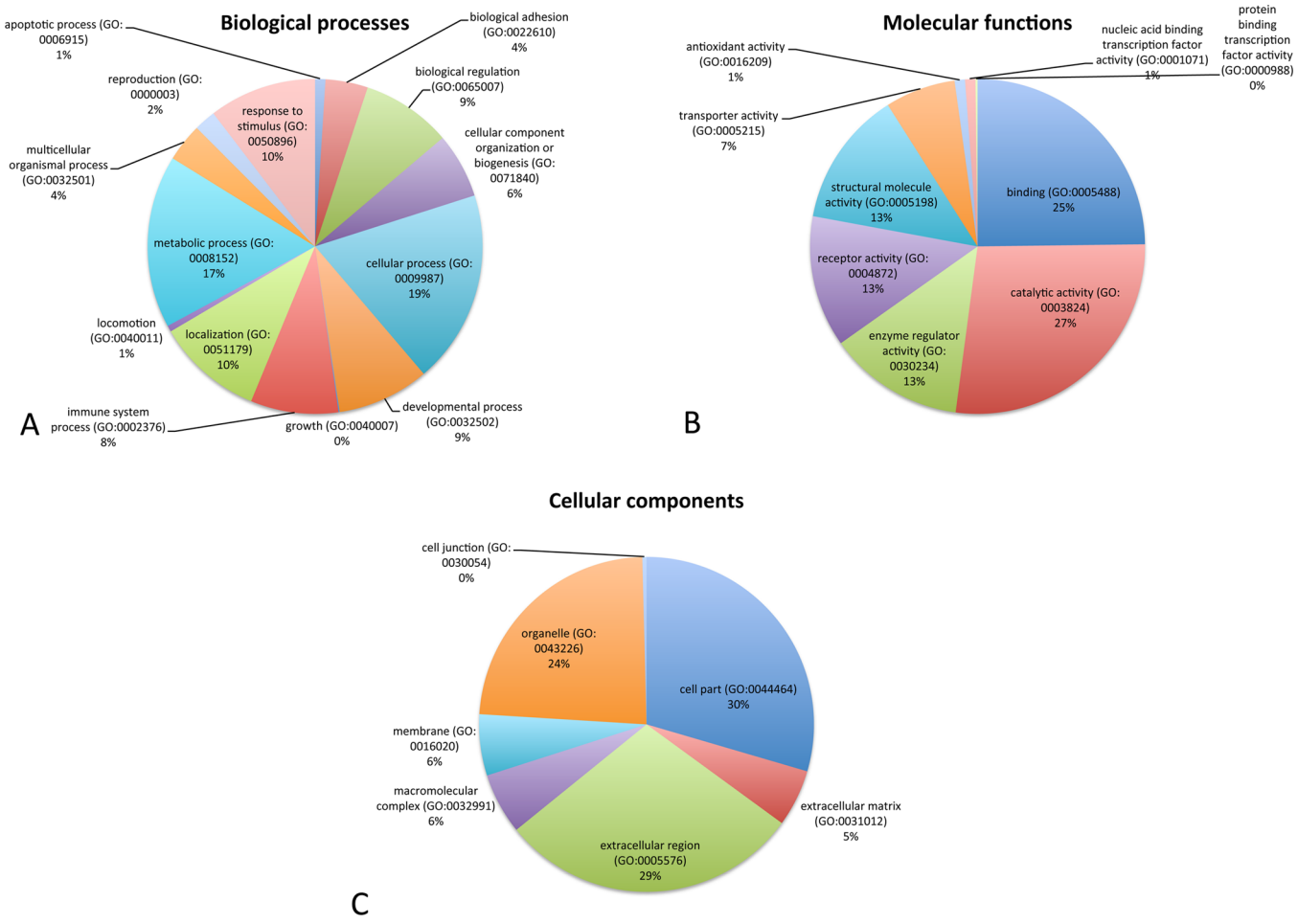

Figure 3. Functional classification of the proteins identified with at least two peptides. Pie charts represent the assigned biological processes (A), molecular functions (B), and cellular localizations (C) of the protein hits.

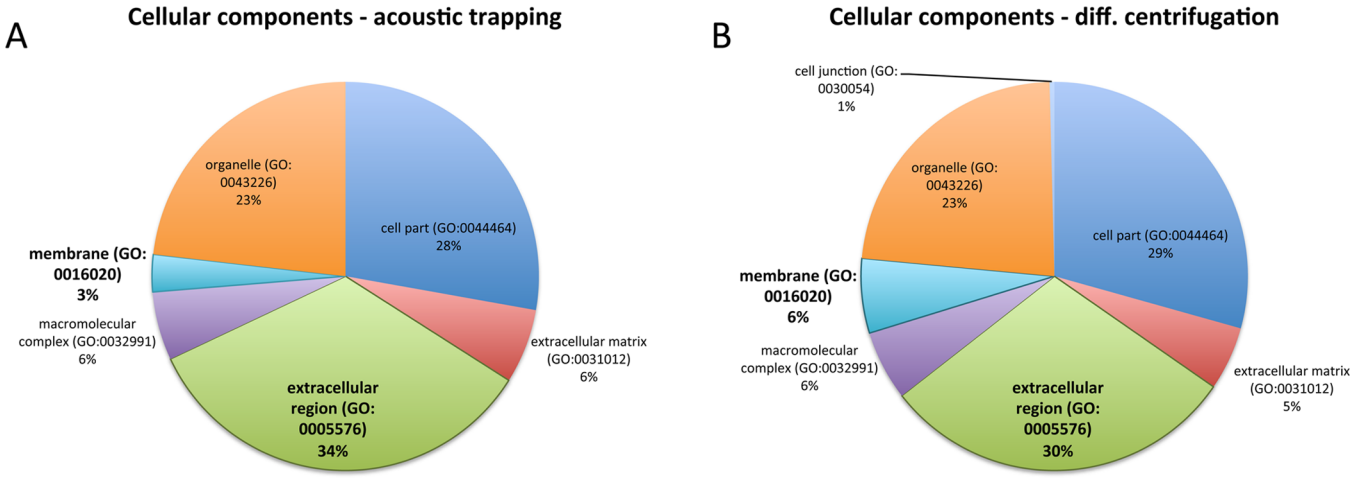

Figure 4. Comparison of the cellular localization of proteins originating from ECV samples prepared with acoustic trapping (A) or differential centrifugation (B).

with earlier MS studies, we obtained very similar arrangement with respect to cellular localization. Previous publications showed that roughly 70% of the ECV proteins correspond to cell parts and organelles, which is close to our findings (54%) (Figure 3C); however, the extracellular region is fairly dominant in our data set, and this is even more prominent when only "core" proteins are considered (Supplementary Figure 1).

The DAVID system provided statistical value to functional annotations overrepresented in the set of proteins confidently detected in extracellular vesicles compared with those of the entire human proteome. Quite expectedly, among cellular component Gene Ontology terms, annotations such as

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Table 1. List of Differentially Expressed Proteins in ECV Samples Isolated by Acoustic Trapping or Differential Centrifugation

	protein ID	gene name	protein name	*p*-value	$\log_2$ fold ratio (trapping/diff. centrifugation)
			↑ Differential Centrifugation		
1	P02730	*SLC4A1*	Band 3 anion transport protein	2.28×10^{-03}	−5.00
2	P04406	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	1.73×10^{-02}	−5.00
3	P05556	*ITGB1*	Integrin beta-1	1.11×10^{-02}	−5.00
4	P16671	*CD36*	Platelet glycoprotein 4	3.72×10^{-02}	−5.00
5	P23229	*ITGA6*	Integrin alpha-6	7.64×10^{-03}	−5.00
6	P62937	*PPIA*	Peptidyl-prolyl cis−trans isomerase A	4.47×10^{-03}	−5.00
7	P68366	*TUBA4A*	Tubulin alpha-4A chain	4.88×10^{-02}	−5.00
8	Q15404	*RSU1*	Ras suppressor protein 1	1.68×10^{-02}	−5.00
9	Q15942	*ZYX*	Zyxin	2.86×10^{-02}	−5.00
10	Q31610	*HLA-B*	HLA class I histocompatibility antigen, B-81 alpha chain	8.85×10^{-04}	−5.00
11	Q6ZR08	*DNAH12*	Dynein heavy chain 12, axonemal	1.99×10^{-04}	−5.00
12	Q8NAA6	*C15orf53*	uncharacterized protein C15orf53	2.94×10^{-02}	−5.00
13	Q9HBI1	*PARVB*	beta-Parvin	4.43×10^{-02}	−5.00
14	P01598	N/A	Ig kappa chain V−I region EU	3.37×10^{-05}	−2.96
15	P60709	*ACTB*	Actin, cytoplasmic 1	3.40×10^{-04}	−2.08
16	P02655	*APOC2*	Apolipoprotein C−II	5.64×10^{-05}	−1.54
17	P07359	*GP1BA*	Platelet glycoprotein Ib alpha chain	3.07×10^{-02}	−1.40
18	P04275	*VWF*	von Willebrand factor	8.22×10^{-05}	−1.22
19	P02649	*APOE*	Apolipoprotein E	2.13×10^{-05}	−1.11
20	P12259	*F5*	Coagulation factor V	9.62×10^{-04}	−0.99
21	P04114	*APOB*	Apolipoprotein B-100	3.15×10^{-04}	−0.92
22	P06732	*CKM*	Creatine kinase M-type	2.00×10^{-02}	−0.88
23	P00488	*F13A1*	Coagulation factor XIII A chain	6.15×10^{-05}	−0.82
24	P02656	*APOC3*	Apolipoprotein C−III	1.86×10^{-03}	−0.81
25	Q9Y6R7	*FCGBP*	IgGFc-binding protein	4.54×10^{-02}	−0.77
26	P05154	*SERPINA5*	Plasma serine protease inhibitor	2.27×10^{-04}	−0.56
27	P02654	*APOC1*	Apolipoprotein C−I	2.32×10^{-04}	−0.56
28	P18428	*LBP*	Lipopolysaccharide-binding protein	7.83×10^{-03}	−0.55
			↑ Acoustic Trapping		
29	P01620	N/A	Ig kappa chain V−III region SIE	2.95×10^{-04}	0.51
30	P25311	*AZGP1*	Zinc-alpha-2-glycoprotein	7.68×10^{-08}	0.64
31	P01766	N/A	Ig heavy chain V−III region BRO	3.93×10^{-03}	0.64
32	P02766	*TTR*	Transthyretin	3.62×10^{-02}	0.85
33	P01625	N/A	Ig kappa chain V−IV region Len	2.74×10^{-06}	0.85
34	P01624	N/A	Ig kappa chain V−III region POM	1.30×10^{-02}	5.00
35	P01743	N/A	Ig heavy chain V−I region HG3	1.33×10^{-04}	5.00
36	P06311	N/A	Ig kappa chain V−III region IARC/BL41	8.92×10^{-07}	5.00
37	Q8WZ42	*TTN*	Titin	1.86×10^{-02}	5.00
38	Q9NQ79	*CRTAC1*	Cartilage acidic protein 1	1.20×10^{-06}	5.00

extracellular region (202 proteins, *p*-value = 1.5×10^{-80}), membrane-bound vesicle (55, 3×10^{-17}), cytoskeleton (80, 3.7×10^{-12}), and high-density lipoprotein particle (19, 6.3×10^{-24}) were visible. Among molecular function Gene Ontology terms, annotations such as peptidase inhibitor activity (35 proteins, *p*-value = 1.3×10^{-24}), peptidase activity (35, 2.1×10^{-7}), and polysaccharide binding (19, 1×10^{-8}) were most striking. Lastly, among biological process Gene Ontology terms, annotations such as blood coagulation (36 proteins, *p*-value = 1×10^{-32}), inflammatory response (80, 1.6×10^{-35}), cell adhesion (37, 2×10^{-6}), and lipid transport (22, 7.5×10^{-12}) led the overrepresented annotation list. Very interestingly, our set of ECV proteins from healthy donors and STEMI patients was, according to DAVID analysis, significantly enriched in proteins from BIOCARTA's "Acute Myocardial Infarction" pathway (8 proteins, *p*-value = 1.8×10^{-6}, 11-fold over-representation). This corroborates the fact that protein content of ECVs appears to be a useful source of biological information linked to myocardial infarction.

Comparison of the Different ECV Extraction Techniques: Acoustic Trapping vs Differential Centrifugation. The total number of identified proteins with at least 2 peptides was higher in ECVs prepared with centrifugation (395 protein groups) compared to that of trapped samples (339 protein groups). Thirteen proteins were detected only in trapped samples; 69 were found only in samples prepared by differential centrifugation, and 80% (326) of the proteins were detected with both types of preparation. Membrane-associated proteins are overrepresented in centrifuged ECV samples relative to trapped samples (6 vs 3%), and the proportion of extracellular proteins is a little higher in trapped samples compared to that in centrifuged samples (34 vs 30%) (Figure 4). With respect to biological processes and molecular functions, there was no difference between the ECV preparation methods (Supplementary Figure 2).

LFQ intensities that reflect the relative amounts of the proteins across the samples were determined with the help of MaxQuant software; in total, 416 protein groups were

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quantified in the 40 samples. The samples were divided into two groups based on ECV extraction technique and regardless of the disease category, and the two groups were subsequently pairwise compared. When comparing the protein quantities, the two extraction methods show high similarities (Supplementary Figure 3). The average LFQ intensities measured in the two sample groups are highly correlated (r = 0.99). Altogether, 38 proteins significantly differing in abundance were found by comparing the two ECV extraction methods, from which 28 were enriched in the centrifuged and 10 in the trapped samples (Table 1).

Considering the proteins enriched in centrifuged samples (vs trapping), 13 out of the 28 were not at all detected in trapped samples. Among the most significant hits, we can find several apolipoproteins (*APOB*, *APOC1*, *APOC2*, *APOC3*, and *APOE*), complement factors (*F5* and *F13A1*), and von Willebrand factor. In contrast, among the proteins whose amount was higher in trapped ECV samples (vs diff. centrifugation), numerous immunoglobulins can be found besides zinc-alpha-2-glycoprotein or transthyretin.

With both preparation methods, we isolated diverse populations of extracellular vesicles from plasma. Additionally, the protein composition of the different types of ECVs is still incomplete; therefore, the evaluation of the contaminants in such a heterogeneous matrix is challenging. However, it has been reported that lipoprotein particles, in particular HDL[35,36] and just recently LDL,[37] are probably copurified with ECVs by various isolation techniques, even with density gradient centrifugation, and therefore appear as contaminants.

As we demonstrated, the two extraction methods resulted in highly similar protein profiles; however, several lipoproteins were more abundant in centrifuged than in trapped samples. It is a known phenomenon that lipid particles display a negative acoustic contrast factor in aqueous fluids,[38] i.e., the acoustic force drives these to the acoustic standing wave antinodes, thereby not coming into the vicinity of the seed particles, and become subject to enrichment. This is most likely true for lipoproteins as well, which could explain why the use of acoustic trapping results in less contaminant.

Differential Proteomics Analysis of STEMI and CTRL Plasma ECVs. It is known from previous publications that the level of circulating ECVs is increased in cardiac patients.[39−41] In accordance with these observations, we obtained the same results in our previous study by using the acoustic trapping platform.[25] In the present work, only the protein content of the isolated ECVs was investigated, and we did not find a significant difference in the total protein content between the subject groups using either preparation method. However, regardless of the ECV extraction method, a higher number of proteins were found and quantified in STEMI samples than in controls. Altogether, 257 proteins were common in both subject groups; 138 were found only in patient samples, and 21 were found only in control samples (Figure 5).

As the analyses of the centrifuged and trapped samples resulted in different protein lists, we decided to compare the two subject groups separately according to the preparation methods. We obtained two different significant protein lists by handling the centrifuged and trapped samples separately (Table 2); out of the 19 proteins that were overexpressed in STEMI samples, six (*HBA*, *HBB*, *HBD*, *MB*, *LDHB*, and *CKM*) were found to be significant in both sample types, and two proteins (*CD5L* and *IGHM*) were common from the five that were enriched in control samples (Figure 6).

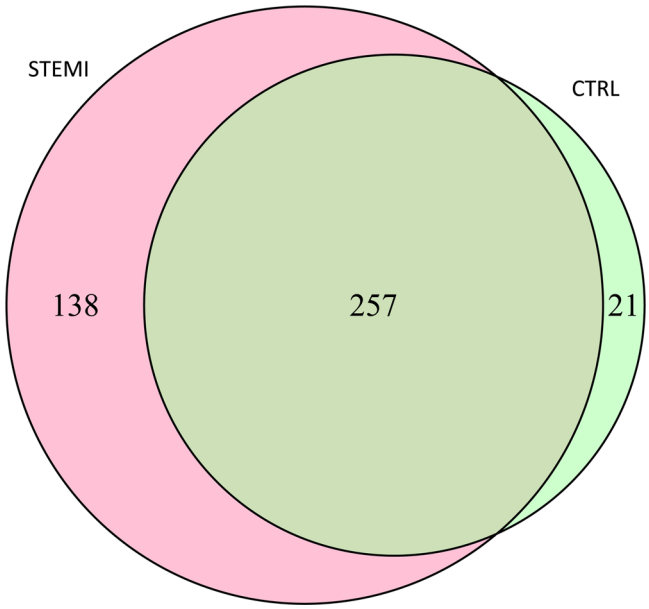

Figure 5. Comparison of the number of proteins identified and quantified in STEMI and control ECV samples.

The 24 proteins that were differentially regulated between STEMI and control samples (see Table 2) were analyzed in Ingenuity Pathways Analysis and found to be tightly linked by biological relationships, mostly protein−protein interactions (see Figure 7). The main hub of this small protein set is fibronectin (*FN1*). Fibronectin is an extracellular matrix protein with various functions in wound healing, embryogenesis, and cell adhesion but also plays an important role in platelet thrombus formation and in cardiac repair after myocardial infarction.[42]

Most of the 24 proteins are already linked to CV diseases and/or are biomarkers of those diseases. Among them, there is myoglobin, a biomarker of myocardial ischemia,[43] actin and zyxin, which were noted as upregulated in acute coronary syndrome (ACS),[44] and IGHM protein, which is involved in myocarditis.[45] Creatine kinase M-type, which is an established biomarker for myocardial infarction, was increased in the ECV fraction in STEMI patients regardless of isolation method. Its presence in ECVs has to our knowledge not been shown before and might represent a population of cardiac-derived ECVs present in circulation as a result of cardiac injury. The presence of hemoglobin subunits in the ECV fraction is indicative of erythrocyte microparticles, which are laden with heme.[46] Consistent with our finding, it has been shown that growing thrombi release erythrocyte microparticles and that these ECVs are elevated in the circulation of STEMI patients.[47] Platelet basic protein (or CXCL7) was found to be increased in STEMI ECVs isolated by acoustic trapping. It is a marker of platelet activation and has previously been shown to be present in platelet microparticles.[12] The fact that it was detected in ECVs isolated by acoustic trapping but not in differential centrifugation suggests an enrichment of this specific type of ECV by acoustic trapping.

CONCLUSIONS

We have used an nLC−MS/MS method to examine the protein composition of plasma-derived extracellular vesicles (ECVs) isolated by acoustic trapping and by conventional differential centrifugation. The proteomic profile of differently isolated

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Table 2. List of Differentially Expressed Proteins in ECV Samples from STEMI Patients and Controls

			diff. centrifugation		acoustic trapping	
protein ID	gene name	protein name	*p*-value	$\log_2$ fold ratio (STEMI/CTRL)	*p*-value	$\log_2$ fold ratio (STEMI/CTRL)
			↑ STEMI			
O75636	*FCN3*	Ficolin-3	2.71×10^{-03}	0.59		
P07195	***LDHB***	**L-Lactate dehydrogenase B chain**	$\mathbf{2.17 \times 10^{-02}}$	**0.62**	$\mathbf{1.94 \times 10^{-02}}$	**5.00**
P01701	N/A	Ig lambda chain V−I region NEW	1.11×10^{-02}	0.76		
P01876	N/A	Ig alpha-1 chain C region			4.61×10^{-02}	0.80
P06889	N/A	Ig lambda chain V−IV region MOL			4.82×10^{-02}	0.83
P60709	*ACTB*	Actin, cytoplasmic 1			3.12×10^{-02}	1.38
P02775	*PPBP*	Platelet basic protein			2.88×10^{-02}	2.18
P68871	***HBB***	**Hemoglobin subunit beta**	$\mathbf{6.38 \times 10^{-04}}$	**2.56**	$\mathbf{3.75 \times 10^{-04}}$	**3.11**
P69905	***HBA1/HBA2***	**Hemoglobin subunit alpha**	$\mathbf{7.71 \times 10^{-05}}$	**2.89**	$\mathbf{3.43 \times 10^{-04}}$	**3.31**
P01719	N/A	Ig lambda chain V−V region DEL	3.80×10^{-02}	5.00		
P02042	***HBD***	**Hemoglobin subunit delta**	$\mathbf{7.30 \times 10^{-03}}$	**5.00**	$\mathbf{7.51 \times 10^{-04}}$	**5.00**
P02144	***MB***	**Myoglobin**	$\mathbf{7.77 \times 10^{-04}}$	**5.00**	$\mathbf{3.72 \times 10^{-05}}$	**5.00**
P06732	***CKM***	**Creatine kinase M-type**	$\mathbf{6.90 \times 10^{-03}}$	**5.00**	$\mathbf{8.14 \times 10^{-04}}$	**5.00**
Q15942	*ZYX*	Zyxin	2.86×10^{-02}	5.00		
Q8NAA6	*C15orf53*	uncharacterized protein C15orf53	2.94×10^{-02}	5.00		
P14151	*SELL*	L-Selectin			1.38×10^{-02}	5.00
P14618	*PKM*	Pyruvate kinase PKM			2.63×10^{-03}	5.00
P37802	*TAGLN2*	Transgelin-2			8.94×10^{-03}	5.00
P63104	*YWHAZ*	14-3-3 protein zeta/delta			4.60×10^{-02}	5.00
			↑ CTRL			
P02751	*FN1*	Fibronectin			5.59×10^{-04}	−0.97
P01871	***IGHM***	**Ig mu chain C region**	$\mathbf{1.28 \times 10^{-02}}$	**−0.87**	$\mathbf{2.31 \times 10^{-02}}$	**−0.76**
Q96KN2	*CNDP1*	Beta-Ala-His dipeptidase	2.11×10^{-02}	−0.84		
O43866	***CD5L***	**CD5 antigen-like**	$\mathbf{2.19 \times 10^{-02}}$	**−0.81**	$\mathbf{1.39 \times 10^{-02}}$	**−0.79**
Q16610	*ECM1*	Extracellular matrix protein 1			2.86×10^{-03}	−0.54

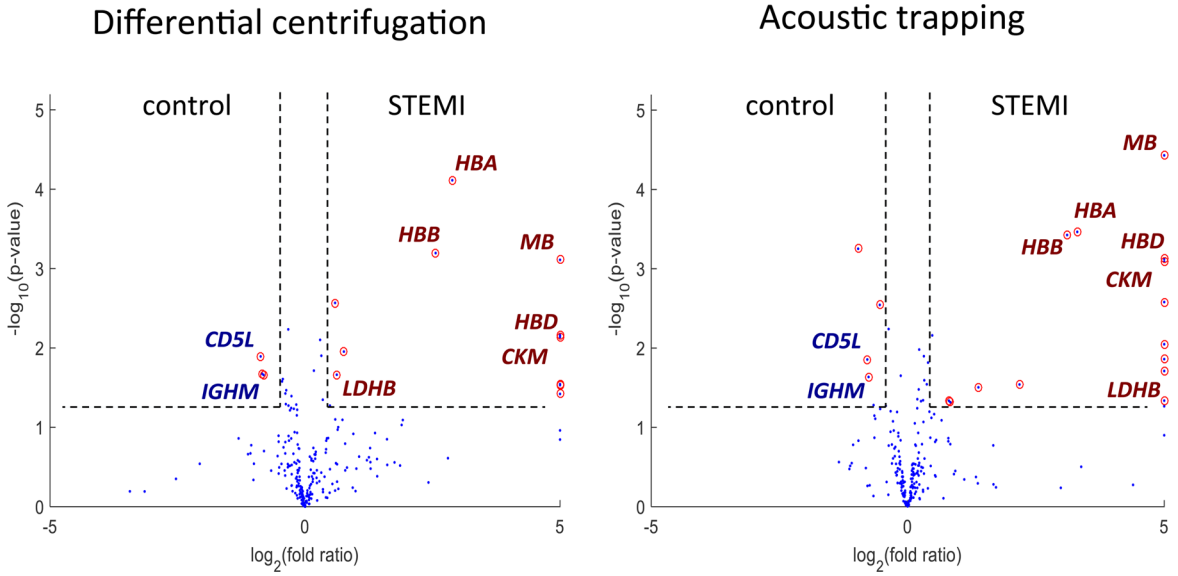

Figure 6. Protein $\log_2$ fold changes (on the *x* axis) and the corresponding adjusted $-\log_{10}$ *p*-values (on the *y* axis) are summarized in volcano plots for ECV samples isolated by acoustic trapping or differential centrifugation. The dots marked in red represent a *p*-value lower than 0.05 and an absolute $\log_2$ fold change higher than 0.5.

ECV samples showed great similarity, i.e., 80% of the identified proteins were found in both sample types. ECV samples originating from ST-elevation myocardial infarction (STEMI) patients and healthy controls were also compared using a label-free LC−MS quantification approach. Twenty-four proteins were identified as differentially regulated between STEMI and control samples, many of which are already linked to CV diseases, such as myoglobin, platelet basic protein, and creatine kinase M-type. Of these, eight proteins were found to be significant with both ECV isolation techniques. In conclusion, the present study demonstrated that acoustic trapping is suitable to isolate ECVs from small plasma volumes in a rapid

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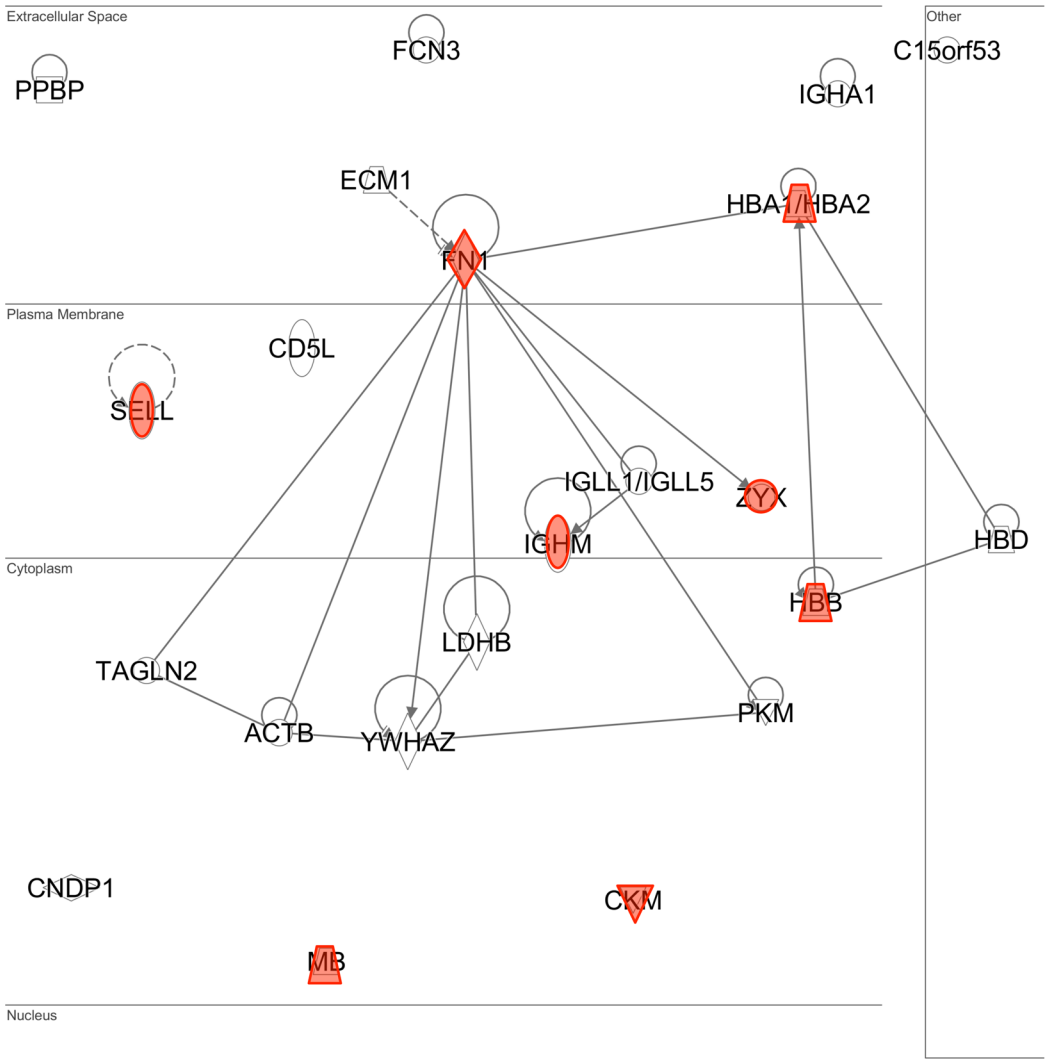

Figure 7. Ingenuity network showing functional relationships between the 24 proteins differentially regulated between STEMI and control samples. Proteins marked in red are implicated in cardiovascular diseases.

and automated way and, most importantly, that the protein composition of trapped ECVs is very similar to those that were isolated by the conventional differential centrifugation method.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b01694.

Functional classification of the ECV "core" proteins, comparison of the different ECV isolation techniques with regards to functional annotation of the identified proteins and protein LFQ intensities, and list of identified ECV proteins (PDF)

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Notes

The authors declare no competing financial interest.

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