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Proteomic and Biochemical Analysis of Extracellular Vesicles Isolated from Blood Serum of Patients with Melanoma

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Abstract: Background: Malignant melanoma is the most serious type of skin cancer with the highest mortality rate. Extracellular vesicles (EVs) have potential as new tumor markers that could be used as diagnostic and prognostic markers for early detection of melanoma. Methods: EVs were purified from the blood serum of melanoma patients using two methods—ultracentrifugation and PEG precipitation—and analyzed by mass spectrometry and immunoblot. Results: We identified a total of 585 unique proteins; 334 proteins were detected in PEG-precipitated samples and 515 in UC-purified EVs. EVs purified from patients varied in their size and concentration in different individuals. EVs obtained from stage II and III patients were, on average, smaller and more abundant than others. Detailed analysis of three potential biomarkers—SERPINA3, LGALS3BP, and gelsolin—revealed that the expression of SERPINA3 and LGALS3BP was higher in melanoma patients than healthy controls, while gelsolin exhibited higher expression in healthy controls. Conclusion: We suggest that all three proteins might have potential to be used as biomarkers, but a number of issues, such as purification of EVs, standardization, and validation of methods suitable for everyday clinical settings, still need to be addressed.

Keywords: melanoma; extracellular vesicles; purification; ultracentrifugation; PEG precipitation

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1. Introduction

Malignant melanoma is a malignancy of pigment-producing cells called melanocytes, which are located primarily in the skin. Despite being the least common form of skin cancer, it has one of the highest mortality rates and the worldwide incidence of cutaneous melanoma has been increasing annually at a more rapid rate compared to any other type of cancer [1,2]. One possibility for an early detection of melanoma is liquid biopsy. Liquid biopsy is the analysis of tumor-derived biomarkers from peripheral blood such as circulating tumor cells, circulating tumor DNA and extracellular vesicles, and their genomic or proteomic assessment [3]. Tumor markers, molecules that indicate the presence of cancer or provide information about the likely future behavior of cancer, are playing an increasingly important role in cancer detection and management [4–6].

Extracellular vesicles (EVs) are membrane vesicles released into the extracellular environment by different cells. They represent an important mode of intercellular communication by serving as transfer vehicles between different cells carrying proteins, lipids, and RNA [7]. EVs have been identified in a diverse range of human biofluids including serum, plasma, urine, saliva, breast milk, amniotic fluid, ascites fluid, cerebrospinal fluid, and bile, and as such have potential to be used as liquid biopsies [7–9]. EVs can be broadly divided into three main types: exosomes, microvesicles, and apoptotic bodies. 'Exosome' is used for 40–100 nm vesicles released as a consequence of multivesicular endosome fusion with the plasma membrane, while 'microvesicle' refers to EVs that are shed from the plasma membrane [7,10,11]. Microvesicles are generally larger, up to ~1000 nm in diameter, but

small vesicles of 100 nm may also bud from the cell surface [12]. Apoptotic bodies are formed when cells undergo programmed cell death. Large oncosomes represent an additional class of tumor-derived EVs atypically with a large size and abundant oncogenic cargo. Their formation is more evident in highly migratory aggressive tumor cells and they are specifically released by tumor cells, whereas their detection in benign systems is negligible [13,14].

Serum and plasma are attractive sources of EV-based biomarkers, as blood sample acquisition is a minimally invasive procedure and tumor cells release circulating EVs into the bloodstream [15]. However, isolation and purification of EVs from serum is complicated due to high serum viscosity, high abundance of serum proteins, and non-EV lipid particles. The choice of EV isolation procedure significantly impacts EV yield from human serum, together with the presence of lipoproteins and protein contaminants [9]. Ultracentrifugation (UC) is a classical method for EV purification that yields a high amount of EVs. UC uses centrifugal force to separate and purify EVs by high centrifugal speed. Another centrifugation step can be added to further purify EVs from non-EV particles, but this results in reduced particle yield due to loss and damaged EVs. The problems with UC are a high work load and the need for specialized equipment and personnel. As such, it is not a high throughput method that could be easily used in an everyday clinical laboratory setting. On the other hand, polymer-based precipitation, for example, with polyethylene glycol (PEG), is a robust and high throughput method and as such could theoretically be used in an everyday clinical setting. This method is based on using volume-excluding polymers that reduce the solubility of EVs and other similarly sized proteins and particles. After precipitation, EVs are isolated using low speed centrifugation. The main problems arise from a large amount of co-precipitated proteins that could "hide" the EV yield [9].

The aim of the current study is to investigate the potential of EVs to serve as diagnostic and prognostic markers for early detection of melanoma. For this, EVs from melanoma patients in different stages of disease were purified from blood serum with two different methods and protein content was determined using proteomics. Three possible biomarkers purified with UC were analyzed further to determine their possible diagnostic potential.

2. Materials and Methods

2.1. Patients

Human sera were collected from 48 patients with melanoma attending the North Estonian Medical Centre (Tallinn, Estonia). Six sera were obtained from healthy donors from the Estonian Blood Bank. All these sera were previously used for studying of antibodies against MAGEA proteins [16]. All samples were handled by standard procedures and stored at −80 °C. Approval for the use of blood samples for the study was obtained from the Tallinn Medical Research Ethics Committee (Tallinn, Estonia).

2.2. Isolation and Purification of Extracellular Vesicles

Isolation and purification of extracellular vesicles was done with two methods: ultracentrifugation (UC), and precipitation with 10% of polyethylene glycol (PEG).

Ultracentrifugation (UC): Up to a 100 µL of each blood serum sample was diluted to a total volume of 1 mL with PBS (Phosphate Buffered Saline, pH = 7.4) and samples were centrifuged at 1200× *g* for 30 min at 4 °C (Pico 21, Thermo Scientific) to remove cells and debris. Then, 3 mL of PBS was added to the supernatant and it was further centrifuged at 120,000× *g* for 90 min at 4 °C using the Optima™ L-90K Ultracentrifuge with rotor SW55Ti. The EVs were resuspended in 300 µL of Dulbecco's PBS (DPBS) (Sigma-Aldrich, St. Louis, MO, USA). The EV sample concentrations were measured with the Bradford Protein Assay (Bio-Rad Laboratories; Hercules, CA, USA) using BSA (bovine serum albumine) as a standard.

Precipitation with 10% of polyethylene glycol (PEG): Up to 100 µL of each sample was centrifuged at 10,000× *g* for 10 min at 4 °C (Pico 21, Thermo Scientific) to remove insoluble material. Then, 20 µL of 50% PEG MW 6000 (final concentration, 10%) and 1.5 µL of 5M NaCl was added per 100 µL of sample and precipitated overnight at 4°C. EVs were centrifuged at 10,000× *g* for 10 min and the pellet was resuspended in 200 µL of PBS. 100 µL of the sample (diluted in 4 mL of PBS) was ultracentrifuged at 120,000× *g* for 1.5 h with the Beckman-Coultier SW55Ti rotor. The pellet was resuspended in 200 µL of Dulbecco's PBS (DPBS) (Sigma-Aldrich, St. Louis, MO, USA). Bradford Protein assay was used to determine protein concentration.

2.3. Analysis of EVs by NTA

NTA (Nanoparticle tracking analysis) was performed with a ZetaView nanoparticle analyzer (Particle Metrix GmbH; Inning am Ammersee, Germany). Before each session, the machine was calibrated using 102 nm polystyrene beads. In all cases, 11 measurements were recorded twice and averaged in at least one dilution in Dulbecco PBS (Corning Inc., New York, NY, USA), and were analyzed using the ZetaView Software 8.04.02 (Particle Metrix GmbH; Inning am Ammersee, Germany) using default image evaluation settings and the following camera acquisition settings: sensitivity 85, shutter 70, and frame rate 30. Each sample was measured three times.

2.4. Proteomic Analysis

Twenty PEG and 18 UC samples were selected for proteomics analysis. Based on sample protein concentration, UC samples were pooled into stages with 260 µg per pool. 4 mL of PBS was added and the mixture was ultracentrifuged at 120,000× *g* for 90 min at 4 °C using the Optima™ L-90K Ultracentrifuge with rotor SW55Ti. The pellet was resuspended with 100 µL of PBS and sent for analysis to the Proteomics Core Facility. For PEG samples, 20 µL of each sample was pooled into a stage pool, 4 mL of PBS was added and the mixture was ultracentrifuged at 120,000× *g* for 90 min at 4 °C using SW55Ti rotor and the Optima™ L-90K Ultracentrifuge to remove the serum albumin. The pellet was resuspended in 100 µL of PBS and 10 µg of sample was sent to Proteomics Core Facility for analysis.

Proteomic analysis was performed as described [17] in the Proteomics Core Facility of Institute of Technology, University of Tartu (Tartu, Estonia). Briefly, proteins from EVs were precipitated and digested with trypsin, and obtained peptides were detected with an LTQ Orbitrap XL (Thermo Fisher Scientific; Waltham, MA, USA) mass-spectrometer (MS). Mass-spectrometric raw data were analyzed with MaxQuant 1.4.0.8. Data were searched against UniProtKB (accessed on 15 September 2020, www.uniprot.org) sequences. Criteria for identification were specified as following: one peptide, minimum length of seven residues, and false discovery rate of <1% using a target decoy approach.

2.5. Western Blot

EVs were lysed and proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred by a semidry blotting method to a polyvinylidene difluoride (PVDF) membrane (MilliporeSigma, Burlington, MA, USA). The first samples were lysed in SDS-sample buffer, heated at 100 °C for 5 min, and loaded onto the 10% SDS-PAGE gel. After electrophoresis the proteins were transferred onto a PVDF membrane and blocked for 30 min to overnight with 2% non-fat dry milk in buffer (20 mM Tris pH 7.5, 0.15 M NaCl, 0.01% Tween20). Primary antibodies used were anti-GELSOLIN (ab109014, Abcam, dilution 1:10,000), anti-SERPINA3 (HPA002560, Atlas antibodies, dilution 1:2000) and anti-LGALS3BP (HPA000554, Atlas antibodies, dilution 1:500). Secondary goat anti-rabbit antibody (LabAS, Tartu, Estonia) was used in a 1:10,000 dilution. The signal was visualized by adding the ECL substrate (Amersham ECL Western Blotting Detection Reagents) for one minute and the films were exposed for either 10 (SERPINA3) or 60 min (GELSOLIN, LGALS3BP), after which they were scanned using Epson Expression 1680 (Epson, Suwa, Nagano, Japan). After reading the image, western blot images were measured with

Image J software. where each sample was given an arbitrary value based on blot intensity minus background intensity multiplied by blot size. To further normalize the measured signals, all Image J arbitrary values were divided by protein concentration obtained with Bradford assay, and a relative value was used in statistical analysis.

2.6. Statistical Analysis

Statistical analysis was carried out in R Studio using the Kruskal–Wallis rank sum test to find potential significant differences between different populations. If the Kruskal–Wallis was below 0.2, the Wilcoxon rank sum test with continuity correction was used to screen through all population pairs to find statistically important differences (p-value ≤ 0.05).

3. Results

In total, the blood serum from 54 individuals was used, including 48 patients with melanoma and six healthy controls. Patients belonged to five different melanoma stages: nine patients with stage 0 and stage IV disease, and ten patients with stages I, II, or III. The melanoma stage was assigned based on tumor thickness, ulceration, and the involvement of lymph nodes or organs. Overall, most patients were elderly females, median age 51.5 to 73 years, who had been diagnosed with melanoma less than five years ago, with a median disease duration from 1 to 4 years. There is no information about the age or sex of healthy controls. The characteristics of patients are shown in Table 1.

Table 1. Patient overview.

Group		Stage 0	Stage I	Stage II	Stage III	Stage IV	Healthy Controls
Number		9	10	10	10	9	6
Gender							
	Male	2 (22%)	1 (10%)	2 (20%)	5 (50%)	3 (33%)	
	Female	7	9	8	5	6	
Disease duration							
	<5 years	8	7	7	8	8	Unknown
	≥5 years	1 (11%)	3 (30%)	3 (30%)	2 (20%)	1 (10%)	
	Mean (range)	3 (0–13) years	3 (0–11) years	4 (0–10) years	2 (0–8) years	2 (0–6) years	
	Median	2 years	2 years	4 years	1 year	1 year	
Age							
	Mean (range)	55 (26–83) years	53.3 (28–78) years	67 (33–90) years	62.5 (50–82) years	64.5 (35–92) years	
	Median	60 years	51.5 years	72 years	60.5 years	73 years	

3.1. Biophysical Characterization of EVs Purified from Blood Serum

EVs were purified by ultracentrifugation from the blood serum of 54 individuals. In addition, EVs from the blood samples of twenty patients were purified by precipitation with PEG. There was an overlap of seven patients whose material was purified with both methods. The biophysical characteristics as the diameter and amount of purified EVs analyzed with NTA revealed that the median size as well as the profile of vesicles were similar for EVs purified either by UC or PEG precipitation (Figure 1A,B).

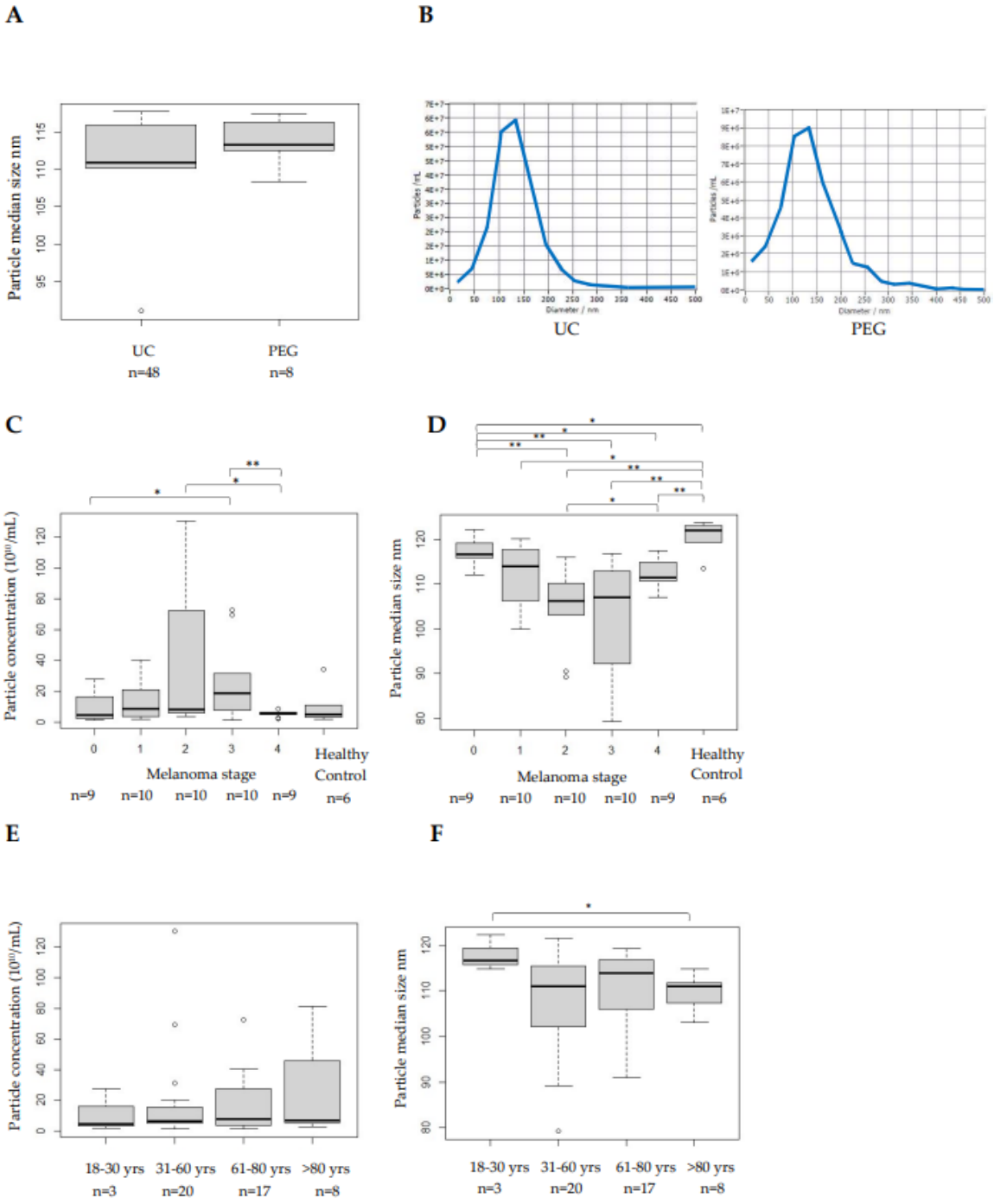

Figure 1. Biophysical characterization of EVs. (**A**) The particle median size measured by nanotracking analysis (NTA) for both ultracentrifugation (UC) and polymer precipitation (PEG) samples. (**B**) The profiles of EVs obtained with NTA. (**C**) Particle concentration measured by NTA in UC purified samples grouped by melanoma stages. (**D**) Particle median size measured by NTA in UC purified samples grouped by melanoma stages. (**E**) The particle concentration measured by NTA in UC purified samples grouped by age. (**F**) Particle median size measured by NTA in UC purified samples grouped by age. N—number of patients analyzed. The results indicated with * have a statistically significant difference compared to the value of the PBS-treated sample: * p-value < 0.05; ** p-value < 0.01.

EVs purified with UC were divided into subgroups according to the melanoma stage, and their amount and median diameter were compared (Figure 1C,D). The lowest particle concentration was seen in stage IV patients, 5.4×10^{10}/mL, while the peak was in stage II at 38.4×10^{10}/mL. Stage 0 had an average particle concentration of 10×10^{10}/mL, stage I, 24.3×10^{10}/mL, and stage III, 26.8×10^{10}/mL. Healthy controls had a similar concentration to stage 0, at 10.1×10^{10}/mL. There were statistically important differences between stage 0 and III ($p = 0.05$), between stage II and IV ($p = 0.03$), and between stage III and IV ($p = 0.01$). EVs isolated from stage II patients had the greatest variability in the amount of EVs (Figure 1C). When comparing the median diameter of EVs between different stages, the largest particles were detected in healthy controls at 120.6 nm. The second largest were in stage 0, at 117 nm, followed by stage I, at 114.4 nm. The smallest particles were detected in stage III patients, at 103.7 nm, followed by stage II, at 104.4 nm. There were statistically important differences between particle sizes between healthy controls and stage I ($p = 0.01$); stage 0 ($p = 0.05$); stage II patients ($p = 0.0005$); and stage IV ($p = 0.007$). Stage 0 melanoma in situ patients had also a statistically different particle size from stage I patients ($p = 0.0002$); stage III ($p = 0.003$); and stage IV ($p = 0.01$). There was also a significant difference in median diameters of stage II and IV ($p = 0.01$). NTA-measured median diameters of EV particles are shown in Figure 1D. There was no statistically significant difference in the amount of EVs isolated from the blood serum of patients with melanoma with different age; however, there was a statistically relevant difference between the particle size of patients aged 18–30 years and over 80 years old ($p = 0.02$, Figure 1E,F). The younger age group had a median particle size of 117.9 nm, while the older age group had smaller sized EVs with median diameter of 111.2 nm.

3.2. Proteomic Analysis of EVs

Eighteen EV samples purified by UC and twenty purified by PEG precipitation were pooled into stage pools and their protein content was analyzed using LC-MS/MS–based proteomics. We identified a total of 585 unique proteins; 334 proteins were detected in PEG-precipitated samples and 515 in UC-purified EVs (Figure 2A). Identified proteins were divided into twelve groups according to their biological and cellular function using the UniProt database (accessed on 15 September 2020, www.uniprot.org). The list of proteins depending on the purification method is shown in Table 2 and Figure 2A–C. In UC-purified samples, significantly more CD markers, cytoskeleton, and motor proteins, as well as pathway proteins, were detected (Figure 2D). Identified extracellular surface markers such as CD31, CD151, and different HLA-A, B, and C molecules have previously been used to purify EVs by surface markers in microarray settings [18], suggesting that our UC-purified samples contained a considerable amount of EVs. EV marker CD9 was present and calnexin was not in analyzed EV samples. PEG-precipitated samples were enriched in immunoglobulins, lipoproteins, and clotting or innate immunity proteins. We performed extensive research of literature to find which of these proteins have been linked to melanoma. In total, 159 proteins previously linked to melanoma in in vivo experiments, immunohistochemistry, melanoma cell lines, murine models, in silico proteomics analysis, or gene expression studies were found (marked bold in Table 2).

Table 2. Proteins found with proteomics with both purification methods. All proteins that have been linked to melanoma in previously published literature are in bold. Three proteins that were used in western blot are red.

Group	Number in Group	Gene Names		
		Found with Both Purification Methods	Found Only with UC Purification	Found only with PEG
CD markers	24	**CD91 (LRP1)**	**CD9, CD10 (MME), CD13 (ANPEP)**, CD16b (FCGR3B), **CD26 (DPP4), CD29 (ITGB1), CD31 (PECAM1), CD36**, CD41 (ITGA2B), CD42a (GP9), CD42c (GP1BB), **CD49b (ITGA2)**, CD49f (ITGA6), **CD59, CD61 (ITGB3), CD71 (TFRC), CD148 (PTPRJ), CD151, CD156c (ADAM10), CD167b (DDR2)**, CD233 (SLC4A1), CD235a (GYPA), CD321 (F11R)	none
Cytoskeleton and motor proteins	72	**ACTB**, ACTG1, ACTG2, GSN, TTN	ACTBL2, **ACTN1**, ACTN4, ACTR2, ACTR3, ANK1, ARL14EP, ARPC1B, **ARPC3**, CALD1, **CAP1**, **CAPZA1**, CAPZB, **CDC42, CENPF**, CEP126, **CFL1**, CNTD1, CORO1A, **CORO1C, DMD**, DNAH17, DNAH2, DSP, DSTN, **FHOD1, FLNA**, GRIPAP1, ITSN1, JUP, KIF1A, **LIMS1, MSN**, MYH9, MYL6, **MYO7A**, NEDD1, NIN, PAFAH1B1, **PFN1**, PLEK, **PPP2R1A, RHOC**, SDPR, SWAP70, SYNE3, TLN1, **TMSB4X**, TNNC1, TPM3, TPM4, **TUBA1A**, TUBA1B, TUBA3C, TUBA4A, **TUBB, TUBB1**, TUBB3, **TUBB4B, VASP, VCL**, WDR1	**DOCK1**, CEP152, OBSCN, PPP4C, SPAG17
Pathway	118	**PPBP**, UBB	AKT1, **ALDOA**, ANKRD12, ANKRD16, **ANXA1**, ANXA11, ANXA3, ANXA4, **ANXA7, ARHGDIB**, ARIH2, ARMC5, ATP2A2, **ATP5A1**, ATP5B, **BIRC6, CALR**, CASP9, CCDC81, **CERK**, CSN2, CSTA, CYFIP2, DDA1, EFTUD1, EHD1, EHD3, ENO1, FGD6, **GAPDH**, GBX2, GDI2, **GLIPR2, GNAI2**, GNB1, GUCY1B2, **H2AFJ**, HAAO, HEATR5B, HIST1H4A, HSPA8, **ILK**, KIAA0922, **LATS2, LDHB, LGALS7**, LINC00523, LRRK1, MCM5, MGA, MIPEP, MKX, MON2, NCOR2, NOP2, PDIA3, PGK1, **PKM, PPIA**, PTTG1IP, RAB11A, **RAB1B, RAB27B**, RAB6B, **RAB7A, RALB, RAP1B**, RAP2B, **RBM10**, RDH5, REPIN1, RSU1, RUFY1, SCAMP1, SCAND2P, SDCBP, SH3BGRL3, **SOD2, SRC**, STXBP2, TAF9, TAGLN2, TBC1D31, TBC1D8B, TMF1, **TPI1**, TRIM7, TSPAN9, TTK, **TXN**, USP29, **WNT2, YWHAB**, YWHAE, YWHAG, YWHAH, **YWHAQ, YWHAZ**, ZC3H14	**CAMP**, CAND2, CHCHD3, CLEC3B, LRRC37B, **MST1**, NKAIN1, PCYOX1, PHAX, PLIN5, PRMT9, RBM15B, RGAG1, SASH1, SLC25A52, SZT2, ZKSCAN2

Table 2. *Cont.*

Group	Number in Group	Gene Names		
		Found with Both Purification Methods	**Found Only with UC Purification**	**Found only with PEG**
Channel	16	PIGR	**AQP1**, CACNA2D1, **CLCN7, CLIC1**, FCHSD2, GFPT2, GJD2, GRIN1, SLC22A23, **SLC2A1**, SLC2A3, **SRI**, STOM	DLG2, PDZK1
Other proteins	65	A1BG, A2M, ADIPOQ, **AGT**, AHSG, **ALB**, AMBP, APCS, CALML5, CD5L, **CLU, CP**, CSN1S1, **DCD**, DEFA3, **ECM1**, FCGBP, **FLG2, GC**, HBA, HBA1, HBB, HBD, HP, HPR, HPX, HRG, HRNR, ITIH1, ITIH2, ITIH4, LTF, ORM2, PZP, RBP4, S100A7, **TF, TTR**	ATRN, **FLG, FTL**, LCA5L, LCN1, MUC16, OSCP1, **S100A8, S100A9**, SMIM4, SOGA3, SPDYE3, SPP2	AFM, **CRP, DMBT1**, IGFALS, ITIH3, MUC5B, PRG4, SAA1, **SAA2**, SAA4, SEPP1, **SERPINF1**, SPERT, TPRN
Innate immunity	34	**C1QA, C1QB, C1QC**, C1R, C1S, **C3**, C4A, C4B, C4BPA, C4BPB, C5, C6, C7, C8A, C8B, C8G, C9, CFB, **CFH**, CFHR1, CFHR2, CFP, FCN2, FCN3, LBP, MASP1, MBL2, SERPING1	MASP2	BPIFA1, BPIFB1, C4, CFI, COLEC11
Keratin	25	KPRP, **KRT1, KRT2**, KRT5, KRT6A, KRT6B, KRT6C, KRT8, **KRT9, KRT10**, KRT14, KRT16, KRT17, KRT76, KRT78, KRT83, KRT85, KRT86	KRT13, KRT31, KRT33A, KRT34, KRT36, KRT82, KRT87P	none
Clotting	23	**F2**, F5, F9, F11, **FGA, FGB, FGG**, KLKB1, **KNG1, PF4**, PF4V1, **PLG**, PROS1, SERPINC1, SERPIND1, SERPINF2, VWF	F13A1	F12, F5, F13B, HABP2, SERPINA4
Enzyme	21	**CAT, LYZ**, PON1, Protease 1, SERPINA1, **SERPINA3**, Trypsin	**BCHE, CA1**, ENPP7, MPO, **PRDX2**, RELN, SERPINA5, SERPINB3	CPB2, CPN1, CPN2, GPX3, HGFAC, PGLYRP2
Lipoprotein	16	**APOA1**, APOA2, APOA4, APOB, APOC1, APOC3, **APOD, APOE**, APOH, APOL1, LPA	none	APOC4, APOC2, APOF, APOM, PLTP
Cell adhesion	12	**FN1, LGALS3BP, THBS1, VTN**	**DSG1, FERMT3**, OLFM4, PARVB, PCDHB8, **PPFIBP1**	**FBLN1, MLLT4**
Immunoglobulins	159	Heavy chains: **IGHA1**, IGHA x2, IGHG x9, IGHM x3, IGHV x44. Ig joining chain: IGJ x1. Light chains: IGKV x44, IGL x2, IGLC x3, IGLV x23.	Heavy chain: IGHV x2. Light chains: IGKV x3, IGLV x7. MHC class I molecules: **B2M, HLA-A, HLA-A, HLA-B, HLA-B, HLA-C**	Heavy chain: IGHD x1, IGHV x2. Light chains: IGKV x5, IGLV x1

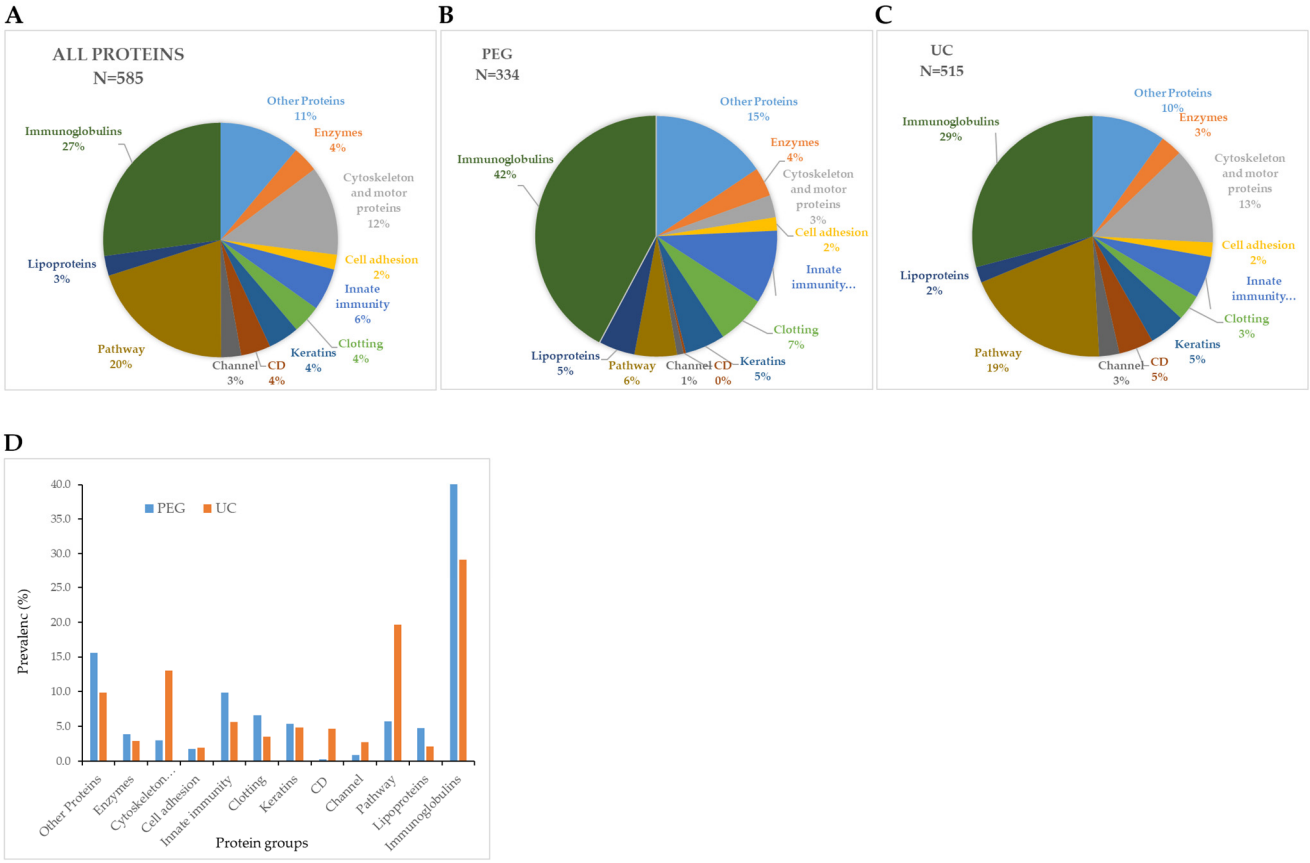

Figure 2. Proteomic analysis of EVs. (**A**) In total, 585 unique proteins were found from EVs purified with ultracentrifugation (UC) and polymer precipitation (PEG). These 585 proteins were divided into 12 groups and are represented here as the prevalence of the protein group. (**B**) The prevalence of protein groups identified from EVs purified by PEG precipitation. (**C**) The prevalence of protein groups identified from EVs purified by ultracentrifugation. (**D**) Comparison of the prevalence of proteins purified by PEG precipitation and ultracentrifugation.

3.3. Expression of SERPINA3, LGALS3BP and Gelsolin in EVs

Next we followed the expression of three potential biomarkers—SERPINA3, LGALS3BP and gelsolin—in EVs purified with UC using the immunoblot analysis. The upregulation of SERPINA3, alpha-1-antichymotrypsin, has been shown to correlate with worse patient outcomes, as it has pro-migration and pro-invasion functions in melanoma cells [19]. The expression of LGALS3BP, a galectin-3-binding protein, is altered in a variety of human carcinomas. It promotes integrin-mediated cell adhesion and may stimulate host defense against viruses and cancer cells [20]. Gelsolin, an actin-depolymerizing protein, is expressed both in extracellular fluids and in the cytoplasm of cells. Its extracellular isoform, called plasma gelsolin is an abundant plasma protein and has been recognized as a potential biomarker of inflammatory-associated medical conditions [21]. Gelsolin occurs in acidic exosomes and its expression is highest in metastatic melanoma patients [22].

As shown in Figure 1C, the amount of EVs varied in different samples. The total concentration of proteins was measured and correlated with the amount of EVs obtained by NTA. The Pearson coefficient of 0.65 showed a positive correlation and allowed us to use the protein concentration for calculating the relative values of protein expression (Figure 3A). The relative expression of proteins depending on the melanoma stage of patients is shown in Figure 3B–D. For SERPINA3, the lowest protein expression was detected in EVs of healthy controls and the highest in stage II and III patients, followed by stage IV patients (Figure 3B). There were statistically significant differences in signal strength between healthy controls

and stage IV ($p = 0.002$), stage II ($p = 0.005$), and stage 0 patients ($p = 0.05$). The expression of LGALS3BP showed a similar pattern; however, in this case, EVs from both healthy control and in situ (stage 0) patients showed a very low expression (Figure 3C).

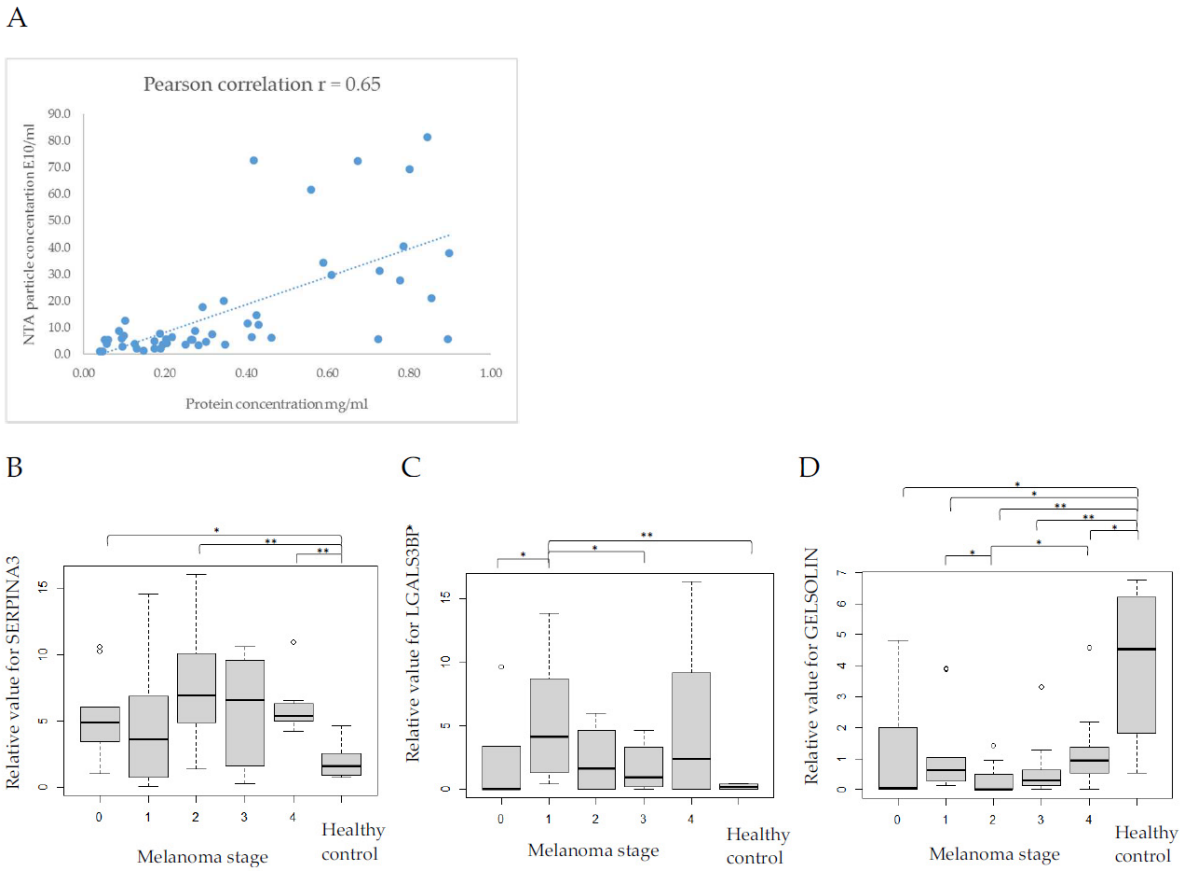

Figure 3. Analysis of EVs purified from patients with melanoma using ultracentrifugation. (**A**) Correlation analysis of the concentration of EVs measured by NTA and the total protein concentration measured by Bradford protein assay. (**B**) The relative values of the SERPINA3 signal obtained from the immunoblot analysis of EVs grouped by melanoma stages. (**C**) The relative values of the LGALS3BP signal obtained from the immunoblot analysis of EVs grouped by melanoma stages. (**D**) The relative values of gelsolin signal obtained from the immunoblot analysis of EVs grouped by melanoma stages. N—number of patients analyzed. The results indicated with * had a statistically significant difference compared to value of PBS-treated sample: * p-value < 0.05; ** p-value < 0.01.

The highest signals were detected for stage I and stage IV patients. Statistically significant changes were seen between stage I and healthy controls ($p = 0.003$), and stage I and III patients ($p = 0.05$). Gelsolin showed a completely different expression pattern, with the highest median signal detected in healthy controls (Figure 3E). Healthy controls had a significantly higher gelsolin signal when compared to stage I ($p = 0.003$), stage II ($p = 0.002$), stage III ($p = 0.005$), and stage IV patients ($p = 0.04$). There was also a significant difference between stage II and stage IV patient signals ($p = 0.03$). In summary, the expression of SERPINA3 and LGALS3BP was higher in melanoma patients than healthy controls, and gelsolin exhibited higher expression in healthy controls compared to melanoma patients.

4. Discussion

In the current study, we analyzed the biophysical and proteomic characteristics of EVs isolated from the blood serum of patients with melanoma using two different approaches: ultracentrifugation, which is the classical method for purification of EVs, and PEG precipitation, a more rapid and robust way to isolate EVs from biological fluids. Our data showed

that EVs can be purified by both methods; however, UC resulted in samples that were more enriched in EVs than PEG-precipitated material. PEG-purified material contained a considerable amount of lipoproteins, immunoglobulins, and clotting enzymes, and was not suitable for downstream protein analysis in our experiments. For EVs to be actually used in routine clinical analysis, they need to be purified by a high-throughput method that can be fully validated by ISO 15189 quality standards [13,23]. At the current moment, neither of the methods used fully comply with the requirements. Even though UC had a higher yield of EVs than PEG precipitation, we agree with others that its drawbacks are expensive equipment, skill dependency, time consuming nature, and low purity, as shown by co-purified serum proteins. However, PEG precipitation showed even lower yield and a higher co-precipitation rate, so its advantages such as a lower processing time and a lower required g force are not enough to choose it over UC [24,25]. Another problem is its inability to always produce a similar EV population across different analytical platforms and methods. A lack of such stability leads to a high measurement of uncertainty, which might reflect as an overlap between health and disease [23].

Purified EVs varied in their size and concentration in different individuals. The material obtained from stage II and III patients was the most heterogenous and differed the most from that of the healthy controls. EVs obtained from stage II and III patients were on average smaller and more abundant than others. This may reflect the status of the disease. EVs purified by UC are a mixture of different vesicles and co-purified proteins, including exosomes, microvesicles, apoptotic bodies, and oncosomes [13,14]. In addition, lipoparticles such as chylomicrons, VLDL, LDL, and HDL are probably co-purified. Depending on the cancer stage, the proportion of different vesicles and their relative amount in EVs may be different. Malignant cells secrete more EVs than nonmalignant cells of the same type and tumor microenvironment characteristics can further increase EV secretion rates [26,27]. In our study, the highest concentration of EVs was found in stage III patients, which was statistically different from in situ patients; and the lowest concentration was found in stage IV patients.

In our proteomic screen, we identified different EV markers such as CD9 and WNT2 for exosomes and CD31 for microvesicles. as well as the exosome-associated epithelial cell adhesion markers CA125 and CD41b [14,25,28]. We also found multiple proteins that are involved in exosome formation such as RAB7A, RAB11, and RAB27A [28,29], as well as extracellular surface markers such as CD31, CD151, and different HLA-A, B, and C molecules which have previously been used to purify EVs in microarray settings [18]. On the other hand, multiple new approaches for EV purification were built upon using so called pan-EV markers such as CD63, CD81, and TSG101 [18,24,25,30]. In our study, none of these markers were identified in the proteome of EVs. As such, a downside of immunocapture methods using EV markers could be the loss of EV material that does not contain classical EV markers.

The isolation and purification of EVs from serum has its challenges due to a high serum viscosity and high abundance of serum proteins and non-EV lipid particles [9]. Consistent with previous studies, we also identified co-purified serum proteins such as apoB as well as a large amount of immune and inflammatory response proteins [31]. In additions, a wide range of innate immunity complement proteins as well as complement regulatory protein CD59, the tissue factor TF, and clotting factors were detected. All of these might suggest that some microvesicles are platelet-derived and appeared in the sample during pre-analytical sample taking procedures, as our material was serum where no platelets should be present [13,18,31]. A high amount of lipoproteins, which might have been co-isolated with EVs from serum, have been previously shown [18]. However, a co-purified blood protein albumin, which can be found in quantities around 0.05 g per ml in serum, may also be part of EVs through a surface interaction to extend EV half-life [18,31].

Out of 585 proteins detected by mass spectrometry, 159 have been linked to melanoma in different studies. We detected multiple proteins shown to be upregulated in melanoma cell-derived EVs such as NOTCH2, TLN1, PGK1, SERPINF2, WDR, CSGP4, and YWHAE, as well as proteins linked with progressive disease such as ITHI3, MSN, THBS1, and TUBB [32]. In addition, EV markers linked to other tumors were also found. PIGR has

been shown in EVs from ultracentrifuged cholangiocarcinoma patient sera [33], and ALK1, CD151, and ECM1 have been found in non-small cell lung cancer patient EVs as potential protein biomarkers, while CD91 has been found to be a powerful surface biomarker for the same disease [24,34]. The upregulation of SERPINA3 has been shown to correlate with worse patient outcomes, as it has pro-migration and pro-invasion functions in melanoma cells [19]. Our results are consistent with previous findings as healthy controls had a significantly lower SERPINA3 signal when compared to stage II, III, and IV melanoma patients. The highest signal of SERPINA3 was found in stage II patients when the invasion of melanoma cells starts and small nests of melanoma start to form in the papillary dermis, the lowest level of the epidermal skin [35]. LGALS3BP expression was highest in stage I patients, which was statistically different from stage 0 patients and healthy controls. LGALS3BP has potential to be a good early biomarker of melanoma. LGALS3BP expression is altered in the serum of patients with a variety of human carcinomas, and a down regulation of the protein it binds to, galectin-3, is related to increased aggressiveness of tumors [36]. In melanoma, the galectin-3 protein overexpression correlates with metastatic progression and with negative clinical outcome [37].

One of the largest challenges of translating EVs into suitable clinical biomarkers is to offer fully validated clinical test that are up to ISO 15189 quality standards. Validation would offer the quality assurance that tests perform at an adequate level and as such could be used in clinical diagnostics [23]. Currently the largest challenges in using EVs as biomarkers are the lack of standardization for sample collection, sample processing, and sample analysis [13]. As such, the large variability between different EV samples might lead to problems distinguishing between health and diseased patients [23].

5. Conclusions

EVs has a great potential to be a source of protein biomarkers, however, a lot of work is still needed to fully understood and characterize the complex nature of EVs found in human blood and to work out verified isolation methods.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data and materials presented in this study are available on request from the corresponding author.

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