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Exploration of the platelet proteome in patients with early-stage cancer

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ABSTRACT

Platelets play an important role in tumor growth and, at the same time, platelet characteristics are affected by cancer presence. Therefore, we investigated whether the platelet proteome harbors differentially expressed proteins associated with early-stage cancer. For this proof-of-concept study, patients with early-stage lung ($n = 8$) or head of pancreas cancer ($n = 4$) were included, as were healthy sex- and age-matched controls for both subgroups. Blood samples were collected from controls and from patients before surgery. Furthermore, from six of the patients, a second sample was collected two months after surgery. NanoLC-MS/MS-based proteomics of gel-fractionated platelet proteins was used for comparative spectral count analyses of patients to controls and before to after surgery samples. The total platelet proteome dataset included 4384 unique proteins of which 85 were significantly (criteria Fc > 1.5 and $p < 0.05$) changed in early-stage cancer compared to controls. In addition, the levels of 81 platelet proteins normalized after tumor resection. When filtering for the most discriminatory proteins, we identified seven promising platelet proteins associated with early-stage cancer. In conclusion, this pioneering study on the platelet proteome in cancer patients clearly identifies platelets as a new source of candidate protein biomarkers of early-stage cancer.

Biological significance: Currently, most blood-based diagnostics/biomarker research is performed in serum or plasma, while the content of blood cells is usually neglected. It is known that especially blood platelets, which are the main circulating pool of many bioactive proteins, such as growth factors, chemokines, and cytokines, are a potentially rich source of biomarkers. The current study is the first to measure the effect of early-stage cancer on the platelet proteome of patients. Our study demonstrates that the platelet proteome of patients with early-stage lung or head of pancreas cancer differs considerably compared to that of healthy individuals of matched sex and age. In addition, the platelet proteome of cancer patients normalized after surgical resection of the tumor. Exploiting platelet proteome differences linked to both tumor presence and disease status, we were able to demonstrate that the platelet proteome can be mined for potential biomarkers of cancer.

1. Introduction

Timely discovery and subsequent treatment of cancer drastically improves the efficacy of treatment and overall survival of patients [1]. During the past decades researchers have attempted to identify potential blood biomarkers of early-stage malignancy in plasma and/or serum of cancer patients. Unfortunately, this strategy has not yet led to clinically relevant blood biomarkers that can be applied to detect localized cancer [2–4]. Recently, it was suggested that platelets, which contain vast amounts of proteins, are a potential but unexplored source for blood biomarker research [5].

Platelets are generally known for their role in hemostasis and initiation of wound healing [6]. However, they also play an important role in tumor angiogenesis, growth and metastasis [7,8]. Platelets contain a great deal of proteins including growth factors, chemokines and proteases, which are synthesized by megakaryocytes or absorbed

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from the blood by the platelets themselves [7,9,10]. In addition, platelets are able to secrete their rich content within prothrombotic tumor vessels, supporting tumor angiogenesis and vessel stability [11,12], tumor cell proliferation and migration [13,14], and the induction of epithelial to mesenchymal-like transition processes promoting cancer cell metastasis [15–17].

In vivo studies in mice have demonstrated that the growth factor content of circulating platelets already changes during early tumor development [10,18,19]. In addition, it has been shown that platelets of cancer patients contain altered concentrations of growth factors and mRNA compared to healthy individuals [20,21]. Furthermore, preliminary data show that a combination of platelet characteristics (platelet count, volume, activation and content) can be used to distinguish patients with early-stage cancer from healthy individuals [22]. Overall, this suggests that in patients harboring cancer lesions, platelets and their molecular content can serve diagnostic biomarker functions.

We hypothesize that the platelet proteome is changed in patients with early-stage cancer as compared to healthy controls. The aim of the current study is to identify differentially expressed platelet proteins associated with early-stage cancer that have the potential to be applied as biomarkers in early diagnosis of cancer. In this prospective study, we included patients with early-stage lung cancer or head of pancreas cancer, and a sex- and age-matched control group. From all patients, blood samples were collected one day before surgical removal of the tumor. In some patients, a second sample was collected two months after surgery. Our data demonstrate that the platelet proteome in patients with early-stage cancer is different from that of their healthy counterparts. Moreover, surgical removal of the malignant tumor appeared to normalize the platelet proteome.

2. Materials and methods

2.1. Chemicals

Acetonitrile, methanol and formic acid were purchased from Biosolve (Biosolve, Valkenswaard, The Netherlands). LDS (lithium dodecyl sulfate) sample buffer and pre- casted gradient gels were acquired from Invitrogen (Invitrogen Carlsbad, CA). Dithiotreitol, phosphoric acid, ammonium sulfate, ammonium bicarbonate, and iodoacetamide were purchased from Sigma (Sigma-Aldrich, St Louis, MO), and sequence-grade trypsin was purchased from Promega (Promega, Madison, WI). Coomassie brilliant blue R-250 dye was acquired from ThermoFisher scientific (Landsmeer, The Netherlands).

2.2. Study population

In accordance with the declaration of Helsinki, study approval by the medical ethical committee of Maastricht University Medical Center + (MUMC+) was obtained. Patients with histologically proven early-stage lung ($n = 8$) or head of pancreas cancer ($n = 4$) were included. Almost all patients were diagnosed with either stage I or II cancer. After surgery, it became clear that one patient with head of pancreas cancer had stage III cancer without any organ metastasis. Also a healthy sex- and age-matched control group for each cancer group was included (total $n = 11$; Table 1); one female control served as match for both cancer groups. Exclusion criteria were: neoadjuvant chemo- or radiotherapy, previous history of cancer, use of platelet-influencing drugs, blood or platelet transfusion during the previous 14 days, active inflammatory disease, fractures, or non-healing ulcers. Cancer staging was performed in accordance with the TNM classification (version 7) of the Union for International Cancer Control [23].

2.3. Blood collection and sample preparation

Blood samples were collected from patients one day before surgical removal of the tumor. From six patients (five with lung and one with head of pancreas cancer), a second sample was collected two months after surgery. This time frame was chosen to minimize the potential effect of surgery itself on the platelets. Blood from healthy controls was collected once. In both patients and healthy individuals, blood samples were obtained from the antecubital vein and immediate sample preparation was performed in such a way that platelet-activating effects were minimal, as described before [24]. The first 5 ml of blood was discarded, after which BD Vacutainer tubes containing 3.2% buffered sodium citrate (Becton Dickson, Breda, The Netherlands) were used to collect the blood samples (20 ml). After several washing steps, the platelet count in all samples was normalized (3×10^{11}/L). Subsequently, platelets were pelleted at 1600 *g* for 2 min. This platelet pellet was dissolved in NuPAGE LDS sample buffer containing DTT (10 mM dithiothreitol in 50 mM ammonium bicarbonate), heated to 99 °C for 5 min, and stored at −80 °C until use.

Table 1
Patient and healthy control characteristics.

	Patients ($n = 12$)	Controls ($n = 11$)
Age		
Years (SD)	66.8 (7.4)	65.1 (7.3)
Gender		
Male	5	5
Female	7	6
Type of cancer		
Lung	8	–
Pancreatic	4	–
TNM Stage		
I	4	–
II	7	–
III	1	–
IV	0	–
Histology		
Adenocarcinoma	10	–
Sarcomatoid	1	–
Neuroendocrine	1	–

2.4. Protein electrophoresis

After thawing, samples were heated at 99 °C for 3 min. Twenty μl was applied to a 12% acrylamide SDS-PAGE gel (BioRad, Hercules, CA), and run for 45 min at 200 V. Next, gels were washed with Milli-Q water and fixed in 50% EtOH/1% phosphoric acid for 12 min. After washing with Milli-Q water, the gels were stained overnight with 1% Coomassie brilliant blue R250 in 40% MeOH/1% phosphoric acid containing 1.5 M ammonium sulfate. Next, the gels were destained with Milli-Q water, and scanned using a digital scanner (Hewlett-Packard, Palo Alto, CA).

2.5. In-gel digestion

In-gel digestion was performed as described previously [25]. In short, gel processing and in-gel digestion was performed in a keratin-free laminar flow cabinet. Gel slices were cut from the Coomassie-stained SDS-PAGE gel and washed with 50 mM ammonium bicarbonate/50% acetonitrile. Next, the gel was diced on a clean glass plate into 1-mm^3 cubes using a scalpel. Subsequently, gel cubes were transferred to a 1.5-ml microcentrifuge tube, vortexed in 400 μl 50 mM ammonium bicarbonate for 10 min, and pelleted. The supernatant was removed and the gel cubes were vortexed in 400 μl 50 mM ammonium bicarbonate/50% acetonitrile for 10 min. After pelleting and removal of the supernatant, this wash-step was repeated. Subsequently, gel cubes were reduced in 50 mM ammonium bicarbonate supplemented with 10 mM DTT at 56 °C for 1 h. After cooling down and pelleting, the supernatant was removed and gel cubes were alkylated in 50 mM ammonium bicarbonate supplemented with 50 mM iodoacetamide for 45 min at room temperature in the dark. Next, gel cubes were washed

with 50 mM ammonium bicarbonate/50% acetonitrile and 50 mM ammonium bicarbonate/50% acetonitrile as described above, dried in a vacuum centrifuge at 50 °C for 10 min, and covered with trypsin solution (6.25 ng/μl in 50 mM ammonium bicarbonate). Following rehydration with trypsin solution and removal of excess trypsin, gel cubes were covered with 50 mM ammonium bicarbonate and incubated overnight at 25 °C. After digestion, peptides were extracted from the gel cubes with 100 μl of 1% formic acid (once) and 100 μl of 5% formic acid/50% acetonitrile (twice). All extracts were pooled and stored at −20 °C until use. Prior to LC-MS, the extracts were concentrated in a vacuum centrifuge at 50 °C, volumes were adjusted to 50 μl by adding 0.05% formic acid, and the solution was transferred to an LC autosampler vial while filtering through a 4-mm, 0.45-μm PVDF filter (Merck Millipore).

2.6. NanoLC-MS/MS

Tryptic peptide extracts were separated utilizing an Ultimate 3000 nanoLC-MS/MS system (Dionex LC-Packings, Amsterdam, The Netherlands) harboring a 20-cm fused silica column (75 μm inner diameter) custom packed with 3-μm ReproSil-Pur C18-AQ silica particles (120-Å pore diameter; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). First, peptides were trapped on a 10-mm trap column (100 μm inner diameter) packed with 5-μm ReproSil-Pur C18-AQ silica particles (120-Å pore diameter) at 6 μl/min and 2% buffer B (buffer A, 0.5% acetic acid (Fischer Scientific); buffer B, 80% acetonitrile, 0.5% acetic acid), and separated at 300 nl/min in a 10–40% buffer B gradient in 60 min (90 min inject-to-inject). Eluting peptides were ionized at a potential of +2 kV into a Q Exactive mass spectrometer (Thermo Fisher, Bremen, Germany). Intact masses were measured in the orbitrap at a resolution of 70.000 (at m/z 200), using an AGC target value of 3×10^6 charges. The top 10 peptide signals (excluding charge state 1+) were submitted for MS/MS in the HCD cell (Higher-Energy Collisional Dissociation, 4-amu isolation width, 25% normalized collision energy). MS/MS spectra were acquired in the orbitrap with a resolution of 17.500 (at m/z 200), using an AGC target value of 2×10^5 charges and an underfill ratio of 0.1%. Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 30 s.

2.7. Protein identification

MS/MS spectra were searched against a Uniprot human reference proteome FASTA file (release January 2014, 61,552 entries) using MaxQuant 1.5.2.8 software [26].

Enzyme specificity was set to trypsin and up to two missed cleavages were allowed. Cysteine carboxamidomethylation was treated as fixed modification, and methionine oxidation and N-terminal acetylation as variable modifications. Peptide precursor ions were searched with a maximum mass deviation of 4.5 ppm and fragment ions with a maximum mass deviation of 20 ppm. Peptide and protein identifications were filtered at an FDR of 1% using the decoy database strategy. The minimal peptide length was 7 amino acids, the minimum Andromeda score for modified peptides was 40, and the minimum delta score was 6. Proteins that could not be differentiated based on MS/MS spectra alone were grouped to protein groups (default MaxQuant settings). Searches were performed with the label-free quantification option selected. The mass spectrometry data have been submitted to the ProteomeXchange Consortium [27] via the PRIDE partner repository, with the dataset identifier PXD005921.

2.8. Protein quantification and statistical analysis

Spectral counts, i.e. the number of identified MS/MS spectra for a given protein, were used as a proxy for protein abundance [28]. Raw counts were normalized on the basis of the sum of spectral counts for all identified proteins in a particular sample, relative to the average sample sum determined with all samples. To find statistically significant differences in normalized counts between sample groups, we applied the beta-binomial test, which takes into account within-sample and between-sample variation using an alpha level of 0.05 [29]. For hierarchical clustering analysis, protein abundance data were normalized to zero mean and unit variance, and Euclidian distance and Ward linkage were used for clustering. All analyses were performed using dedicated R scripts.

2.9. Protein network analysis

Human gene symbols for identified proteins were uploaded to the web-based STRING tool (version 10.0, http://string.embl.de) to retrieve protein–protein association data [30]. The data were imported, visualized, and annotated in Cytoscape version 3.1.1 [31]. Gene ontology analysis was performed using the BiNGO app for Cytoscape to retrieve overrepresented biological process terms (corrected p-value < 0.05) [32].

3. Results

3.1. Identification and comparison of platelet proteins in patients with early-stage cancer and healthy individuals

Platelets from patients with early-stage lung cancer (n = 8) or head of pancreas cancer (n = 4) and from a healthy control group (n = 11) were isolated and processed for proteomics involving protein gel prefractionation (Supplemental Fig. 1) followed by combined fractionation and analysis on a nano LC-MS/MS platform including an orbitrap mass spectrometer. This label-free proteomics workflow was previously extensively benchmarked for its reproducibility [33,34]. Patient and healthy control characteristics are presented in Table 1. A total of 4595 database entries linked to 4384 unique proteins were identified (Supplemental Table 1), with an average of 3403 identified entries (3321 unique proteins) for cancer patient platelet samples, and 3380 identified entries (3298 unique proteins) for platelet samples from healthy individuals. Unsupervised hierarchical cluster analysis using normalized spectral count data for all identified proteins (Supplemental Fig. 2) did not show clear clustering of healthy versus cancer platelet proteomes.

Among the 4595 database entries (4384 unique proteins) identified, 105 entries (104 unique proteins) exhibited a significant difference in abundance ($p < 0.05$). Of these, 61 entries (61 unique proteins) were at least 1.5-fold more abundant (Supplemental Table 2), and 24 entries (24 unique proteins) were at least 1.5-fold less abundant in early-stage cancer platelets compared to platelets of healthy individuals (Supplemental Table 3). Importantly, supervised cluster analysis including all differential entries revealed separate clustering of cases and controls, indicating substantial changes in the platelet proteome of patients with early-stage cancer compared to that of healthy controls with the same sex and age (Fig. 1). Notably, a separate cluster analysis of patient subgroups classified according to cancer type also indicated a significant difference between platelets from cancer patients and healthy controls (Fig. 2). The platelet proteomes of patients with early-stage lung cancer (Fig. 2A) or head of pancreas cancer (Fig. 2B) are clearly different from that of the healthy sex- and age-matched control groups; this difference appears to be more evident in the pancreatic patient group.

3.2. Functional data mining of differentially expressed proteins

Next, we pursued the identification of biological functions that are associated with the proteins that exhibited changed abundance in platelets from patients with early-stage cancer relative to platelets from healthy controls. For this analysis, we focused on proteins with an abundance in cancer platelets that was at least 1.5-fold higher

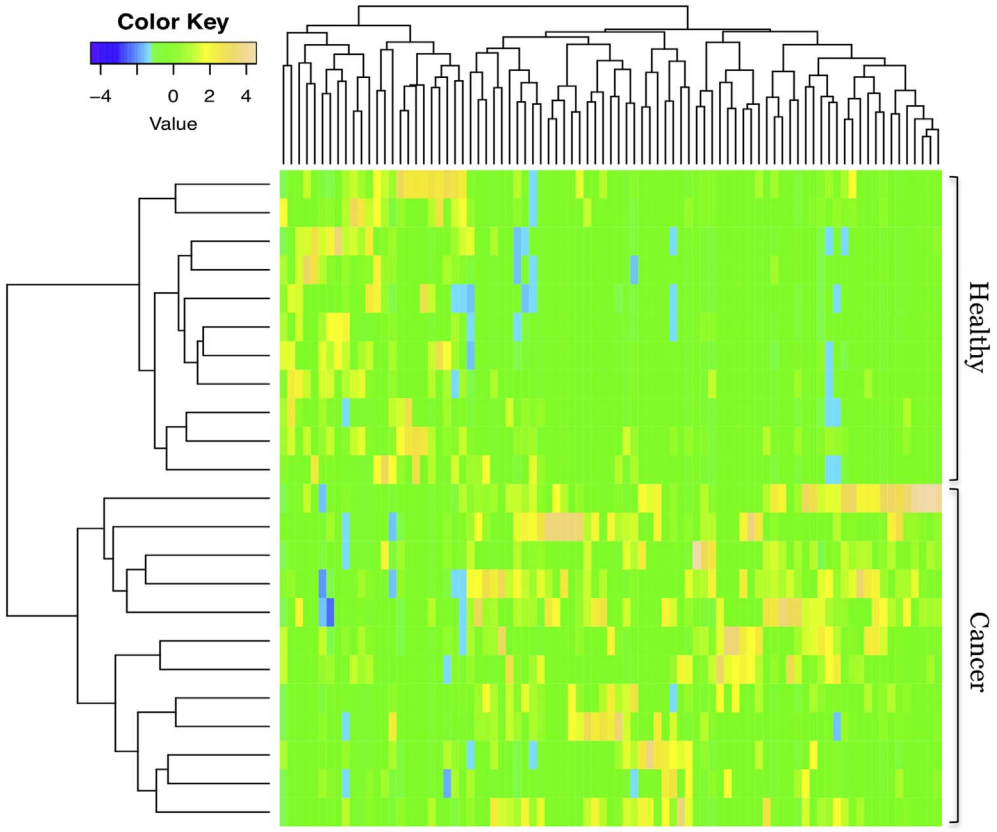

Fig. 1. Heat map and cluster analysis using protein expression data from platelets of patients with early-stage cancer and healthy sex- and age-matched controls. Supervised cluster analysis clearly separates patients with early-stage cancer from healthy controls and shows a distinct heat map pattern for up- and downregulated proteins.

('upregulated') or lower ('downregulated') compared to control platelets, and were detected in at least 6 of the 12 patient samples or 5 of the 11 control samples, respectively. This resulted in a list of 31 upregulated (Table 2) and 18 downregulated proteins (Table 3). Protein networks were generated for both protein sets using protein-protein association data from the STRING database [30]. These networks were subsequently imported and visualized in Cytoscape (Fig. 3). Also gene ontology mining using the BiNGO Cytoscape app was performed (Table 4). Among the upregulated proteins, subsets were primarily linked to inflammation, immune response and cytoskeleton organization and transport. Subsets of downregulated proteins were involved in antigen processing and presentation.

3.3. Platelet proteome normalizes after surgical tumor resection

The platelet proteome of six patients (five with lung cancer and one with head of pancreas cancer) was compared before and after surgical resection of the tumor. Resection led to significant (at least 1.5-fold) downregulation of 21 proteins and upregulation of 17 proteins (Table 5). The downregulated proteins after surgical resection were

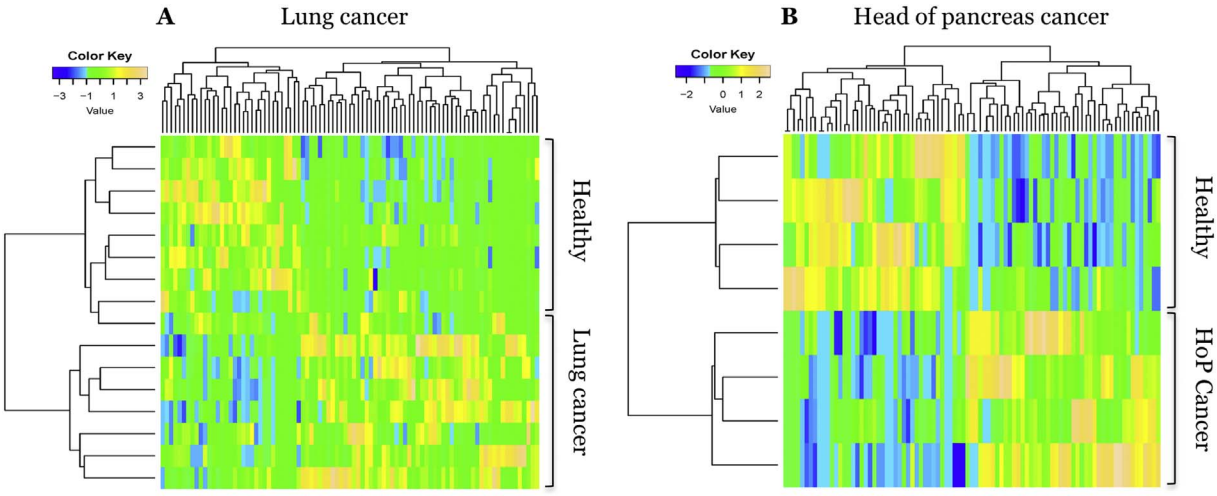

Fig. 2. Heat map and supervised cluster analysis of protein expression data from platelets of patients with early-stage lung or head of pancreas (HoP) cancer compared to healthy sex- and age-matched controls. Cancer type-specific supervised cluster analysis clearly separates the platelet proteome of patients with early-stage lung cancer (A) or head of pancreas cancer (B) from healthy controls.

Table 2
List of 31 upregulated proteins in platelets from patients with early-stage cancer compared to platelets from healthy controls ($p \leq 0.05$, fold change ≥ 1.5, detected in at least 6 of 12 cancer samples).

Accession number	Gene symbol	Approved name (HGNC)	Fold change	p-Value	Positive cancer samples (of 12)	Positive control samples (of 11)
J3KQ66	RELN	Reelin	17,64	0,00	7	1
Q96N66-3	MBOAT7	Isoform 3 of Lysophospholipid acyltransferase 7	8,66	0,01	6	1
Q6UWE0	LRSAM1	E3 ubiquitin-protein ligase LRSAM1	7,64	0,02	6	1
H0YMV8	RPS27L	40S ribosomal protein S27	6,72	0,04	7	1
Q96FN4	CPNE2	Copine-2	6,41	0,04	6	1
Q9NPA8-2	ENY2	Isoform 2 of Transcription and mRNA export factor	6,12	0,01	8	2
P56181	NDUFV3	NADH dehydrogenase [ubiquinone] flavoprotein 3	5,96	0,04	6	1
Q9Y6K5	OAS3	2–5-oligoadenylate synthase 3	5,57	0,01	8	2
J3KS81	TMEM199	Transmembrane protein 199	5,16	0,02	7	1
G5E933	SBF1	Myotubularin-related protein 5	4,42	0,03	8	2
Q86VR2	FAM134C	Protein FAM134C	4,34	0,02	7	1
P22626-2	HNRNPA2B1	Isoform A2 of Heterogeneous nuclear ribonucleoproteins A2/B1	3,83	0,01	10	5
P33908	MAN1A1	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	3,66	0,02	8	4
P08311	CTSG	Cathepsin G	2,88	0,01	11	5
P03973	SLPI	Antileukoproteinase	2,73	0,05	9	4
Q9NZJ9	NUDT4	Diphosphoinositol polyphosphate phosphohydrolase 2	2,55	0,04	9	5
P62805	HIST1H4A	Histone H4	2,49	0,02	12	10
P62263	RPS14	40S ribosomal protein S14	2,45	0,03	12	6
H7C1J4	UHRF1BP1	Chromosome 6 open reading frame 107, isoform CRA_b	2,44	0,03	11	8
P04083	ANXA1	Annexin A1	2,39	0,00	12	11
P02792	FTL	Ferritin light chain	2,38	0,02	11	6
P02549-2	SPTA1	Isoform 2 of Spectrin alpha chain, erythrocytic 1	2,09	0,03	12	11
Q6ZWT7	MBOAT2	Lysophospholipid acyltransferase 2	2,09	0,02	11	7
P13796	LCP1	Plastin-2	2,08	0,03	12	11
Q9UPN4-2	AZI1	Isoform 2 of 5-azacytidine-induced protein 1	2,00	0,03	12	10
Q9Y5P4-2	COL4A3BP	Isoform 2 of Collagen type IV alpha-3-binding protein	1,78	0,02	12	11
Q63HN8	RNF213	E3 ubiquitin-protein ligase RNF213	1,70	0,00	12	11
P06702	S100A9	Protein S100-A9	1,66	0,01	12	11
P12955	PEPD	Xaa-Pro dipeptidase	1,60	0,03	12	11
P05109	S100A8	Protein S100-A8	1,58	0,03	12	11
J3KNL6	SEC16A	Protein transport protein Sec16A	1,55	0,04	12	11

Table 3
List of 18 downregulated proteins in platelets from patients with early-stage cancer compared to platelets from healthy controls ($p < 0.05$, fold change ≥ 1.5, detected in at least 5 of 11 control samples).

Accession number	Gene symbol	Approved name (HGNC)	Fold change	p-Value	Positive cancer samples (of 12)	Positive control samples (of 11)
Q9NPB1	NT5M	5(3)-deoxyribonucleotidase	Only in healthy	0,01	0	5
P49788	RARRES1	Retinoic acid receptor responder protein 1	−14,04	0,01	1	5
Q15904	ATP6AP1	V-type proton ATPase subunit S1	−7.19	0,04	1	5
Q86VX2-2	COMMD7	Isoform 2 of COMM domain-containing protein 7	−6.83	0,04	1	5
P30499	HLA-C	HLA class I histocompatibility antigen, Cw-1 alpha chain	−4.61	0,01	1	6
P28907	CD38	ADP-ribosyl cyclase 1	−4.44	0,03	2	7
Q8WTR2	DUSP19	Dual specificity protein phosphatase 19	−2.93	0,02	4	8
M0R2G3	KXD1	KxDL motif-containing protein 1	−2.69	0,04	5	8
Q95365	HLA-B	HLA class I histocompatibility antigen, B-38 alpha chain	−2.55	0,04	5	8
Q5VZ66	JAKMIP3	Janus kinase and microtubule-interacting protein 3	−2.28	0,02	8	10
P20472	PVALB	Parvalbumin alpha	−2.21	0,02	5	9
Q9H4L5-2	OSBPL3	Isoform 1b of Oxysterol-binding protein-related protein 3	−2.01	0,02	9	10
G8JLE0	STX16	Syntaxin-16	−1.71	0,05	12	11
P04196	HRG	Histidine-rich glycoprotein	−1.63	0,00	12	11
Q9UKW4	VAV3	Guanine nucleotide exchange factor VAV3	−1.60	0,04	12	11
E9PGF5	TNS1	Tensin-1	−1.59	0,04	10	11
Q08426	EHHADH	Peroxisomal bifunctional enzyme	−1,58	0,03	11	11
Q9NZ08	ERAP1	Endoplasmic reticulum aminopeptidase 1	−1,55	0,03	12	11

linked to defense responses (Supplemental Table 4). Supervised cluster analysis (Supplemental Fig. 3) revealed a good separation of samples before and after resection, except for one lung cancer patient whose platelet proteome did not significantly change after resection and clustered with its 'before resection' samples. Strikingly, most proteins (58 out of 61) that were significantly up- or downregulated (23 out of 24) in platelets of patients with cancer compared to platelets of healthy controls, normalized their expression after surgical resection of the tumor. Altogether, these data show that surgical tumor resection significantly changes the platelet proteome in most patients.

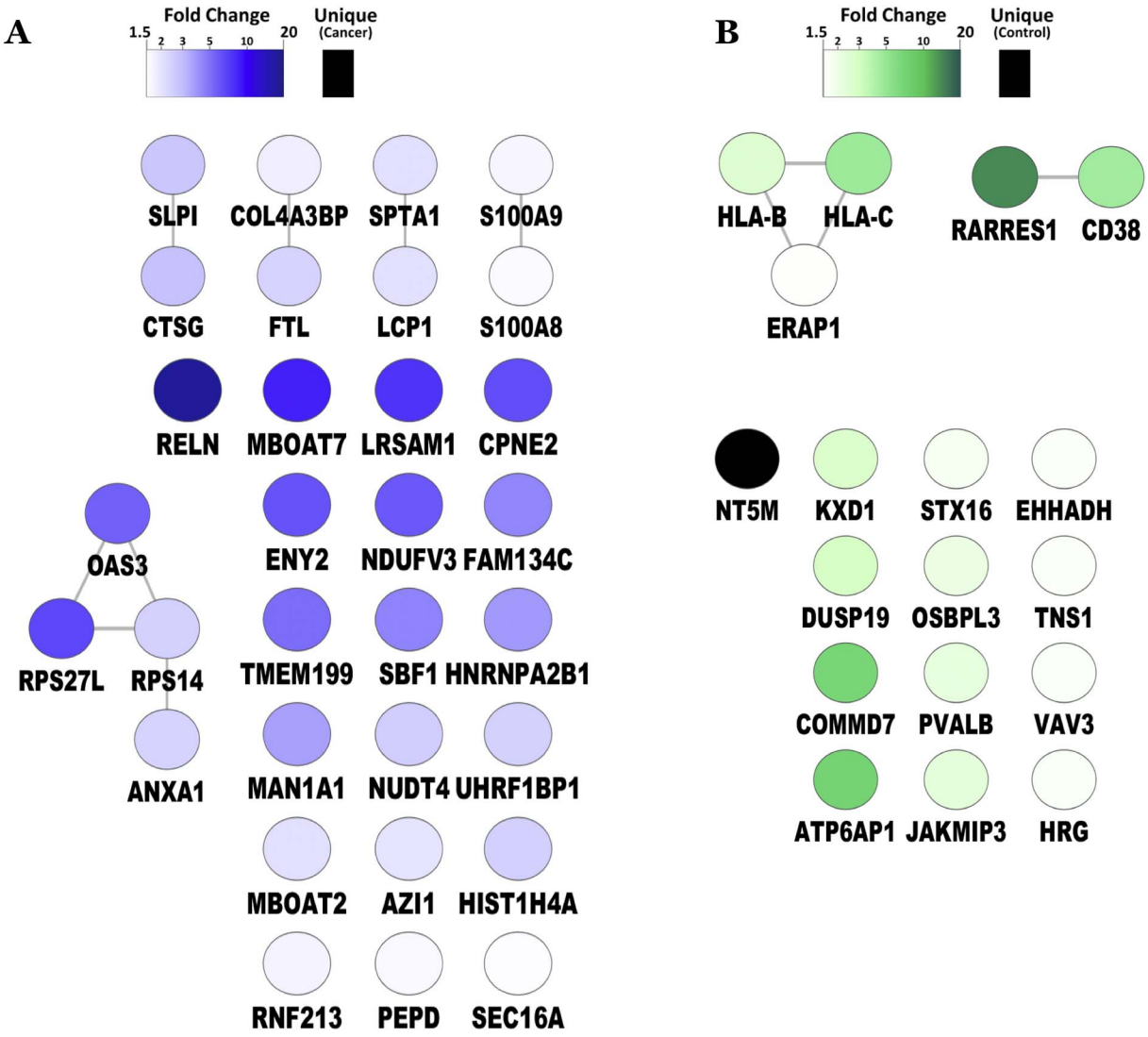

Fig. 3. Protein-protein networks of significantly upregulated (A) and downregulated (B) proteins in platelets of cancer patients compared to platelets of healthy individuals ($p < 0.05$, fold change ≥1.5, detected in at least 6 of 12 cancer samples or 5 of 11 control samples). The networks were generated using default settings in String and visualized using Cytoscape.

Table 4
Overrepresented gene ontology (biological process) terms associated with differential proteins in cancer patients compared to healthy individuals ($p < 0.05$, fold change ≥1.5, detected in at least 6 of 12 cancer samples or 5 of 11 healthy controls)a.

Protein regulation in cancer	# proteins	Biological process	Associated molecules
↑	31	Inflammation response	ANXA1, S100A9, S100A8
		Immune system process	SPTA1, RPS14, COL4A3BP, OAS3, CTSG, HLA-A, LCP1 and S100A9, ANXA1
		Cytoskeleton organization and transport	SPTA1, LCP1, S100A9, RPS14, COL4A38P
↓	18	Antigen processing and presentation	TRAF6, ERAP1, HLA-C

3.4. Singling out differentially expressed proteins associated with early-stage cancer

To select differentially expressed proteins associated with early-stage cancer, two different approaches were utilized. First, the upregulated proteins were carefully explored. Nine proteins were at least five times upregulated in platelets of patients with early-stage cancer compared to healthy controls (Table 2). Four of these proteins, namely

Table 5
List of 38 differential proteins in platelets from patients with early-stage cancer after surgical tumor removal ($p < 0.05$, fold change ≥1.5, detected in at least 3 of 6 relevant samples).

Regulation	Accession number	Gene symbol	Approved name (HGNC)	Fold change	p-Value	Positive before surgery (of 6)	Positive after surgery (of 6)
↓	I3L3X5	PLSCR3	Phospholipid scramblase 3	Only before	3.3E-02	3	0
↓	P10153	RNASE2	Non-secretory ribonuclease	Only before	3.8E-02	3	0
↓	P01023	A2M	Alpha-2-macroglobulin	−6.2	4.5E-03	4	1
↓	F5H5N0	CDC42BPA	Serine/threonine-protein kinase MRCK alpha	−5.7	2.1E-02	5	1
↓	Q5R372-7	RABGAP1L	Isoform 7 of Rab GTPase-activating protein 1-like	−5.0	3.7E-02	5	1
↓	H9KV70	LCN2	Neutrophil gelatinase-associated lipocalin	−4.8	6.0E-03	4	1
↓	P24158	PRTN3	Myeloblastin	−3.8	8.9E-03	4	3
↓	E7ER44	LTF	Kaliocin-1	−3.3	1.1E-03	6	6
↓	P05164-2	MPO	Isoform H14 of Myeloperoxidase	−3.2	4.1E-02	4	4
↓	Q8IW45	CARKD	ATP-dependent (S)-NAD(P)H-hydrate dehydratase	−2.8	2.3E-02	6	4
↓	P08311	CTSG	Cathepsin G	−2.6	1.0E-02	6	5
↓	Q9H7Z7	PTGES2	Prostaglandin E synthase 2	−2.5	4.8E-02	6	3
↓	P61073	CXCR4	C-X-C chemokine receptor type 4	−2.3	3.6E-02	5	4
↓	O43865	AHCYL1	Putative adenosylhomocysteinase 2	−2.2	3.1E-02	6	6
↓	Q8IZ81	ELMOD2	ELMO domain-containing protein 2	−2.2	4.7E-02	6	5
↓	B0I1T2	MYO1G	Unconventional myosin-Ig	−2.0	2.2E-02	5	5
↓	Q6UXV4	APOOL	Apolipoprotein O-like	−2.0	1.4E-02	6	6
↓	E5RJ99	ZFAND1	AN1-type zinc finger protein 1	−2.0	2.5E-02	6	5
↓	P53597	SUCLG1	Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha	−1.7	4.5E-02	6	6
↓	P22105	TNXB	Tenascin-X	−1.7	2.2E-02	6	6
↓	P06702	S100A9	Protein S100-A9	−1.6	4.7E-03	6	6
↑	G3V5I3	SERPINA3	Alpha-1-antichymotrypsin	1.5	4.1E-02	6	6
↑	Q15118	PDK1	[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 1	1.5	4.2E-02	6	6
↑	A2ACR1	PSMB9	Proteasome subunit beta type	1.7	4.3E-02	6	6
↑	Q8IYB5-3	SMAP1	Isoform 3 of Stromal membrane-associated protein 1	1.9	4.4E-02	6	6
↑	O95352	ATG7	Ubiquitin-like modifier-activating enzyme	2.0	3.7E-02	6	6
↑	Q5VY30	RBP4	Plasma retinol-binding protein(1-182)	2.1	2.4E-02	6	6
↑	Q9GZQ3	COMMD5	COMM domain-containing protein 5	2.3	4.3E-02	4	5
↑	Q9UHD1	CHORDC1	Cysteine and histidine-rich domain-containing protein 1	2.4	4.1E-02	5	6
↑	Q7L2H7	EIF3M	Eukaryotic translation initiation factor 3 subunit M	2.4	3.9E-02	5	6
↑	Q7Z2W9-2	MRPL21	Isoform 2 of 39S ribosomal protein L21	3.8	3.8E-02	1	5
↑	G3V1S6	GALNT2	Polypeptide N-acetylgalactosaminyltransferase 2 soluble form	3.8	1.9E-02	3	5
↑	Q6IC75	GGA1	ADP-ribosylation factor-binding protein GGA1	4.0	2.4E-02	2	6
↑	J3QLC8	RPL17	60S ribosomal protein L17	4.6	3.5E-02	1	4
↑	K7ER00	FARSA	Phenylalanine--tRNA ligase alpha subunit	5.4	1.5E-02	2	6
↑	P10114	RAP2A	Ras-related protein Rap-2a	Only after	1.4E-02	0	5
↑	Q15904	ATP6AP1	V-type proton ATPase subunit S1	Only after	2.7E-02	0	5
↑	Q96AQ8	MCUR1	Mitochondrial calcium uniporter regulator 1	Only after	4.7E-02	0	4

RELN (reelin), LRSAM1 (Leucine Rich Repeat And Sterile Alpha Motif Containing 1), RPS27L (ribosomal protein S27-like) and ENY2 (transcription and export complex 2 subunit) are already known to be upregulated and/or involved in cancer development and progression [35–40]. Interestingly, after surgical resection of the tumor, the concentrations of these four proteins in platelets of patients normalized towards control levels.

Secondly, we filtered for proteins exhibiting an abundance that was not only significantly higher in cancer versus healthy platelets (Table 2), but also in pre-surgery versus post-surgery cancer platelets (Table 5). In doing so, we focused on proteins that are known to be specifically linked to the presence of a malignant tumor. This strategy identified six proteins, four of which were already discovered using the first strategy. In addition, the second strategy pinpointed two other candidates, cathepsin G (CTSG) and S100A9 that may be of interest for the detection of early-stage cancer. The CTSG protein exhibited a 2.9-fold higher abundance in blood platelets from cancer patients when compared to platelets isolated from healthy individuals, and showed a 2.6-fold reduction in abundance after surgical tumor removal. The S100A9 protein, in turn, exhibited 1.7-fold difference in both comparisons. CTSG is known for its role in inflammatory processes and has also been linked to cancer [41–43]. Likewise, S100A9 has several links to malignant diseases.[42]

In addition, we also searched for proteins that were downregulated in platelets of patients with cancer compared to the platelets of healthy individuals (Table 3), but also in pre-surgery versus post-surgery platelets (Table 5). As a result, we found V-type proton ATPase subunit S1 (ATP6Ap1). The ATP6Ap1 protein was 7.2 fold less abundant in blood platelets of patients with cancer compared to platelets from healthy individuals. Moreover, it was upregulated in platelets of patients with cancer after surgical removal of the tumor (in the pre vs. post surgery samples only detectable after surgery). ATP6p1 is a protein against which, in cancer patients, autoantibodies are generated [45].

4. Discussion

In the current study, we showed that the platelet proteome of patients with early-stage lung or head of pancreas cancer differs considerably compared to that of healthy individuals of matched sex and age. In addition, the platelet proteome of cancer patients normalized after surgical resection of the tumor. Exploiting platelet proteome differences linked to both tumor presence and disease status, we were able to select seven differentially expressed platelet proteins associated with early-stage cancer.

Previous studies have demonstrated that tumors can affect platelet characteristics and content in patients with cancer [20–22]. Platelets contain vast quantities of bioactive proteins in their granules, which they can secrete upon activation [7,46]. A proteomics study in animals

demonstrated that platelets sequester regulatory proteins secreted from clinically undetectable tumors, resulting in significantly higher concentrations of these proteins in platelets of tumor-bearing mice compared to healthy controls [10]. While proteomics is increasingly being used to investigate disorders associated with impairments in platelet function [47], thus far, human studies investigating the effect of cancer presence on the platelet proteome were lacking. Yet, as platelets are easily accessible through venapuncture, their exploration represents an attractive avenue to detect and diagnose cancer compared to radiology and/or pathological analysis of tissue biopsies. The importance of platelets as a potential diagnostic tool in the field of oncology was highlighted by the study of Best et al. [21], who demonstrated that changes in the platelet RNA profile of patients with cancer could be used to discriminate patients with cancer from healthy individuals. Comparison of the differentially expressed proteins of the current study with the differentially regulated RNA's found in the paper of Best et al., revealed that several genes showed similar up (HNRNPA1, ATP2B1, EPB42, MITD1, RPS14, ANXA1, LCP1 and RNF213) or down regulation (NT5M, MRPL37, ATP6AP1 and COMMD7), while other genes exhibited an opposite expression pattern (SRRM2, HIST1H2Bj, CEP44, TMEM2, NDUFV3, UHRF1BP1, FLT, MBOAT2 and S100A8). None of the seven most discriminatory platelet derived cancer proteins of the current study was differentially regulated in the paper of Best et al. This discrepancy could be due to the difference in methods used in both papers (nanoLC-MS/MS vs. RNA sequencing), but it could also be caused by the difference in gender and age distribution and/or the presence of advanced stage cancers in the study of Best et al.

In the current study, the changes in platelet proteome of patients with cancer are already apparent before the cancer has metastasized, and offer new opportunities for the early detection of cancer. We identified a total of 4384 non-redundant proteins in platelets, of which 61 were at least 1.5-fold more abundant in platelets of patients with early-stage cancer compared to healthy volunteers. These proteins are either synthesized by the megakaryocytes that produce the platelets or absorbed by the circulating platelets from the blood, in which case these proteins may originate from the tumor [5,10]. The upregulated proteins were largely linked to inflammation, immune response and cytoskeleton organization and transport. This is in agreement with previous in vitro, in vivo and human studies, which show that cancer presence induces an immune and inflammation response from the host body [48]. Also, in pathological states, such as in malignant diseases, there is an altered activity of cellular cytoskeleton machinery and morphogenesis [49,50]. We also detected downregulated proteins in platelets of patients with early-stage lung or head of pancreas cancer. These bioactive proteins are mostly involved in antigen presentation/processing and membrane protein proteolysis. Previous studies have demonstrated that downregulation of antigen processing machinery is one of the mechanisms tumors use to evade immune surveillance [51]. Together, these data indicate that the effect of an active tumor in the body could be reflected inside platelets. The effect of early-stage head of pancreas cancer on the platelet proteome appears to be different compared to that of early-stage lung cancer. This supports the notion of tumor type-dependency of platelet changes, an effect that may be due to differences in cancer cell secretome and/or tumor localization [52].

Two different strategies were exploited to select differentially expressed proteins associated with early-stage lung or head of pancreas cancer. First, we selected proteins that were at least five times upregulated in platelets of patients with early-stage lung or head of pancreas cancer compared to platelets of healthy individuals, and are also known to be upregulated and/or involved in cancer progression. This selection resulted in four proteins of interest; RELN (reelin), LRSAM1 (Leucine Rich Repeat And Sterile Alpha Motif Containing 1), RPS27L (ribosomal protein S27-like) and ENY2 (transcription and export complex 2 subunit) [35–41]. RELN is a glycoprotein, which is upregulated in several cancer types and is involved in the regulation of cell migration and thereby stimulates cancer metastatic potential [35,53]. LRSAM1 (also known as Tsg101-associated Ligase) is an ubiquitin-protein ligase that mediates ubiquitination and inactivation of tumor suppressor TSG101 (Tumor susceptibility gene 101) [54,55]. The inactivation of TSG101 by LRSAM1 stimulates cancer progression. RPS27L is involved in rRNA assembly and protein synthesis, and is -in physiological conditions- an inhibitor of P53 that determines cell fate by modulating DNA damage response [39,56,57]. ENY2 expression is increased in several types of cancer such as breast and colorectal cancer [40] and its depletion inhibits tumor growth [58]. In summary, all four proteins stimulate tumor aggressiveness and cancer progression. Their increased concentration in platelets of patients with cancer seems to be associated with the presence of a malignant tumor, as surgical resection normalizes their expression in platelets.

As a second strategy, we explored whether any proteins, that were upregulated in platelets of patients with early-stage lung or head of pancreas cancer compared to healthy individuals, showed reduced or normalized expression after surgical removal of the tumor. This strategy again identified the four proteins that were selected during the first strategy. In addition, it resulted in two other proteins, CTSG and S100A9. These proteins are known to have an increased expression in early-stage cancer patients as compared to healthy controls, making them potential biomarkers of early-stage cancer. Both CTSG and S100A9 have been implicated in cancer progression [43,44]. S100A9 is a calcium-binding protein, which usually exists as a heterodimer with S100A8 [59], and is known for its involvement in inflammatory responses and cancer development and progression [44]. S100A9 is shown to be differentially expressed in various types of cancer such as breast, colon, liver, gastric and non-small cell lung cancer and is crucial in promoting cancer growth by recruitment of myeloid derived suppressor cells [44]. S100A9 is also described to be higher in serum of patients with cancer [60,61]. Interestingly, S100A9 is an important platelet protein and is secreted by platelets upon activation [46,62]. CTSG or Cathepsin G, which is a potent platelet activator [63], plays an important role in eliminating intracellular pathogens and breaking down tissues at inflammatory sites [41]. In addition, CTSG is also upregulated in various types of tumors and has been linked to an increase in TGF-beta signaling and tumor angiogenesis and metastasis [42,43]. Altogether, both S100A9 and CTSG seem to have important links to cancer development and progression, so their identification as proteins associated with early-stage cancer also has a functional foundation.

In addition, we also found V-type proton ATPase subunit S1 (ATP6Ap1) as a potential marker. ATP6Ap1 is significantly downregulated in platelets of patients with early-stage cancer, but this expression reverses after surgical resection of the tumor. ATP6AP1 is an ATPase component involved in various intracellular processes such as protein sorting, but also acidification of secretory granules [45]. It has been reported that in cancer patient's autoantibodies are generated against ATP6Ap1 [64], which could explain the low expression of this protein within platelets.

Overall, we were able to select seven differentially expressed proteins potentially associated with early-stage lung or head of pancreas cancer in platelets of patients. These proteins either originate from the megakaryocytes that produce the platelets or are absorbed by circulating platelets from the blood [5,10].

In biomarker discovery studies, it is important to validate potential biomarkers in a larger and independent cohort of patients. While this was outside the scope of this feasibility study, we did perform a subset analysis, as a proof-of-concept validation set, on both cancer patient groups separately. This evaluation confirmed that most of the differentially expressed proteins potentially associated with early-stage cancer, that we initially selected, were significantly changed in early-stage lung (RELN, LRSM, ENY2, CTSG and S100A9) and pancreatic (RELN, CTSG and S100A9) cancer.

This proof-of-concept study demonstrates that the platelet proteome significantly changes when malignant disease materializes, even in patients with early-stage and localized disease. Hence, the platelet

proteome can be mined for potential cancer biomarkers. Future research is needed to validate our current findings in independent, larger and possibly more diverse cohorts of patients and to further investigate their clinical relevance. Also, the effect of different types of cancer treatment (e.g. chemo- and radiotherapy) on the platelet proteome of cancer patients should be investigated to establish whether platelets can be used to monitor response to treatment. Thus, blood platelets offer a promising new source of potential biomarkers and should be further studied in future blood-based biomarker research.

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Conflicts of interest

There are no conflicts of interest.

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