

# Accelerated endoplasmic reticulum and coagulation factor processing in platelets are linked with the hypercoagulable state of patients with lung cancer

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## Research

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## Abstract

## Background

The risk of venous thromboembolism in cancer is nine times higher than in the general population and the second leading cause of death in these patients. Platelets play a key role in tumour growth, metastasis, and cancer-associated thrombosis. Despite this widely observed functional role of platelets in the prothrombotic state of certain cancers, the underlying molecular mechanisms are largely unknown.

## Methods

In order to comprehensively expose cancer-related biochemical changes, we compared the platelet proteome of two types of cancer with a high risk of thrombosis (22 patients with brain cancer, 19 with lung cancer) to 41 matched healthy controls using unbiased two-dimensional differential in-gel electrophoresis. Cancer-related platelet proteins were statistically characterised by adjusted one-way ANOVA and planned comparisons post hoc tests. Significantly changed platelet proteins were identified by mass spectrometry. Afterward, selected cancer-associated platelet proteins were validated by 1-D and 2-D Western blots and/or functional assessments were made by association analysis with central haemostatic plasma parameters.

## Results

The examined platelet proteome was unchanged in patients with brain cancer, but considerably affected in lung cancer with 15 significantly altered proteins. Amongst these, the endoplasmic reticulum (ER) proteins CALR, HSPA5 and P4HB were significantly elevated. Accelerated conversion of the fibrin stabilising factor has been detected in platelets of patients with lung cancer by elevated levels of a F13A1 55 kDa fragment. A significant correlation of this F13A1 cleavage product with plasma D-dimer levels suggests an enhanced degradation in platelets by the fibrinolytic system. Functional protein association network analysis showed that lung cancer-related protein profiles were involved in platelet degranulation and upregulated ER protein processing. The latter is important in the glycosylation of coagulation factors. As a possible outcome, plasma FVIII, an immediate end product for ER-mediated glycosylation, correlated significantly with the ER-executing chaperones CALR and HSPA5. In addition, several lung cancer-related platelet proteins such as decreased levels of ITGA2B are significantly linked to the plasmatic haemostasis factors D-dimer and fibrinogen.

## Conclusions

These new data on the differential behaviour of platelets in various cancers revealed F13A1 and ER chaperones as potential novel diagnostic and therapeutic targets in lung cancer patients.

## Introduction

Patients with cancer have an increased risk to develop venous thromboembolism (VTE) (1). This complication occurs in up to 20% of affected individuals per year (2) and is associated with high morbidity and mortality (3, 4). Malignancies with the highest incidence of VTE are brain and lung cancer (5). Platelets are thought to play an important role in the prothrombotic state and the development of cancer-associated VTE as well as arterial thromboembolism (6). Besides their crucial functions in primary haemostasis, platelets are also important players in the progression of VTE. Various platelet associated parameters were already identified to be linked with increased risk of VTE in patients with cancer such as high platelet counts (7) and exposed phosphatidylserine on platelets (8). High plasma levels of soluble P-selectin (sCD62P) indicate cancer-associated platelet activation (9). In a previous study of patients with different types of cancer, we found a statistical association of increased VTE risk and poor survival rate with lower surface expression of receptors important for platelet activation, namely CD62P and glycoprotein IIb/IIIa, a complex of integrin alpha IIb (ITGA2B) and beta 3 (ITGB3) (5). The decline of these platelet receptors is supposed to be caused by shedding during continuous platelet activation, stronger ongoing in cancer patients with increased

mortality and VTE rate (5). Platelet activation is also suggested to play a role in the progression of cancer and metastasis formation, as shown by experimental studies *in-vitro* (10) and in knock-out mice with depleted peripheral platelets (11). Furthermore, in clinical studies anti-platelet therapy with aspirin reduced the risk of cancer and metastasis development (12, 13). Although many studies have shown connections between platelets, cancer and thrombosis, the molecular mechanisms are not entirely understood. Despite this recognized importance of platelets in cancer (14, 15) and in many other prothrombotic conditions, clinical studies with comprehensively and unbiased analysis of the platelet proteome in particular from well-defined high thrombosis risk patient cohorts have scarcely been performed. In a recent study, we investigated the platelet proteome of Lupus anticoagulant (LA) positive patients, who are at high risk to develop thrombosis. These investigations revealed changes of functional important platelet proteins which uncover new prothrombotic triggers of primary haemostasis (16).

Due to the lack of a nucleus platelets have a rather small and rapidly declining amount of mRNA and in line with that also a low rate of protein synthesis. Consequently, regulatory mechanisms are mostly detectable on the level of pre-existing proteins and their post translational modifications (PTMs). A proteomic investigation of these processes might help to elucidate the development of cancer-associated VTE and may contribute substantially to better diagnostics, prevention, and therapy, and thus ultimately to patient survival.

The proteomics technology of fluorescence-based two-dimensional differential gel electrophoresis (2D-DIGE) was selected to reveal in one analytical process qualitative and quantitative cancer-related differences of proteins and corresponding PTMs (17). This gel-based “top-down” proteomics technology remains still the preferred method for parallel quantitative characterisation of intact proteins and their corresponding PTMs (= proteoforms) (18, 19). Protein spots of interest were worked up for proteomics analysis by mass spectrometry (MS) to obtain protein identities.

Despite the important role of platelets in development of cancer-associated VTE, no prospective systemic study investigating the respective platelet proteome have been performed so far. To address this, we investigated the platelet proteome in patients with two different types of cancer to identify potential mechanisms of increased risks for VTE and/or mortality and possible molecular pattern that are specific for the cancer type. Our data demonstrate that several proteins and/or corresponding proteoforms were significantly changed in the platelet proteome of patients with lung cancer, but not in brain cancer.

## Material And Methods

### Study design

Forty-one patients with cancer (lung and brain) enrolled in framework of the Vienna Cancer and Thrombosis Study (CATS) were included in this analysis. CATS is a prospective, observational, single-centre cohort study started in October 2003 at the Medical University of Vienna. Patients included in the present study were recruited between 2014–2017. All detailed inclusion and exclusion criteria were previously described (20, 21). Briefly, adult patients ( $\geq 18$  years) with newly diagnosed cancer or cancer progression after partial or complete remission were enrolled in the study. Patients who were not treated with anticoagulant drugs in the past three months, did not undergo chemotherapy in the last 3 months or receive radiotherapy or surgery within the past 2 weeks, were informed about the study design and gave written consent. At study inclusion, patient history was documented with a structured interview and furthermore, patients enrolled in the CATS study were monitored with a structured questionnaire on a three-month basis for occurrence of VTE and death. For this study and its hypothesis generating approach we used a case control design and included a matched healthy control cohort. Follow ups were made after 3 and 6 months and two years.

Forty-one age and sex matched healthy volunteers were included after giving written informed consent. Venous blood was drawn from cancer patients and healthy controls for platelet proteome investigations, routine and coagulation parameter analysis as well as for further experimental approaches. Data on the study populations can be found in Table 1.

This study was approved by the Ethics Committee of the Medical University of Vienna in accordance with the Declaration of Helsinki (EC no. 126/2003 and 039/2006)

### Blood collection, washed platelet and plasma isolation

For platelet isolation, blood was drawn from an antecubital vein into 3.5 mL vacuum tubes containing CTAD (0.129 mM trisodium citrate, 15 mM theophylline, 3.7 mM adenosine, 0.198 mM dipyridamole; Greiner Bio-One, Kremsmünster, Austria) as anticoagulant. The first tube drawn was discarded to avoid any contaminations. Immediately following blood draw, CTAD tubes was centrifuged at 120 xg for 20 min without brake at room temperature. Obtained platelet rich plasma (PRP) was carefully transferred into a fresh tube containing prostacyclin I<sub>2</sub> (0.8 µM, PGI<sub>2</sub>; Sigma-Aldrich, St. Louis, MO, USA) to prevent platelet aggregation and degranulation during the following washing steps. PRP was pelleted by centrifugation for 3 min at 3000 xg at room temperature and the protein pellets were washed twice in phosphate-buffered saline (w/o: Ca<sup>2+</sup> and Mg<sup>2+</sup>) containing PGI<sub>2</sub> (0.8 µM). Before the last centrifugation step, platelet count was determined with a Sysmex XN-350 hemacytometer (Sysmex, Kobe, Japan). After the last centrifugation step the supernatant was completely taken off and the platelet pellet was snap-frozen in liquid nitrogen and stored at -80°C until processing.

For preparation of plasma blood was drawn into 3.5 mL vacuum tubes containing sodium citrate (0.129 mM citrate; Greiner Bio-One, Kremsmünster, Austria). The blood was centrifuged at 2500 xg for 15 min at 15°C to separate the cellular fraction and the plasma supernatant was stored at -80°C.

## Platelet preparation for 2D-DIGE analysis

The frozen platelet protein pellets were resolubilized in urea-sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-HCl pH 8.68) and incubated for 2 h at 4°C under agitation (800 rpm). Protein quantitation of individual samples was done in triplicate with a Coomassie brilliant blue protein assay kit (Pierce, Thermo Scientific, Rockford, IL, USA). The internal standard (IS) was made by pooling the same protein amounts from each platelet samples of all included study participants. Platelet protein samples and IS were aliquoted and stored at -80°C.

## Platelet proteome analysis by two-dimensional fluorescence differential gel electrophoresis (2D-DIGE)

Prior electrophoresis, proteins were labelled with fluorescent cyanine dyes (5 pmol of CyDyes per µg of protein; GE Healthcare, Uppsala, Sweden) according to previous concentrations (16). The IS was always labelled using Cy2, while Cy3 and Cy5 were used alternately for study samples. Briefly, IPG-Dry-Strips (24 cm, pH 4–7, GE Healthcare, Uppsala, Sweden) were rehydrated for 11 h with 450 µL rehydration buffer (7 M urea, 2 M thiourea, 70 mM DTT, 0.5% pH 4–7 ampholyte; Serva, Heidelberg, Germany) mixed with a total of 36 µg (2x 12 µg sample + 1x 12 µg IS) of alternatively Cy-labelled sample. Isoelectric focusing (IEF) was performed on a Protean I12 IEF unit (Biorad) until 30 kVh was reached.

After IEF, the strips were first equilibrated with gentle shaking in 12.5 mL of equilibration buffer 1 (1% DTT, 50 mM Tris-HCl pH 8.68, 6 M Urea, 30% glycerol and 2% SDS) for 20 min followed by incubation in equilibration buffer 2 (2.5% iodoacetamide, 50 mM Tris-HCl pH 8.68, 6 M Urea, 30% glycerol and 2% SDS) for 15 min. Each of the IPG strips was transferred on 11.5% acrylamide gel (26x 20 cm, 1 mm gel) and sealed with low melting agarose sealing solution (375 mM Tris-HCl pH 8.68, 1% SDS, 0.5% agarose). The SDS-PAGE was performed using an Ettan DALTsix electrophoresis chamber (GE Healthcare, Uppsala, Sweden) under the following conditions: 35 V for 1 h, 50 V for 1.5 h and finally 110 V for 16.5 h at 10°C.

## 2D-DIGE image analysis

For protein spot detection, 2D-DIGE gels were scanned with three different wavelengths of the particular CyDye at a resolution of 100 µm using a Typhoon 9410 Scanner (GE Healthcare, Uppsala, Sweden). Gel images were analysed via the DeCyder™ software (version 7.2, GE Healthcare, Uppsala, Sweden). Spots were matched to a master 2D-DIGE gel (a representative pH 4–7 platelet protein map of the IS images). On average, 500 protein spots were matched manually to the master gel using the DeCyder™ software. Afterwards an automatic spot match was used which achieved an average of 4310 matched spots per gel. The standardised abundance (SA) of every protein spot was calculated by the DeCyder™ software with two normalisation steps. The first step is the in-gel normalisation by dividing each spot with the center volume of the corresponding spot map and a the second one by dividing each normalised spot volumes against the corresponding spot normalised spot value of the IS. (22). Detailed information about the image analysis was published by Winkler *et al.* (23).

## Protein identification via mass spectrometry

For MS-based identifications, 250 µg unlabelled proteins were separated by 2D-DIGE and proteins were visualized by MS-compatible silver staining (24). Protein spots of interest were excised manually from the gels, de-stained, disulfide were reduced as well as derivatized with iodoacetamide and the proteins were tryptically digested. Subsequently, these peptide samples were applied onto a Dionex Ultimate 3000 RSLC nano-HPLC system (Thermo Scientific) and afterward directly subjected to a QqTOF mass spectrometer oTOF compact (Bruker Daltonics) equipped with nano-flow CaptiveSpray ionisation device. Detailed analytical conditions were previously described (25). The protein identification was obtained with database searches against UniProtKB/Swiss-Prot (2020-06) using Mascot v2.7 server.

## One and two-dimensional Western blot analysis

For one-dimensional Western blot (1-D WB), 12 µg total platelet protein were mixed with a sample buffer (150 mM Tris-HCl pH 8.68, 7.5% SDS, 37.5% glycerol, bromine phenol blue, 125 mM DTT) to obtain a final volume of 20 µL. Afterward, samples were boiled for 4 min at 95°C and centrifuged for 3 min at 20,000 xg. Thereafter, the samples were separated on a 11.5% SDS gel (50 V, 20 min and 100 V, 150 min) and blotted (75 V, 120 min) on a nitrocellulose membrane (NC; Pall, East Hills, NY, USA) or polyvinylidene difluoride membrane (PVDF; FluoroTrans® W, Pall, East Hills, NY, USA). For protein quantification, total protein on the membrane was stained using a ruthenium(II)-tris-(bathophenanthroline disulfonate) (RuBPS; dilution 1:100,000 overnight at 4°C; Sigma-Aldrich St. Louis, MI, USA).

For two-dimensional Western blot (2-D WB) analysis, 36 µg Cy2-labeled platelet proteins were separated by IEF on either a 7 cm pH 3–10 or a 24 cm pH 4–7 IPG strip as described for 2D-DIGE gels, and subsequently transferred onto a NC- or PVDF membrane (75 V, 90 min). Afterward, the membrane was blocked with 5% non-fat dry milk (BioRad, Hercules, CA, USA) in 1x PBS containing 0.3% Tween-20 (PBS-T) over night at 4°C under gentle shaking. On the next day, membranes were washed (3x with PBS-T for 5 min, each). For detection, following primary antibodies were used in the corresponding dilutions by incubation for 2 h at room temperature (180 rpm) in PBS-T containing 3% non-fat dry milk: monoclonal anti-Factor F13A1 (ab1834; Abcam, USA) 1:250, polyclonal anti-CD41/Integrin alpha 2b (ab83961; Abcam, Cambridge, UK) 1:200, monoclonal anti-Integrin beta 3 [EPR2342] (ab119992, Abcam, USA) 1:1000, monoclonal anti-Calreticulin [FMC 75] (ab22683; Abcam, USA) 1:500, polyclonal anti-HSPA5 (ab21685; Abcam Cambridge, UK) 1:250 and monoclonal anti-Talin clone 8D4 (SAB4200694, Merck, Germany) 1:300. After washing (3x with PBS-T for 5 min, each), the membranes were incubated with a DyLight 650 conjugated secondary antibody (Novus Biologicals, Littleton, CO, USA), diluted 1:500 or with a horse-radish peroxidase (HRP)- conjugated secondary antibody, diluted 1:20,000 in PBS-T containing 3% non-fat dry milk for 1.5 h in the dark at room temperature (65 rpm). After washing (3x with PBS-T for 5 min, each), the membranes were incubated for 1.5 h in the dark at room temperature (65 rpm). After washing (2x with PBS-T, 1x with 1x PBS for 5 min, each), the antibody fluorescence signals were detected with a Typhoon FLA 9500 imager (GE Healthcare, Uppsala, Sweden) at a resolution of 100 µm. The HRP signal was detected using an Enhanced Chemiluminescent (FluorChem® HD2, Alpha Innotech, CA USA). The 1-D WB antibody signal of F13A1 were normalized by the RuBPS fluorescence signal from the 40 kDa to 100 kDa bands and quantified with ImageQuant 8.0 (GE Healthcare, Uppsala, Sweden).

## Measurement of haemostatic biomarkers in plasma

FXIII activity in citrate plasma was measured by Berichrom Factor XIII chromogenic determination (Siemens Healthcare) according to manufacturers' instructions. Factor VIII activity was measured on a Sysmex CA 7000 analyzer using factor VIII-deficient plasma (Technoclone) and APTT Actin-FS (Dade Behring). D-dimer levels were measured by a quantitative latex assay (STA-LIATEST D-DI; Diagnostica-Stago, Asnieres, France) on a STAR analyser (Diagnostica-Stago) according to the manufacturer's instructions. Fibrinogen was routinely measured in platelet poor plasma according to Clauss (STA Fibrinogen; Diagnostica Stago, Asnieres, France; normal range: 180–390 mg/dL). C-reactive protein (CRP) was measured with a fully automated particle enhanced immunonephelometry (N high sensitivity CRP, Dade Behring, Marburg, Germany). For sCD62P, to obtain definitely platelet-free plasma a second centrifugation step (Eppendorf) at 13,400 xg for 2 min was performed. These plasma aliquots were also stored at – 80°C, until the determination of sCD62P plasma levels in series. Soluble CD62P levels were measured using a human sCD62P immunoassay (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

## Fluorogenic F13A1 activity assay from platelet samples

Washed platelets from 42 patient's samples were lysed by 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and sonicated for 1 min at 4°C. After centrifugation (15,000 xg, 4°C) lysed platelets were transferred to a fresh tube. Protein concentration in the lysate was determined by Coomassie. F13A1 activity in platelets was performed by a fluorogenic FXIIIa enzyme activity kit (Zedira GmbH, #F001, Darmstadt, Germany) essentially according to the manufacturers' protocol with minor modifications. Measurements were performed in triplicate in 96-well flat bottom black microplates (Thermo Scientific, #137101, Denmark). Ten µL of lysed samples was mixed with 90 µL reagent mix solution (modified peptide, 100 NIH Units thrombin, Tris buffer pH 7.5 containing CaCl<sub>2</sub>, NaCl, polyethylene glycol (PEG), glycine methyl ester, clot inhibitor peptide, Heparin antagonist (hexadimethrine bromide) and sodium azide), creating a final volume of 100 µL/well. As a control for specificity of enzymatic F13A1 activity, the irreversible transglutaminase inhibitor T101 (50 µM final concentration) was used. The enzyme kinetic were recorded at 37°C using a Varioskan LUX microplate reader (Thermo Fisher Scientific) with excitation at 313 nm and emission at 418 nm in the kinetic mode, absorbance was read every 36 s for 15 min and the change of absorbance between 0 and 15 min were detected. F13A1 activity was determined on the basis of a standard curve constructed with washed human platelets. Microsoft excel was used for further processing of enzyme kinetic data.

## Biological pathway analysis

Biological data base analysis was made from the fifteen lung cancer-related platelet proteins. The data source for the protein-protein interaction (PPI) networks was the protein query of the STRING database (26), with the settings (active interaction sources: experiments and databases; score = 0.4; maximal additional interactors = 0). For the functional enrichment, the Gene Ontology Biological Processes and KEGG pathway analysis were used for the PPI networks with a specific colour for each biological process and KEGG pathway. The STRING Version 11.0b was used.

In addition, we applied the NetworkAnalyst platform (27, 28) for further analysis. The differentially expressed proteins were uploaded to the web-platform ([www.networkanalyst.ca](http://www.networkanalyst.ca)) and generic protein-protein interaction (PPI) network analysis was performed with the Imex interactome (29) resulting in a first-order network with 722 nodes and 889 edges comprising the 15 lung cancer proteins as seeds. This network was downloaded as graphML-file and imported into the Cytoscape 3.8.2 program. The stringApp was used to "STRINGify" the network, followed by functional enrichment of pathways and gene ontologies, which were sorted according to p-values. Relevant pathways were selected and coloured using the bypass function for node colours.

## Statistics

For statistical analysis, from each 2D-DIGE image only protein spots are included which could be matched by the IS spot map with more than 95% of all 2-D platelet proteome maps of this study. This quality selection limits resulting protein spots to 617 from in average 2720 to the master gel matched spots. One-way and two-way analysis of variance (ANOVA) were calculated for these 617 highly reliable matched spots between the three study groups (healthy controls, patients with lung and brain cancer). Resulting p values of the one-way ANOVA were false discovery rate corrected (FDR) by Benjamini Hochberg for multiple comparisons (30). FDR-corrected one-way ANOVA and unadjusted two-way ANOVA were calculated with the Extended Data Analysis (EDA) module of the DeCyder™ software (version 7.2, GE Healthcare, Uppsala, Sweden). Supervised principal component analysis (PCA) of one-way ANOVA significant spots was also performed by this EDA module to control sample clustering. To assess the effect of mortality on the examined platelet proteom between cancer type groups, the two-way ANOVA main effects "mortality", "cancer type group" and interaction term "cancer type group \* mortality" were included. Significant differences between control group and each cancer group were analysed by planned post-hoc contrasts analysis with unpaired Student's t-Test in SPSS and corrected for multiple comparison by the online FDR calculator (31). For a direct comparison of different parameters the effect size was calculated by Cohen's D =  $(\text{mean}^1 - \text{mean}^2) / \text{standard deviation}^{\text{pooled}}$ . Graphs were created with GraphPad Prism 6 (GraphPad Software, Inc. San Diego California, USA).

## Results

### Patient characteristics

To investigate if and how the platelet proteome is affected in patients with cancer and high thrombosis risk, in total 82 study participants were included in this study: Twenty-two patients with brain cancer, 19 patients with lung cancer and 41 healthy age

and sex-matched healthy controls.

During the course of the study, two patients with brain (9.1%) and two patients with lung cancer (10.5%) had a VTE. These thrombotic events included pulmonary embolism (PE; 7.3%) and deep vein thrombosis (DVT; 2.4%). Eight patients with brain cancer (36.4%) and ten patients with lung cancer (52.6%) died during the follow up. Detailed characteristics of the study populations are presented in Table 1.

## Platelet proteome of patients with brain and lung cancer compared to controls

For the identification of differentially regulated platelet proteins in patients with brain and lung cancer and matched healthy controls, the platelet proteome of these samples were analysed by 2D-DIGE in the pH range 4–7 (Fig. 1). After the application of protein spot quality selection criteria (defined in the material and methods section) a total of 617 protein spots were included in statistical analysis.

Platelet counts of patients with lung cancer were significantly higher compared to healthy controls and patients with brain cancer (Table 1). Hence, it may be supposed that changes of the platelet proteomes of patients with lung cancer can also be associated to increased platelet counts. To prove this possible bias of a platelet proteomics study, platelet counts were correlated with the 617 2D-DIGE quantified platelet protein spots from the whole study cohorts. No significant correlation could be detected by these statistical control evaluations. This assessment ensured that cancer-related protein profiles in platelets were not biased by differences in platelet counts

Significant differences in platelet protein levels between the two cancer patients groups with high thrombosis risk and healthy controls were determined by one-way ANOVA. Multiple comparison of variance in the platelet proteome revealed 36 significant protein spot changes between the three study groups (FDR-adjusted  $P < 0.05$ ; Fig. 1; Table 2). Identification of these 36 protein spots by MS showed that these proteoforms correspond to 19 different proteins (Table 2). To get a graphical overview of these cancer-related proteome data, PCA was performed to visualize the overall difference of the 36 significantly altered platelet protein spots from every study participant (Fig. 2a). Furthermore, spots from each study group can be summarized in a graph to enable a straightforward comparison between the three study cohorts (Fig. 2b). PCA analysis revealed a lower variance between patients with brain cancer and healthy participants, but a stronger difference between lung cancer patients and other cohorts.

Adjusted post-hoc contrast calculations of these 36 one-way ANOVA-filtered proteoforms between each cancer type and matched controls confirmed the graphical PCA overview. These further statistical evaluations revealed 27 significant lung cancer-related protein spot changes but no significant brain cancer-related difference (Table 2). Identification of the protein spots by MS showed that the 27 lung cancer related proteoforms belonged to 15 different proteins (Table 2). Therefore, further investigations focused on the lung cancer-related platelet protein changes.

## Pathway analysis of lung cancer-related platelet proteins

To assess coherent protein networks and functional relationships of lung cancer related platelet protein changes, biological database analysis was conducted. Unbiased STRING protein interaction analysis allocated lung cancer-related changes mainly to proteins primary responsible for thrombosis. Based on the altered levels of ITGA2B, ITGB3, TLN1, F13A1, TF and HSPA5 the highest significant functional enrichment was found for the biological process of platelet degranulation ( $p = 3.19 \cdot 10^{-11}$ ) and for platelet activation in KEGG pathways ( $p = 4.47 \cdot 10^{-5}$ ). Additionally, a significantly altered KEGG pathway was protein processing in the endoplasmic reticulum (ER) ( $p = 4.74 \cdot 10^{-6}$ ) (Fig. 3). These findings were further supported by analyses using the *NetworkAnalyst* approach (27), where differentially altered genes or proteins are compared with known networks such as the human protein interactome, followed by the computation of a first-order network, which is then queried against different databases, including KEGG and Reactome of gene ontology databases. This leads to a very robust identification of significantly altered pathways or biological processes (Additional file 1: Fig. S1).

Based on these findings and literature recherche the main stabilizer of the fibrin clot, F13A1, and the ER chaperones P4HB, CALR and HSPA5, important for folding and glycosylation of secretory proteins such as coagulation factors (32, 33), were chosen for

deeper characterisations and functional explorations.

## F13A1 protein processing is changed in platelets of patients with lung cancer

The abundance of a 55 kDa fragment (pI 5.00) from F13A1 was significantly increased in platelets of patients with lung cancer (Table 2; Fig. 1). The fibrin crosslinking protein F13A1 is a transglutaminase with a molecular weight of 83 kDa.

MS-based identification of this 55 kDa fragment of F13A1 was validated by 2-D WB. An antibody, raised against the full length F13A1, recognised the respective 55 kDa proteoform. In addition, the F13A1 antibody bound to three spots with a MW of 83 kDa and pI between 5.85 and 5.65. Subsequent MS analysis confirmed these spots as F13A1 proteoforms (Fig. 4a). Another two F13A1 spots (12 and 12d) were identified by MS in the near neighbourhood (Additional file 2: Fig. S2b). The abundance of all these full-length F13A1 spots were also found to be increased in platelets of patients with lung cancer compared to matched healthy controls, except one spot with a pI of 5.85 (spot 12c; Table 3). Increased levels of the 55 kDa F13A1 proteoform in platelets of patients with lung cancer were confirmed by fluorescence 1-D WB with a significant correlation ( $r_s = 0.843$ ;  $p = 2.0 \times 10^{-6}$ ) to the corresponding 2D-DIGE signals (Additional file 3: Fig. S3a). The abundance of the full-length F13A1 proteoforms were visible as a single band but the lung cancer-related increase of the 83 kDa proteoforms between the pI 5.75 to 5.60 could not be detected by 1-D WB. These unchanged quantitative 1-D profiles of the full-length F13A1 in patients with lung cancer may be based on the significant negative (opposite) correlation of the abundance from the most alkaline F13A1 spot (pI 5.85; spot 12c) to the levels of all other 83 kDa proteoforms from F13A1 (Additional file 4: Table S1). Interestingly, this F13A1 spot 12c with the pI 5.85 was also in the opposite direction changed in patients with lung cancer compared to all other F13A1 proteoforms (Table 3).

To investigate functional outcomes of these altered levels of F13A1 proteoforms in patients with lung cancer, its enzymatic activity was measured in platelets and plasma of the particular study samples. No change of enzymatic F13A1 activity was detectable in platelets of patients with lung cancer (Fig. 4f). However, enzymatic F13A1 activity significantly correlated with the levels of F13A1 spots 12b (pI 5.75), 12 (pI 5.60) and 12a (pI 5.65), abundances of which were significantly increased in patients with lung cancer (Table 3). Once again, the F13A1 level of spot 12c (pI 5.85) attracted attention with an opposite direction by a weak trend of a negative correlation to enzymatic F13A1 activity (Table 3). These observations indicated that a pI shifting PTM regulates the enzymatic activity of these 83 kDa F13A1 proteoforms in platelets, whereby the most alkaline one (spot 12c; pI 5.85) seems to be the inactive variant. The 55 kDa spot 11 of F13A1 did not correlate with enzymatic activity of this transglutaminase (Table 3).

Generally F13A1 is activated by thrombin with the enzymatic removal of a short (2–37) activation peptide (34, 35), which results into the 79 kDa activated F13A1 proteoform. Whereas a 55 kDa fragment of F13A1 is observed to be produced after the enzymatic inactivation of activated F13A1 by further cleavage with e.g. thrombin (36), chymase (37) and plasmin (38). MS analysis of the lung-cancer-related 55 kDa F13A1 cleavage product detected peptides between the amino acid sequence 13 and 492 (Additional file 5: Fig. S4a). For inactivation of F13A1, thrombin cuts at K514 (36), chymase at F574 (37) and plasmin at R492 (38). Accordingly, several of these serine proteases can be responsible for this 55 kDa F13A1 cleavage product in platelets (Additional file 5: Fig. S4b). Interestingly, MS analysis of this platelet 55 kDa F13A1 fragment identified also peptides from the amino acid sequence 13 to 38, which is within the amino sequence of the activation peptide. Thus, the present MS data indicated that this 55 kDa fragment was not cleaved from the activated proteoform of F13A1 in platelets (Additional file 5: Fig. S4a).

A long-standing hypothesis is that activated F13A1 is inactivated by the fibrinolytic system (38). To evaluate these *in-vitro* observations in the clinical situation of this study, the levels of the 55 kDa fragment of F13A1 in platelets of patients with lung cancer and matched controls were correlated with corresponding plasma levels of the fibrinolysis product, D-dimer. This degradation product of cross-linked fibrin is a routine established indicator for VTE and also reflects cancer-associated thrombosis risk (39). The haemostatic biomarker D-dimer correlated significantly ( $r_s = 0.599$ ;  $p_{\text{unadj}} = 1.40 \times 10^{-4}$ ) with the 55 kDa fragment of F13A1 in the platelets of patients with lung cancer and matched healthy controls (Fig. 4d). However, D-dimer levels were significantly increased in patients with brain and lung cancer by an effect size (Cohen's D) of 1.02 and 0.94 respectively.

Interestingly, the lung-cancer-related increase of the 55 kDa degradation product from F13A1 had a stronger effect size with 1.15 compared to the plasma thrombolytic biomarker D-dimer with 0.94.

Because in clinical studies F13A1 are more frequently investigated in plasma, its enzymatic activity was also analysed in the plasma of recruited cancer and matched healthy controls. Plasma F13A1 activity was unchanged in patients with lung cancer and was significantly decreased in patients with brain cancer (Fig. 4e).

### **Chaperones from the KEGG pathway “protein processing in endoplasmic reticulum” are elevated in platelets of patients with lung cancer**

One-way ANOVA-based differential 2D-DIGE analysis identified significantly changed abundance of two P4HB, five CALR spots, and one spot of HSPA5 in platelets between patients with lung and brain cancer and matched healthy controls (Table 2). Post-hoc contrast analysis showed that all these ER chaperones are increased in platelets of patients with lung cancer. Two-dimensional WB analysis confirmed MS identifications of these CALR and HSPA5 spots and identified three more CALR and two more HSPA5 proteoforms (Fig. 5a), which were also all increased in patients with lung cancer.

### **Functional relationships of the platelet proteome with haemostatic plasma laboratory parameters from patients with lung cancer and matched controls**

Interestingly, ER chaperones are frequently described to assist in the final biosynthesis of coagulation factors. The main member P4HB (40) of the protein disulfide isomerase (PDI) family mediates the formation of disulfide bond from FIII (41, 42), FV (43), and FXI (44). This redox based-change of their conformation contributes to enhanced activation of these coagulation factors and consequently for an accelerated generation of thrombin (45) and fibrin. Although plasma D-dimer is an indicator for enhanced fibrinolysis, increased D-dimer levels also indicate the preceding step of an increased fibrin generation. Thus, the functional enhancement on coagulation factor activity (e.g. FV) by more PH4B, may be indirect reflected by the significant correlation of platelet PH4B levels with plasma D-dimer levels of patients with lung cancer and matched controls (spot 28:  $r_S = 0.586$ ;  $p_{\text{unadj}} = 3.10^{-6}$ ).

The ER proteins CALR and HSPA5 are essential chaperones for glycosylation and secretion of proteins and this function is described for the final biosynthesis of coagulation factors (33, 46, 47). Besides F13A1, platelets are also a potential source for coagulation factors e.g. FV (43) and FVIII (48–50). Hence, the FVIII activity, an important activation marker of the haemostatic system, was significantly increased in plasma of patients with lung cancer (Table 1; FC = 1.75;  $p = 0.0002$ ) and to a lesser extent in patients with brain cancer (Table 1; FC = 1.26;  $p = 0.001$ ). In line with the assumption that the processing and release of this glycoprotein may be influenced by the enhanced pathway of “protein processing in ER”, a significant positive correlation was detected between the ER-chaperoning proteins CALR spot abundances (spot 3:  $r_S = 0.557$ ;  $p_{\text{unadj}} = 3.41^{-4}$ ) and HSPA5 (spot 15:  $r_S = 0.628$ ;  $p_{\text{unadj}} = 3.23^{-5}$ ) of platelets with the corresponding plasma values of FVIII from patients with lung cancer and matched controls (Fig. 5d and 5e).

However, these observations of significant correlations of platelet ER glycosylation chaperones with FVIII or the 55 kDa F13A1 fragment and P4HB with plasma D-dimer levels may be biased by literature-based hypothesis generation. To uncover and manifest an unbiased and specific interaction of certain platelet proteins with haemostatic and inflammatory plasma biomarkers, all 617 2D-DIGE quantified platelet proteoforms from patients with lung cancer and matched controls were correlated with their corresponding plasma levels of five well-known cancer-associated VTE plasma biomarkers. These were FVIII activity (51, 52), prothrombin fragment 1 + 2 (53), D-dimer (3), fibrinogen (54) and the acute phase protein CRP. In fact, the strongest correlations of plasma FVIII with these 617 platelet proteins were found with the ER chaperoning proteins P4HB, HSPA5 and CALR allocated to the interactome pathway “protein processing in ER” (Fig. 6d). Remarkably, the substrate for F13A1, plasma fibrinogen, had the strongest correlation with the 55 kDa fragment of fibrin stabilising factor F13A1 of the platelet proteome (Fig. 6e). The platelet 55 kDa fragment of F13A1 had also one of the strongest correlation with the acute phase proteins CRP (Fig. 6c), which indicates the strong interplay of inflammation with essential steps of haemostasis, which finally may result into immunothrombosis. In contrast to the strong association of plasma FVIII with the pathway “protein processing in ER” in platelets, D-dimer, fibrinogen and CRP correlated significantly with proteins from platelet degranulation and platelet activation pathways such as filamin A

(FLNA) and the integrins ITGA2B and ITGB3 (Fig. 6a, 6c and 6e). No strong correlations of the platelet proteome were found with the plasma laboratory values of prothrombin fragment 1 + 2, antithrombin III and plasminogen activator inhibitor 1.

For an additional functional proof, for the specificity of lung-cancer-related platelet protein profiles to platelet activation, the 617 2D-DIGE quantified platelet proteoforms were also correlated to plasma levels of sCD62P, reliable biomarker for platelet activation *in-vivo* (55). Again, platelet degranulation and the ER protein processing pathways were significantly enriched via the sCD62P-correlating platelet proteins by STRING interactome analysis (Fig. 6b). All platelet proteins correlating significantly with haemostatic plasma laboratory parameters are indicated in additional file 10: Fig. S8.

The MS identifications of ITGA2B, ITGB3 and TLN1 were also verified by 2-D WB analysis. These immunological platelet proteome analyses revealed also several additional proteoforms of the respective proteins (Additional file 6: Fig. S5).

## Associations in the platelet proteome with lung cancer and risk of death

Further explorative endpoints of this platelet proteomics study were occurrence of VTE or death during two year follow-up, respectively. Of 41 patients with lung or brain cancer, eighteen died and four developed VTE. To study associations of the platelet proteome with patient outcome the endpoint mortality was selected. An explorative two-way ANOVA analysis and planned post-hoc contrast analysis of the 617 investigated platelet proteins revealed that SERPINB1 (leukocyte elastase inhibitor) had the strongest dependency ( $p = 0.028$ ) on lung cancer and mortality. Previously, we identified that in LA patients a decreased expression of SERPINB1 favoured prothrombotic neutrophil extracellular traps formation (16). The quantitative representation of the actual group effects separately for cancer type and mortality showed that SERPINB1 were significantly decreased in deceased compared to surviving patients with lung cancer ( $FC = 0.82$ ;  $p = 0.03$ ) and healthy controls ( $FC = 0.83$ ;  $p = 0.003$ ). SERPINB1 abundance was independent of mortality in patients with brain cancer (Additional file 7: Fig. S6).

For a small explorative follow-up case-report, we performed 2D-DIGE analysis of platelet samples from four patients with lung cancer after three and six months to evaluate how the identified changes of protein profiles in lung-cancer are associated with patient outcome. These patients were given chemotherapy within 3 months of the first blood draw. One of the traced patients had a PE at two months after the baseline and died 5.5 months after study inclusion.

The platelet SERPINB1 level of this deceased patients stayed decreased ( $SA = 1.10$ ) after three months compared to the corresponding baseline value ( $SA = 1.17$ ) and the mean baseline value of all deceased patients ( $SA_{\text{mean}} = 1.10$ ;  $n = 11$ ) with lung cancer (Additional file 8: Fig. S7). After three months, the SERPINB1 levels decreased also in the surviving patients but returned almost to baseline values after six months and thus almost aligned with the mean values of healthy controls.

Quite similar patterns in the follow up tracing were observed for the lung cancer related proteins ITGA2B, PH4B and 55 kDa F13A1. Thus, these lung cancer related alterations were most pronounced in the deceased patient, who suffered from PE two weeks before the second blood sampling (3 months value). In the surviving patients, the lung cancer-related changes were also fortified after 3 months and returned almost to baseline values after 6 months (Additional file 8: Fig. S7). However, exclusively the abundance of SERPINB1 was already significantly associated with patient outcome at baseline.

## Discussion

Continuous platelet activation may contribute to increased VTE risk in patients with brain and lung cancer. To the best of our knowledge, this is the first study comparing the platelet proteome of these patients and matched healthy controls to elucidate prothrombotic pathways. These investigations showed a predominant influence of lung cancer on the peripheral platelet proteome compared to brain tumours. Altered platelet proteins levels of patients with lung cancer could be linked to enhanced platelet degranulation, changes in conversion of F13A1, and upregulated protein processing in ER presumably reflected by increased plasma levels of FVIII. The identification of these protein changes in platelets expands our knowledge of differential molecular mechanisms for the prothrombotic state in lung and brain cancer.

Interestingly, no brain cancer-related changes were detected by the 2D-DIGE analysis in the platelet proteome. However, in a previous study we identified that high podoplanin expression of brain tumours is functionally linked with increased VTE risks of

this cancer type (56). For the functional examination of this assumption, it is shown that podoplanin on primary human glioblastoma cells induces platelet activation via the specific binding to their C-type lectin receptor type 2 receptors. Since increased intra-tumoral platelet aggregates correlates with VTE events in patients with brain cancer (56), it can be reasoned that platelets are caught by podoplanin of the brain tumour and related proteome changes are not detectable in peripheral blood platelets.

For pathophysiological explorations of platelet proteome changes in patients with lung cancer we concentrated on four candidates described to be involved in the processing of coagulation proteins, these are, F13A1 and the ER proteins P4HB, CALR and HSPA5. An altered conversion of the final clotting factor F13A1, allocated to the pathway "platelet degranulation", was detected by elevated levels of a 55 kDa cleavage product from F13A1 in the platelets of patients with lung cancer. The fibrin stabilizing factor F13A1 is well-known to circulate in the plasma as a heterotetrameric protein complex, consisting of a dimer of the catalytic F13A1 subunits and a dimer of the carrier/inhibitory FXIIB2 subunits. The transglutaminase F13A1 is a critical determinant of venous thrombus characteristics such as thrombus size, stability and red blood cell retention (57, 58). Platelets contain only F13A1. Although, the concentration of F13A1 is around 150-fold higher in platelets compared to plasma (59) its functions in platelets are quite unknown. Only recently it was shown that platelet surface exposed F13A1 contributes to thrombus stability whereas the secreted F13A1 from platelets is functionally irrelevant (60). However, the enzymatic activity of F13A1, exposed on platelets, is temporally limited with a maximum after 5 minutes and completely declines after one hour (60). The mechanisms of F13A1 inactivation are unclear and of substantial interest (61). Our findings that the platelet level of the 55 kDa fragment of F13A1 did not correlate with enzymatic activity of this transglutaminase in platelets suggest that it may be a product of accelerated F13A1 inactivation in patients with lung cancer. The correlation of the platelet 55 kDa fragment of F13A1 with plasma D-dimer would suggest an inactivation by plasmin and thus the involvement of the fibrinolytic system. However, this protease is reported to inactivate only thrombin-activated F13A1. Whereas, the actual platelet proteomics study showed that the 55 kDa lung cancer-related F13A1 fragment originated from its precursor, since peptides from the activation region were also detected by MS analysis. The findings of an inactivation of the F13A1 precursor in platelets becomes functional reliable in the context of previous investigations, showing that the enzymatic activity of F13A1 can also be initiated in platelets by the increase of intracellular  $Ca^{2+}$  without the need of thrombin-mediated activation (62).

The regulations of structural changes of F13A1 during enzymatic activation are less characterized. The most striking phenomenon during the conformational change of activated F13A1 is a high number of lysine sides getting accessible for acetylation in the core domain of the enzyme (63). The current 2D-gel based proteomics study showed that all acidic 83 kDa proteoforms of F13A1 had a significant positive correlation with enzymatic activity of this transglutaminase except the most highly abundant alkaline one. These unique observations inclusively the negative association with enzymatic activity of this latter F13A1 proteoform, fortifies the hypothesis that acetylation, may cause these shifts in their pIs and the regulation of enzymatic F13A1 activity in platelets.

However, no significant change of F13A1 enzymatic activity could be measured in platelets and plasma of patients with lung cancer. Although F13A1 abundance was unchanged in platelets of patients with brain cancer, a significant decrease of enzymatic F13A1 activity was found in the plasma of patients with brain cancer. Decreased plasma levels of F13A1 were previously observed in plasma of patients with thrombosis history with or without cancer. It is assumed that this decrease is caused by an increased consumption of F13A1 due to its inactivation (64). In the actual study the increased levels of the precursor and the 55 kDa fragment from F13A1, may also indicate an increased turnover of F13A1 in the platelets of patients with lung cancer.

Platelets are also of functional importance for the progression of cancer and metastasis formation (65). The observed increased levels of F13A1 and changed processing of F13A1 in platelets of patients with lung cancer may also play a role in metastasis. In F13A1 deficient mice, the metastatic potential was significantly diminished. F13A1 was shown to support metastasis primarily by limiting natural killer cell-mediated clearance of micrometastatic tumour cells (66). F13A1 has been shown to be associated with inferior outcomes in acute promyelocytic leukemia (67) and the activity of F13A1 in plasma was elevated in patients with non-small cell lung carcinoma (68). High expression of F13A1 was also observed in inflammatory monocytes of patients with lung squamous carcinomas which promotes fibrin cross-linking and in turn facilitate metastases of lung carcinoma cells (69).

Elevated levels of ER chaperones P4HB, CALR and HSPA5 indicated the induction of the ER unfolded protein response (UPR) in platelets of patients with lung cancer. An increased abundance of these ER proteins was already previously observed in platelets of LA patients with thrombosis history (16).

Interestingly, the UPR in ER is also associated with malignant transformation of cancer cells (70) as well as with a prothrombotic influence of pancreatic cancer. In this particular study, it was shown that proteins of the UPR in cancer are released from pancreatic cancer cells by extracellular vesicles. Increased levels of the same UPR protein profiles are also detected in the plasma of patients with pancreatic cancer and thrombosis history. These observations indicate a mechanistic link between tumour progression and cancer-associated thrombosis (71). That group also showed that inhibiting enzymatic PDI activity in plasma and platelets by the flavonoid isoquercetin reduced the hypercoagulability of advanced cancer patients (72) as well as that of LA patients (43). Remarkably, in patients with lung cancer as well as in LA positive patient with thrombosis history (16) we identified a highly significant increase of the PDI family member, P4HB, in platelets, which also manifest this ER chaperone as a highly purposive antithrombotic drug target.

The ER proteins HSPA5 and CALR mediate to a great part glycosylation and folding of secretory proteins. Interestingly the function of these ER proteins and the induction of the UPR in the ER are extensively investigated and described for the industrial and therapeutic production of FVIII (46, 73, 74). Increased levels as well as increased activity of FVIII in plasma are generally associated with high VTE risk (75) and predict VTE in cancer patients (51). In the current study FVIII was also significantly increased in the plasma of patients with lung cancer and showed a predominant correlation with the ER proteins HSPA5 and CALR in comparison to all other proteomic investigated platelet proteins. This induction of the UPR and correlations of FVIII with P4HB, HSPA5 and CALR levels may also take place systemically in patients with lung cancer. Thus, platelets may be a peripheral indicator for enhanced glycosylation and secretion from e.g. liver sinusoidal endothelial cell (76). The lung-cancer-related upregulation of ER chaperones in platelets corroborate a functional association of the UPR with FVIII production and thrombosis risks.

The abundance of both integrins of the fibrinogen receptor integrin  $\alpha\text{IIb}\beta\text{3}$ , ITGA2B and ITGB3, were significantly reduced in platelets of patients with lung cancer. These glycoproteins are the two integral parts of the key adhesion receptor of platelets and play a crucial role in fibrinogen-mediated thrombus formation by promoting platelet adhesion. Diminished levels of these integrins in platelets do not functionally correspond to an expected increased adhesion and aggregation of platelets in patients with high thrombosis risks. The decrease of ITGA2B and ITGB3 levels in platelets during this prothrombotic condition of patients with lung cancer and LA with thrombosis history (16) may be caused by continuous shedding of extracellular vesicles, which was recently also described as “membrane pearling” of platelet pseudopodia (77). Platelet extracellular vesicles have in relation to their whole protein content a higher load of ITGA2B and ITGB3 than their corresponding resting platelets (78). Thus, these integrin levels may be declined in exhausted patient platelets, because of an extensive release of extracellular vesicles. In fact, an increase of platelet released extracellular vesicles is observed in plasma of patients with cancer (79) and LA (80).

Accordingly, lower levels of ITGA2B and increased levels of the ER proteins P4HB, CALR and HSPA5 in patients with lung cancer and LA suggest some common affected pathways in their prothrombotic pathology. In contrast to that, the altered proteoforms of F13A1 in platelets appeared to be specific for patients with lung cancer. The data of these two platelet proteomics studies (16) are highly comparable since exactly the same methodology of platelet isolation and 2D-DIGE proteome analysis were used.

Altogether, our study has both limitations and strengths. The selected proteomics technology of 2D-DIGE in the pH range 4–7, does not cover the whole possible pH range of 3–10. Low-abundance and high molecular weight proteins are not captured by 2D-based analysis meaning a lower sensitivity as compared to MS-based shotgun analyses. Hence, the probability is high that we did not detect all protein changes in platelets of patients with lung and brain cancer. However, there is not a single proteomics technology nowadays that can analyse the whole proteome including all PTMs from a biological sample. PTMs are particularly essential for proteomic changes in haemostasis, as primary and secondary haemostasis are tightly coordinated by the PTM “enzymatic cleavage” of coagulation and other factors. Thus, two-dimensional gel electrophoresis should be more versatile to study the prothrombotic phenotype of platelets than the currently mainly applied “bottom-up” shotgun proteomics. Conventional MS-based shotgun proteomics approaches cannot capture the information of regulatory variation of different proteoforms to each other, due to the necessary pre-analytical proteolytic digestion of biological samples (81). Accordingly, 2D-DIGE analysis

had an analytical advantage in the concrete clinical proteomics study. For example, it would not have been possible to reveal the lung-cancer-related changes of F13A1 processing by MS based shotgun analysis, because the preanalytical digestion of the sample would have eliminated the information of different pIs and molecular weights of the respective F13A1 proteoforms.

From the clinical perspective, our study is rather explorative with the inclusion of a relatively small number of patients and matched healthy controls. Due to the limited sample size and number of VTE events it was not possible to perform well-founded statistically subgroup analysis to evaluate comprehensively the influence of VTE or mortality on the investigated platelet proteome.

## Conclusions

Our study delivers novel insights into the differential behaviour of platelets in patients with different types of cancer. These data provide the first evidence that alterations of the platelet proteome are more pronounced in lung cancer compared to brain cancer. Lung-cancer related changes as increased platelet levels of P4HB from the PDI family underscores the importance of this antithrombotic drug target for the hypercoagulable state in certain cancer types [81]. F13A1 might be also involved in development of VTE, as well as in lung cancer progression, and especially this patient group may benefit in multiple ways from antithrombotic treatment such as FXIII inhibitors [82]. Our clinical platelet proteomics study confirms also previously characterised deregulations of excessive malignancies-related platelet activation in lung cancer and furthermore provides novel insights in the mechanisms of thrombosis in lung cancer and its correlation with inflammation. These potential antithrombotic drug targets in platelets should be further investigated in cancer research.

## Abbreviations

1-D: One-dimensional; 2-D: Two-dimensional; 2D-DIGE: Two-dimensional differential gel electrophoresis; ANOVA: Analysis of variance; aPTT: Activated partial thromboplastin time; BC: Patients with brain cancer; C: Healthy control; CALR: Calreticulin; CATS: Cancer and Thrombosis Study; CI: Confidence interval; CRP: C-reactive protein; CTAD: Trisodium citrate, theophylline, adenosine, dipyridamole; DVT: Deep vein thrombosis; EDA: Extended data analysis; ER: Endoplasmic reticulum; F13A1: Coagulation factor XIII A chain; FC: Fold change; FDR: False discovery rate; FVIII: Coagulation factor VIII; FXIII: Coagulation factor XIII; HRP: Horse-radish peroxidase; HSPA5: Endoplasmic reticulum chaperone; IEF: Isoelectric focusing; IPG: Immobilised pH gradient; IQR: Interquartile range; IS: Internal standard; ITGA2B: Integrin alpha-IIb; ITGB3: Integrin beta-3; kDa: Kilo Dalton; KEGG: Kyoto Encyclopedia of Genes and Genomes; LA: Lupus anticoagulant; LC: Patients with lung cancer; M: Marker; MPV: Mean platelet volume; MS: Mass spectrometry; MW: Molecular weight; n.a.: Not applicable; n: Number; NC: Nitrocellulose; P4HB: Protein disulphide isomerase A1; PAI: Plasminogen-activator-inhibitor; PCA: Principal component analysis; PDI: Protein disulphide isomerase; PE: Pulmonary embolism; PGI<sub>2</sub>: Prostacyclin I<sub>2</sub>; pI: Isoelectric point; PPI: protein-protein interaction; PRP: Platelet rich plasma; PTM: Post translational modification; PVDF: polyvinylidene difluoride; QqTOF: Quadrupole time-of-flight mass analyzers; RuBPS: Ruthenium-(II)-tris-(bathophenanthroline disulfonate); sCD62P: Soluble P-selectin; SERPINB1: Leukocyte elastase inhibitor; STRING: Search Tool for the Retrieval of Interacting Genes/Proteins; UPR: Unfolded protein response; VTE: Venous thromboembolism; WB: Western blot; y: Years; λ: Wavelength.

## Declarations

### Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Medical University of Vienna in accordance with the Declaration of Helsinki (EC no. 126/2003 and 039/2006).

### Consent for publication

This manuscript has been read and approved by all the authors to publish and is not submitted or under consideration for publication elsewhere.

### **Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding authors on reasonable request.

### **Competing interests**

All authors declare that they have no competing interests.

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### **Authors' contribution**

IP conceived the work, HE, MZ and IP designed the experiments, EG recruitment of patients and matched controls, EG, LMM, and LH collected and prepared the blood samples, HE made the 2D-DIGE and WB analysis, RH acquired the MS data, HE, IP, JAS and MZ wrote on the manuscript and prepared the figures, LMM, JAS, FM, EG and CA reviewed and revised the manuscript.

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## Tables

Table 1

Characteristic	Healthy controls (n=41)	Healthy controls for brain cancer (n=22)	Healthy controls for lung cancer (n=19)	Brain cancer (n=22)	Lung cancer (n=19)
Median age at study entry, y (IQR)	57 (48-62)	55 (42-60)	61 (56-63)	56 (45-63)	62 (58-67)
Female, n (%)	16 (39)	9 (41)	7 (37)	9 (41)	7 (37)
<b>Median laboratory values (IQR)</b>					
Leukocyte [G/L]	5.9 (5.1-7.1)	5.7 (4.9-7.8)	5.9 (5.2-6.9) \$\$\$	7.1 (5.4-11.8)	9.8 (7.6-11.1)
Hemoglobin [G/dL]	14.5 (13.2-15.4)	14.8 (13.1-15.6)	14.3 (13-15.3) \$	14.3 (13.3-15.2)	12.9 (12.2-14)
Platelet count [G/L]	235 (216-286)	235 (214.5-308.5)	230 (225-286) \$	239 (202-297)	297 (276-350)
MPV [fl]	10.8 (10-11.4)	10.9 (10.1-11.4) #	10.8 (9.9-11.5) \$	10 (9.7-10.7)	10.1 (9.2-10.8)
Neutrophils [%]	58 (51-64.5)	60 (48-61.8) ###	53 (54-68) \$\$\$	73 (56.7-76.1)	82.4 (76-87.7)
CRP [mg/dL]	0.14 (0.06-0.25)	0.16 (0.06-0.23)	0.09 (0.07-0.32) \$\$\$	0.14 (0.06-0.27)	1.16 (0.65-2.8)
aPTT [s]	35 (32.1-37.7)	33 (33.1-38.1) #	36 (31.3-35.8)	34.1 (29.4-36)	33.5 (31.8-40.7)
Fibrinogen [mg/dL]	303 (259-337)	313 (250.5-319.5) ##	282 (280-353) \$\$\$	319 (305-375)	509 (427-639)
Prothrombinfragment [pmol/L]	180 (127-249)	192 (118.5-297)	165 (148.5-225.8)	165 (109-199)	235 (154-349)
FVIII activity [%]	147 (107-173)	165 (103.5-154) ###	135 (117-184) \$\$	208 (167-265)	237 (166-369)
Antithrombin III [%]	103 (97-108)	105 (96.5-108.5) ###	100 (101-108)	122 (111-133)	105 (85-110)
PAI [IU/mL]	1.2 (0.5-5)	1.1 (0.49-6)	1.5 (0.49-4.2)	2.1 (0.53-5.7)	1.4 (0.9-6.6)
Plasma FXIII activity [%]	123 (111-140)	122.5 (104.5-135.4) #	120.4 (115.5-151)	106.8 (91.1-111)	120.2 (95.6-139.4)
D-Dimer [µg/mL]	0.32 (0.27-0.42)	0.33 (0.27-0.41) #	0.3 (0.27-0.42) \$\$\$	0.74 (0.33-0.93)	1.79 (1.13-4.15)
VTE during follow up, n (%)	n.a.	n.a	n.a	2 (9.1)	2 (10.5)
PE	n.a	n.a	n.a	1 (5)	2 (10.5)
DVT	n.a	n.a	n.a	1 (5)	n.a.
Deaths during follow up, n (%)	n.a	n.a	n.a	8 (36.4)	10 (52.6)

Table 2

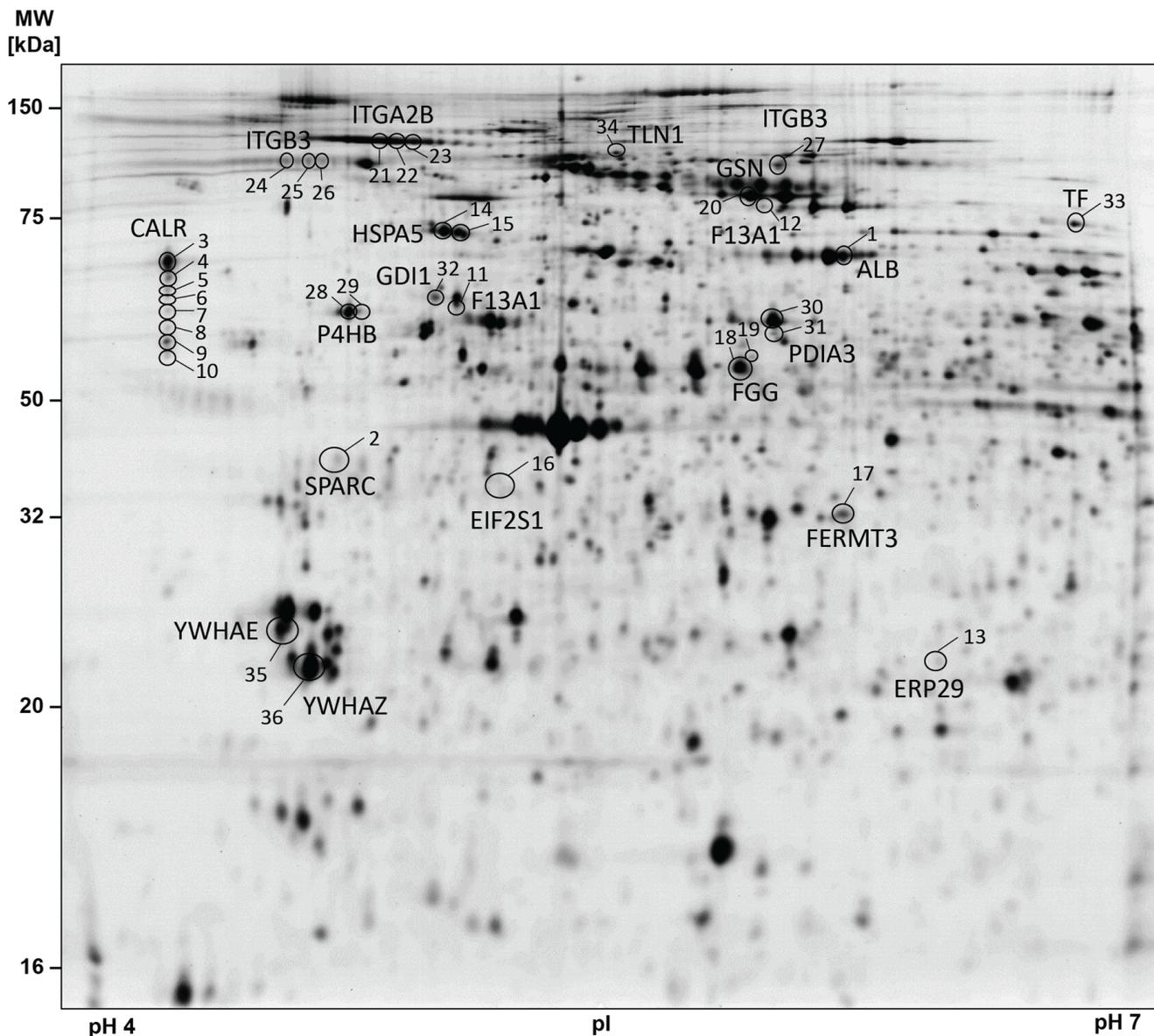
Spot number	Protein name	Gene name	Isoelectric point (pI)	MW [Da]	One-way ANOVA [adjusted]	Brain cancer patients / Healthy controls			Lung cancer patients / Healthy controls		
						Average FC	p-value [unadjusted]	p-value [adjusted]	Average FC	p-value [unadjusted]	p-value [adjusted]
1	Albumin	ALB	6.00	69367	0.0224	0.84	0.0114	0.1136	0.85	0.0281	0.0388
2	Basement-membrane protein 40 (SPARC; Osteonectin)	SPARC	4.75	42014	0.0488	0.96	0.6911	0.8639	0.62	0.0001	0.0013
3	Calreticulin	CALR	4.29	67014	0.0127	1.06	0.1427	0.4926	1.10	0.0315	0.0420
4			4.29	65300	0.0224	1.11	0.0254	0.1451	1.12	0.0524	0.0655
5			4.29	64385	0.0264	1.09	0.0301	0.1505	1.11	0.0616	0.0747
6			4.29	63493	0.0188	1.13	0.0069	0.1053	1.11	0.0156	0.0240
7			4.29	61919	0.0433	1.14	0.0191	0.1273	1.12	0.0123	0.0223
8			4.29	60228	0.0482	1.18	0.0074	0.1053	1.16	0.0515	0.0655
9			4.29	56324	0.0224	1.14	0.0079	0.1053	1.12	0.0054	0.0135
10			4.29	57598	0.0500	1.19	0.0142	0.1136	1.12	0.0144	0.0238
11	Coagulation factor XIII A chain	F13A1	5.00	55000	0.0025	0.95	0.4346	0.7243	1.57	0.0016	0.0080
12			5.75	83267	0.0500	0.90	0.2608	0.5611	1.21	0.0102	0.0194
13	Endoplasmic reticulum resident protein 29	ERP29	5.95	27022	0.0188	1.06	0.2242	0.501	1.20	0.013	0.0212
14	Endoplasmic reticulum chaperone BiP	HSPA5	5.06	73539	0.0488	0.99	0.8875	0.9342	1.07	0.1212	0.1347
15			5.08	73539	0.0224	1.06	0.3828	0.6960	1.15	0.0098	0.0194
16	Eukaryotic translation initiation factor 2 subunit 1	EIF2S1	5.08	43211	0.0188	0.99	0.8726	0.9342	0.83	0.0018	0.0080
17	Fermitin family homolog 3	FERMT3	6.00	39072	0.0019	0.94	0.5676	0.8148	1.11	0.1191	0.1347
18	Fibrinogen gamma chain	FGG	5.74	55146	0.0467	1.03	0.4800	0.7385	1.18	0.0022	0.0080
19			5.58	56048	0.0488	1.45	0.2812	0.5624	1.47	0.0032	0.0091
20	Gelsolin	GSN	5.70	81086	0.0188	1.07	0.0989	0.3956	0.92	0.0221	0.0316
21	Integrin alpha-IIb	ITGA2B	4.80	88938	0.0467	0.94	0.1933	0.5155	0.88	0.0052	0.0135
22			4.92	88938	0.0096	0.93	0.1562	0.4926	0.86	0.0001	0.0013
23			4.95	88938	0.0224	0.91	0.0641	0.2849	0.86	0.0020	0.0080
24	Integrin beta-3	ITGB3	4.68	75464	0.0127	1.00	0.9763	0.9763	0.82	0.0008	0.0053
25			4.70	75464	0.0390	0.95	0.2597	0.5611	0.87	0.0061	0.0144
26			4.73	75464	0.0467	0.96	0.3805	0.6960	0.88	0.0094	0.0194
27			5.63	75464	0.0046	0.90	0.2665	0.5611	0.63	0.0005	0.0040
28	Protein disulfide-isomerase A1	P4HB	4.78	61026	0.0127	1.01	0.8415	0.9342	1.11	0.0016	0.0080
29			4.80	61026	0.0019	0.98	0.5906	0.8148	1.11	0.0211	0.0313
30	Protein disulfide-isomerase A3	PDIA3	5.81	60726	0.0467	1.02	0.4246	0.7243	1.03	0.2144	0.2199
31			5.81	58443	0.0417	1.04	0.1645	0.4926	1.06	0.1529	0.1653
32	Rab GDP dissociation inhibitor alpha	GDI1	4.95	62247	0.0019	1.02	0.6780	0.8639	1.31	0.0002	0.0020
33	Serotransferrin	TF	6.62	76915	0.0272	0.95	0.4797	0.7385	0.79	0.0149	0.0238
34	Talin-1	TLN1	5.31	75981	0.0096	0.97	0.7319	0.8872	1.45	0.0072	0.0160
35	14-3-3 protein	YWHAE	4.60	28953	0.0420	1.01	0.5907	0.8148	0.98	0.3459	0.3459

	epsilon										
36	14-3-3 protein zeta/delta	YWHAZ	4.69	26607	0.0420	0.99	0.8331	0.9342	0.96	0.0673	0.0792

Table 3

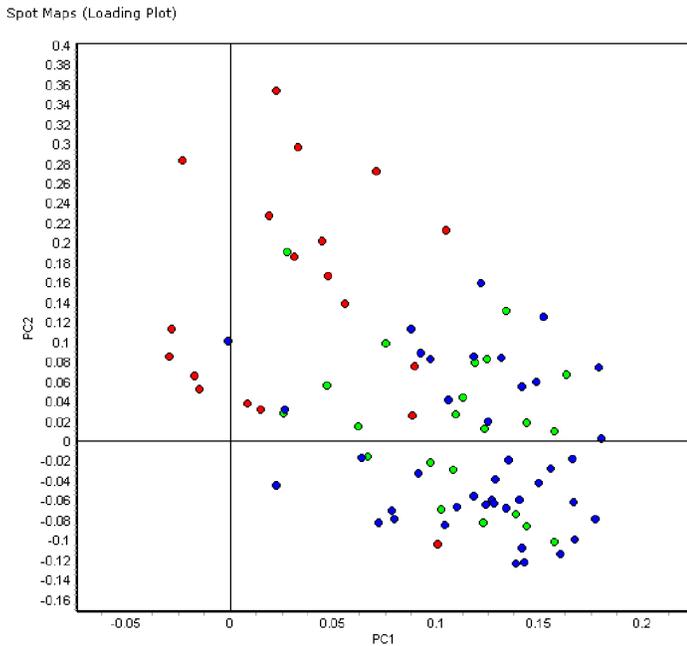
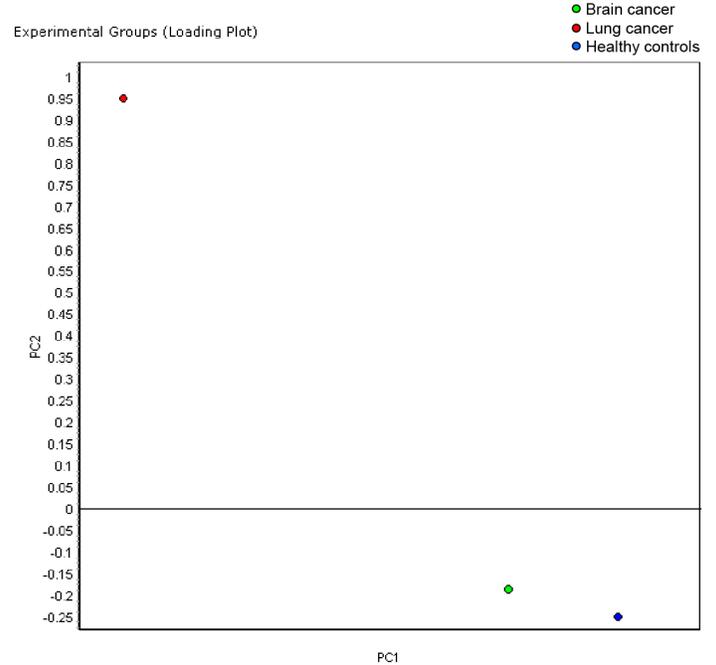
Spot number of F13A1 proteoforms	Isoelectric point (pI)	MW [kDa]	ANOVA [adjusted]	Lung cancer patients / matched controls		Brain cancer patients / matched controls		Correlation: abundance vs. enzymatic activity	
				Average FC	p-value [unadjusted]	Average FC	p-value [unadjusted]	corr. of F13A1 abundance vs. activity @ rho	p-value [unadjusted]
12c	5.85	83	0.998	0.77	0.2652	1.24	0.372	-0.29	0.062
12b	5.75	83	0.410	1.40	0.0019	0.96	0.743	0.43	0.004
12	5.60	83	0.071	1.21	0.0102	0.90	0.261	0.39	0.009
12a	5.65	83	0.198	1.33	0.0369	0.82	0.231	0.50	0.001
12d	6.05	79	0.868	1.26	0.3055	1.05	0.832	0.04	0.807
11	4.95	55	0.006	1.57	0.0016	0.95	0.433	0.14	0.367

## Figures

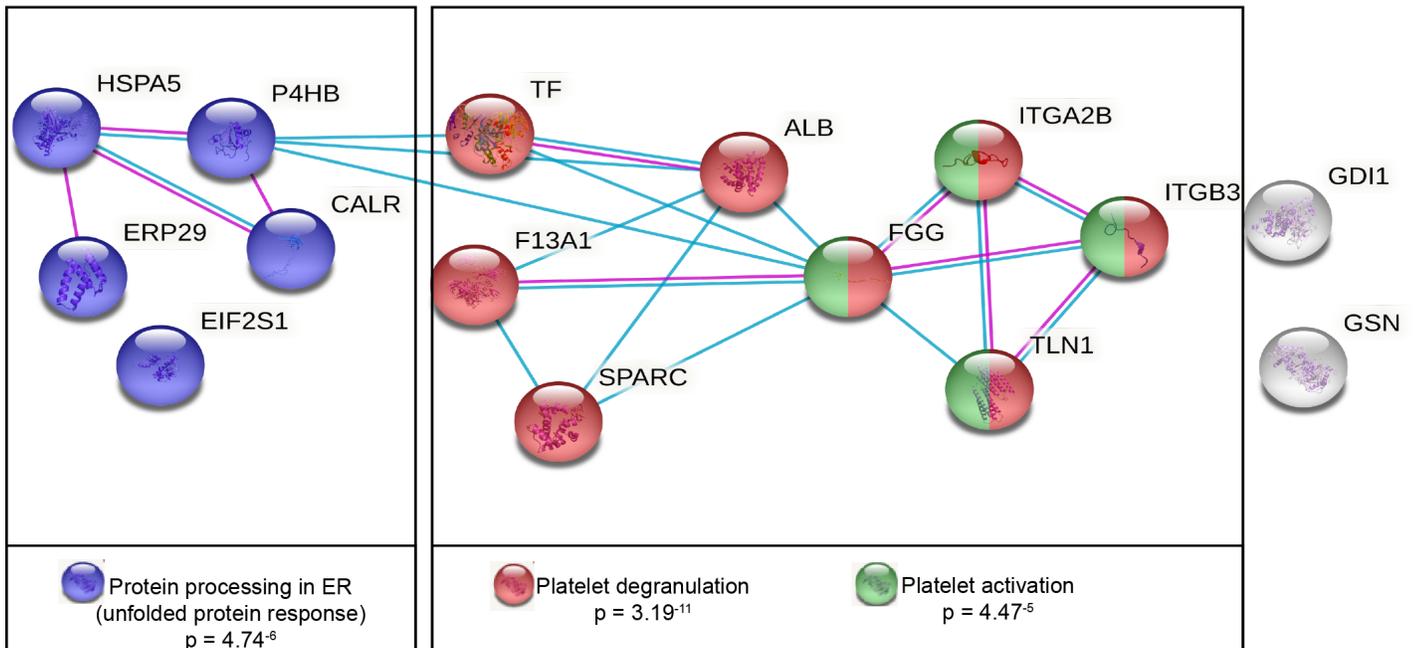


**Figure 1**

2D-DIGE-based proteome analysis of platelets from patients with brain and lung cancer compared to controls. This representative 2D-DIGE image shows all one-way ANOVA filtered significantly altered protein spots between patients with lung (n = 19) and brain (n = 22) cancer compared to healthy controls (n = 41). A total of 36  $\mu$ g (12  $\mu$ g sample Cy3-, 12  $\mu$ g sample Cy5-, and 12  $\mu$ g IS Cy2-labeled) platelet protein extracts were separated according to the isoelectric point (pI) in the pH 4-7 range (separation distance 24 cm) and the molecular weight (MW, separation distance 20 cm). Protein spots identified by MS are circled and labeled with their corresponding gene name and spot numbers are given in Table 1. Protein spots of interest were selected according to (a) protein spots matched >95% of all 2D-DIGE gels and (b) FDR-corrected one-way ANOVA p-value <0.05. Abbreviations: MW – molecular weight; kDa – kilodalton; pI – isoelectric point; 2D-DIGE – two-dimensional differential in-gel electrophoresis; IS – internal standard; MS – mass spectrometry

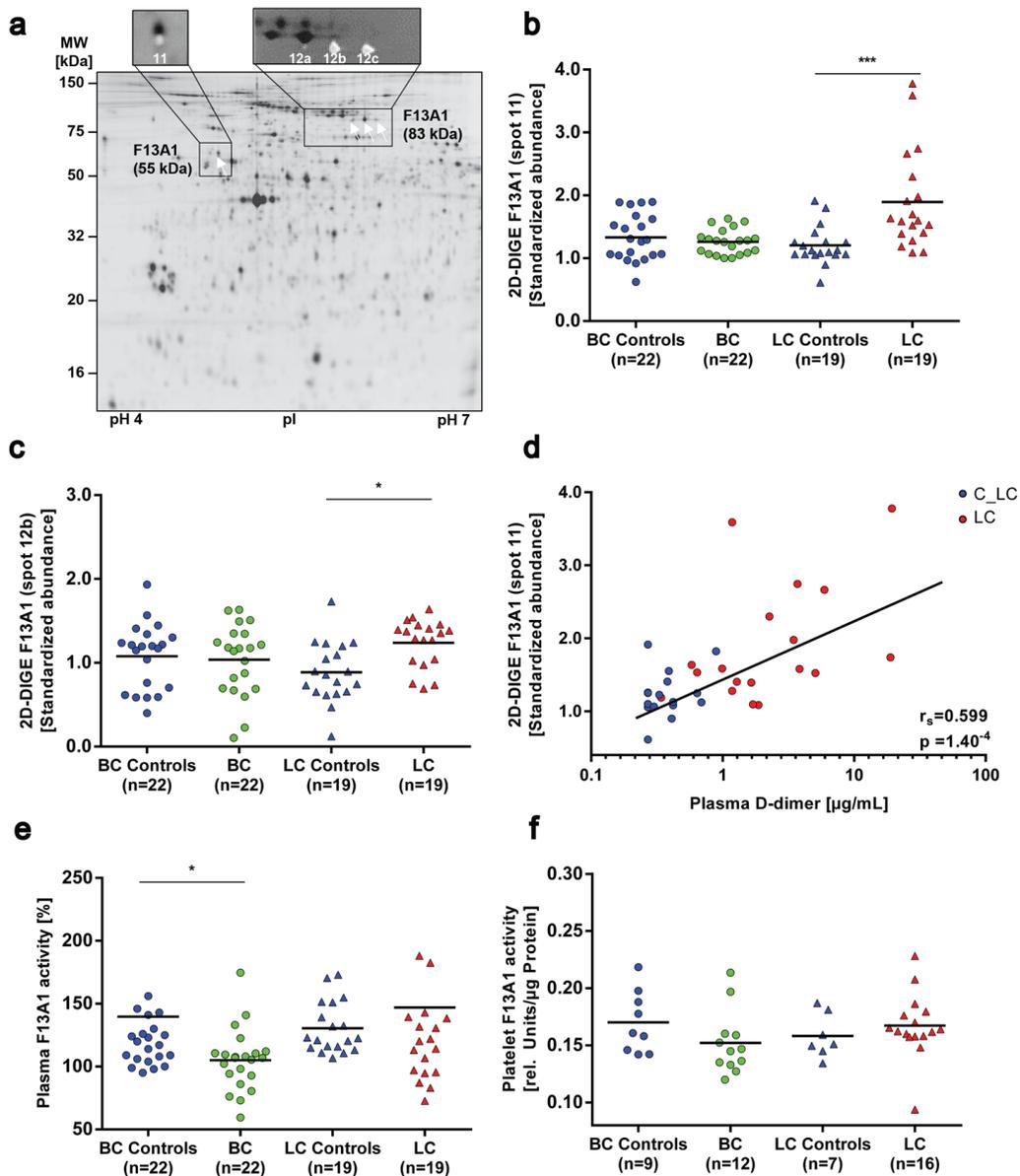
**a****b****Figure 2**

Principle component analysis of variable platelet proteins between controls, brain and lung cancer patients. PCA plot analysis is based on 2D-DIGE analysis and one-way ANOVA filtered platelet protein spot changes between the three study groups. (a) Dots represent the particular platelet samples aggregated from 36 one-way ANOVA-based significant different platelet protein spots from 22 patients with brain cancer, 19 patients with lung cancer and 41 age and sex matched healthy controls. (b) Reduction of all platelet samples from each study group to one value to illustrate simplified the influence of cancer types compared to healthy controls from the acidic platelet proteome (pH range 4-7) Abbreviations: PCA - Principle component analysis, PC1 – Principal Component 1; PC2 – Principal Component 2

**a**

**Figure 3**

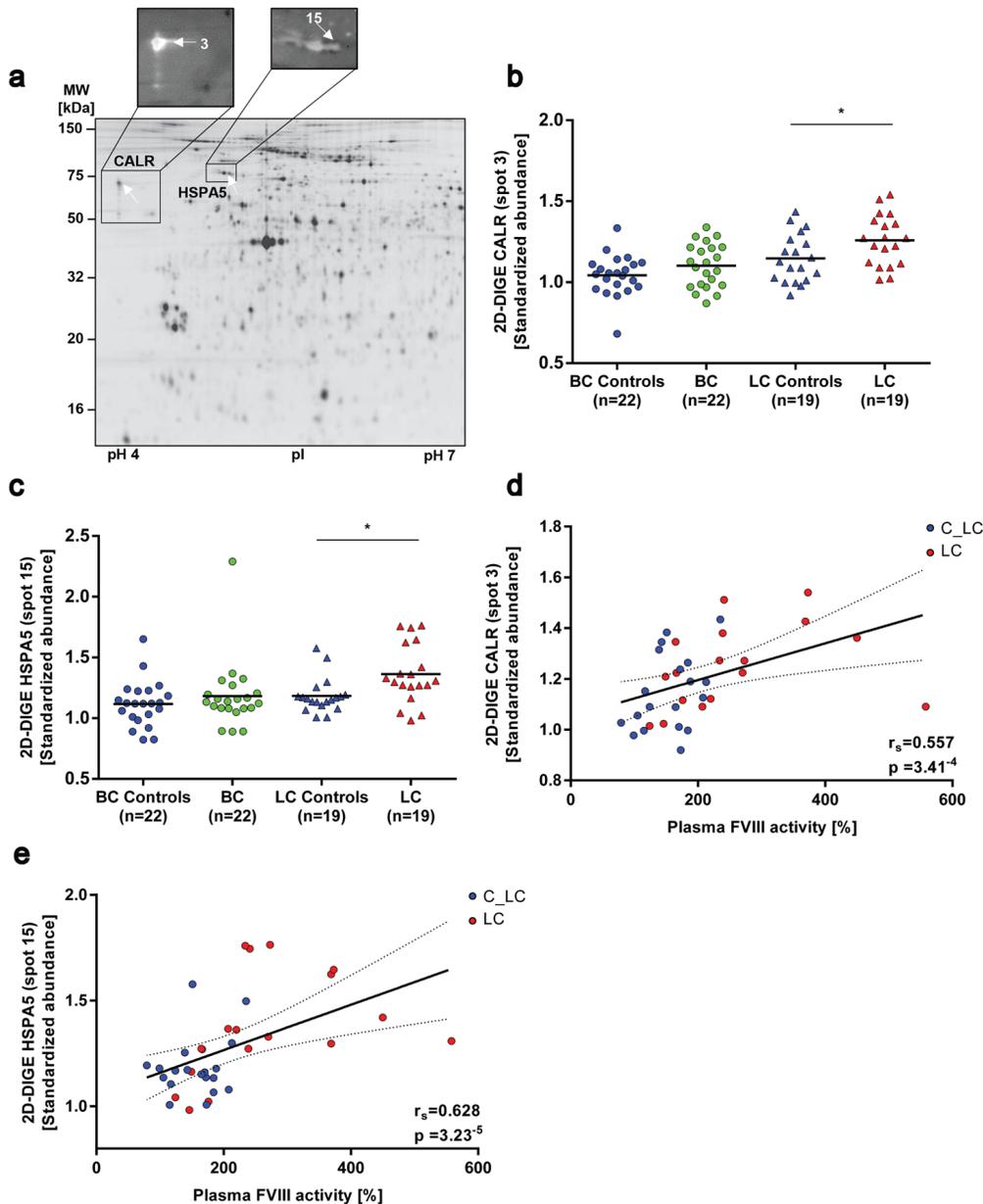
Functional association analysis of lung cancer-related platelet proteins. Network and enrichment analysis shows top pathways obtained upon entering the set of significantly lung cancer-related platelet proteins in STRING database analysis tool [25]. The type of interaction is indicated by colored linear slopes; pink: experimentally determined, blue: from curated databases. The enrichment graphs depict the most significantly enriched GO Biological Process with platelet degranulation in red and two most significant KEGG pathways, in cobalt blue, protein processing in endoplasmic reticulum, and in green, platelet activation. The proteins highlighted in grey were not significantly associated to these quoted functional networks.



**Figure 4**

Platelet F13A1 abundance and F13A1 enzymatic activity in platelets and plasma of the study cohorts. (a) Representative 2-D WB image of the platelet proteome pH 4-7 probed with monoclonal F13A1 antibody. Protein of interest is highlighted with a white arrow. The cut-out region shows an overlay from Cy2-labeled 2D-DIGE gel and specific 2-D WB F13A1 signals, where immun-identified proteins are displayed as white spots and Cy2-labelled spots in black. (b) Protein levels of the 55 kDa F13A1 proteoform (spot 11) and (c) the 83 kDa F13A1 proteoform (spot 12b). (d) Scatter dot plot and correlation analysis of 55 kDa F13A1 standardized platelet protein spot abundances quantified by 2D-DIGE with corresponding plasma D-dimer levels. An

association was assessed by a Spearman's Rank correlation coefficient ( $r_s$ ). (e) F13A1 activity levels in plasma and (f) platelets in patients with lung and brain cancer and matched healthy controls. Protein levels were depicted as single values and mean. \* $p < 0.05$ , \*\*\* $p < 0.001$ . Abbreviations: 2D-DIGE – two-dimensional differential in-gel electrophoresis; WB – western blot; pI – isoelectric point; MW – molecular weight; kDa – kilodalton; confidence interval – CI; BC – patient with brain cancer; LC – patient with lung cancer; C – healthy control



**Figure 5**

Platelet CALR and HSPA5 abundance and their correlations with plasma FVIII activity from patients with lung cancer and matched healthy controls. (a) Representative 2-D WB image of a platelet sample and specific CALR abundance probed with monoclonal anti-Calreticulin and with polyclonal anti-HSPA5 antibodies. Protein spots of interest are highlighted with white arrows. The cut-out region shows an overlay from Cy2-labeled 2D-DIGE gel and specific 2-D WB signals, where immune-identified proteins are displayed as white spots and Cy2 labelled spots are displayed in black. (b) Platelet protein levels of CALR spot 3 and (c) HSPA5 spot 15 quantified by 2D-DIGE analysis. Levels of these ER platelet proteoform were specified as standardized abundance and depicted as mean. (d) Scatter blot and correlation analysis of CALR spot 3 and (e) HSPA5 spot 15 protein levels measured by 2D-DIGE and FVIII analysis (Spearman's Rank correlation coefficient). The solid straight line represent best fitted model with 95% confidence interval (CI) as dashed lines. Abbreviations: WB – Western blot; pI – isoelectric point; MW –

molecular weight; kDa – kilodalton; confidence interval – CI; BC – patient with brain cancer; LC – patient with lung cancer ; C – healthy control

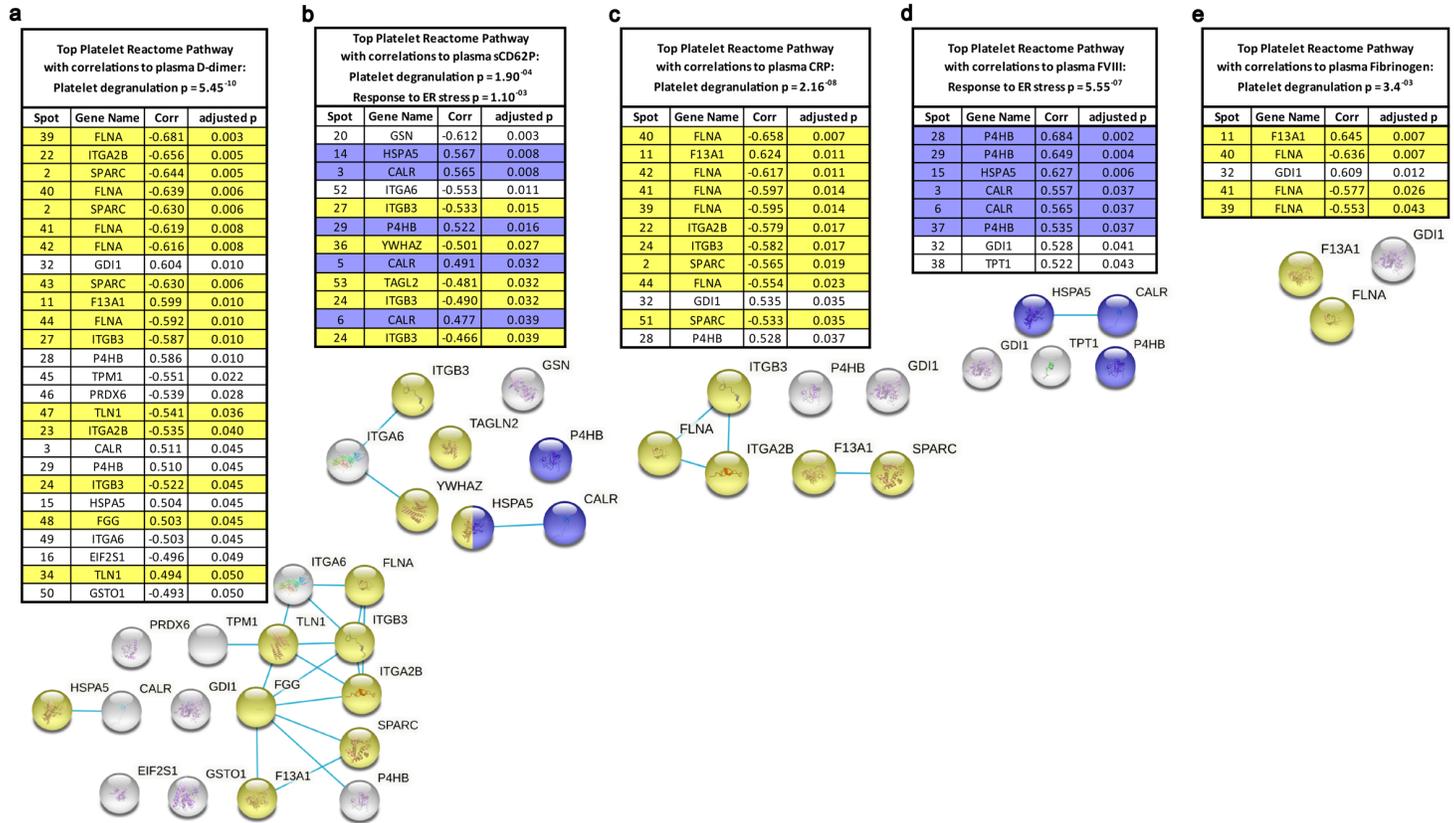


Figure 6

Functional relationships of the platelet proteome with haemostatic plasma laboratory parameters. The standardised abundance of the 617 included platelet protein spots from 2D-DIGE platelet proteome analysis were correlated with the plasma levels of (a) D-dimer [ $\mu\text{g/mL}$ ], (b) sCD62P [ $\text{ng/mL}$ ], (c) CRP [ $\text{mg/dL}$ ], (d) FVIII activity [%] and (e) fibrinogen [ $\text{mg/dL}$ ] by Spearman's Rank correlation coefficient and corresponding adjusted p values are specified. These calculations were made from patients with lung cancer ( $n = 19$ ) and matched controls ( $n = 19$ ). Multiple comparisons were corrected by Benjamini Hochberg. Network and enrichment analysis shows top pathways obtained upon entering the respective set of significantly correlating platelet proteins in STRING database analysis tool. Known interactions are indicated by linear slope from curated databases. The proteins highlighted in grey in the STRING graphs were not significantly associated to these quoted functional networks.

## Supplementary Files

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