

Differential abundance and self-organizing tree algorithm clustering nominate PDGFC and TIMP3 as candidate resection-responsive plasma extracellular vesicle proteins in head and neck cancer

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Abstract

Head and neck squamous cell carcinoma (HNSCC) recurs in a large proportion of patients after multimodal therapy, and no circulating molecular biomarker reliably reports whether tumor-associated signals persist once the primary tumor has been removed. Because tumor-derived extracellular vesicles (EVs) accumulate in the tumor microenvironment and enter the circulation, we asked whether the plasma EV proteome changes with tumor resection and whether such changes carry functional and prognostic weight. Using quantitative proteomics and advanced integrated statistical analysis, we profiled paired plasma EVs obtained from the same patients ($n = 11$) at two vascular sites, peripheral and tumor-draining venous blood, at two time points, before and shortly after resection. Nine proteins were significantly more abundant in peripheral EVs before than after surgery (PDGFB, PDGFC, PPBP, TIMP3, HPSE, COL6A1, LTBP1, PF4 and PF4V1), whereas the local tumor-draining compartment showed only ENO3. Unsupervised Self-Organizing Tree Algorithm clustering independently identified a 24-protein cluster, likewise higher before resection, that converged on platelet-, collagen- and extracellular matrix receptor-related pathways. Data were verified in external patient tissue (TCGA HNSCC) using the Kaplan-Meier Plotter database, where high expression of the pairwise signature (hazard ratio (HR) 1.40, $p = 0.023$), the cluster signature (HR 1.72, $p = 0.0043$) and the two shared proteins PDGFC and TIMP3 (HR 1.43, $p = 0.018$) was associated with poorer 3-year overall survival. Functionally, EV size, morphology, and number were unchanged, but post-resection peripheral EVs stimulated HNSCC cell proliferation significantly less than pre-resection EVs, with no effect on non-malignant keratinocytes. These findings indicate that tumor resection depletes circulating EVs of a platelet- and matrix-associated, growth-promoting protein cargo, and identify PDGFC and TIMP3 as candidate prognostic markers that warrant validation in independent cohorts.

Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) is the 6th most prevalent cancer worldwide¹. The majority of cases are diagnosed at locally advanced stages, which limits treatment options and worsens outcomes². Traditional treatment modalities, including surgery, radiation, chemotherapy, and targeted therapies, are frequently employed in combination to address the complexity of advanced local diseases. Despite the comprehensive approach, the prognosis for advanced HNSCC remains grim, with a substantial portion of patients, approximately 55%, experiencing loco-regional recurrence or metastatic progression within a mere two years following combination therapies³. This distressing reality underscores the limitations of current treatments, leading to a persistently poor five-year overall survival rate. Even with the application of salvage surgery, additional radiation, and immunotherapy in recurrent HNSCC cases, the overarching challenge lies in the fact that a significant proportion of these cases remain incurable^{4–8}. Compounding the complexity, the occurrence of second cancers in HNSCC patients further exacerbates the therapeutic dilemma, with limited options available for those grappling with this dual burden⁹. Surgery, despite being the most common approach for eliminating HNSCC tumor from the oral cavity, does not provide a foolproof solution, as evident from the high rates of recurrence¹. Tumor

progression in HNSCC is driven not only by the malignant cells themselves but also by the surrounding tumor microenvironment (TME), which promotes immune suppression and angiogenesis^{10–12}. Surgical resection removes the tumor together with much of this microenvironment, yet tumor-derived material may already have entered the circulation and persist after the operation. Current molecular biomarkers remain insufficient for diagnosis, prognosis, and therapy guidance, intensifying the search for new candidates using advanced technologies such as proteomics^{13–15}. Consequently, a reliable prognostic circulating biomarker is urgently needed, one that can be measured before and after surgery to indicate whether tumor-related signals still persist in the circulation, and circulating extracellular vesicles (EVs) may serve this role.

Tumor-derived EVs are abundant in the TME and carry nucleic acids, oncogenes and a variety of proteins, including immunosuppressive molecules¹⁶. These vesicles are 30–150 nm in diameter, enclosed by a lipid bilayer, and detectable in body fluids such as blood, saliva, and urine^{17–19}. Common EV surface markers, such as CD9, CD63, and CD81 are expressed on tumor-derived EVs surface^{20,21}. Furthermore, tumor-derived EVs carry protein biomarkers, which may promote tumor progression, immune suppression, and angiogenesis, such as FasL, PD-L1, EGFR, and TGFβ²², and EV-transferred TGFβ type II receptor (TβRII) from stromal fibroblasts can induce TGFβ signaling in SCC cells even in the absence of TGFβ itself²³. Functionally, plasma-derived EVs from HNSCC patients impair CD4 + T cell activation²⁴, while PD-L1 high EVs, whose abundance correlates with disease stage, suppress CD8 + T cell activity²⁵. Additionally, EV numbers are increased in HNSCC patients with active disease²⁶, and immuno-captured CD44v3 + EVs are particularly enriched in EpCAM, PD-L1, FasL, TGF-β1, and CSPG4²⁷. Tumor-derived CD44v3 + exosomes and their immunosuppressive activity decreased significantly after surgical resection in HNSCC patients, indicating that plasma EVs can report on the removal of tumor burden²⁸. Plasma EVs are nonetheless a heterogeneous population released by many cell types, and platelets are among the largest contributors to the circulating pool²⁹. Platelet-derived EVs carry α-granule cargo, including growth factors and matrix regulators, and platelet activation is closely linked to tumor progression, so that the plasma EV proteome reflects both the tumor and the systemic host response to it^{30,31}.

Nevertheless, the EV protein cargo that changes with tumor removal, and whether such changes carry functional and prognostic weight, remains largely unexplored. In this exploratory study, we therefore applied quantitative proteomics to paired plasma EV samples collected from the same patients at two vascular sites - peripheral and tumor-draining venous blood at two time points, before and shortly after tumor resection. Combining pairwise comparison with unsupervised clustering, we asked (i) which EV proteins respond to removal of the tumor, (ii) whether the peripheral and tumor-draining compartments report the same change, and (iii) whether the responsive proteins alter tumor cell proliferation and relate to overall survival.

Materials and Methods

Patient sample collection:

Patients with histologically confirmed HNSCC scheduled for primary tumor resection at the Department of Otorhinolaryngology, University Hospital Basel (Switzerland), were recruited for this study (n = 11; Table I). Staging followed the 8th edition of the TNM classification³², in which T describes the extent and local behavior of the primary tumor, N the absence or presence of regional lymph node metastases, and M the absence or presence of distant metastases. On this basis, tumors were grouped as low grade (WHO stages I and II) or high grade (WHO stages III and IV). The study was approved by the Ethics Committee of Northwest and Central Switzerland (approval no. 2020-02173), and all participants gave written informed consent for the use of their blood samples in research and for anonymized processing of the resulting data.

Blood was drawn from two vascular puncture sites, at two time points, giving four samples per patient. Peripheral puncture venous blood was obtained from an antecubital vein immediately before the start of surgery (PP1) and again 2 h after completion of tumor resection (PP2). Tumor-draining venous blood was sampled intraoperatively by direct puncture of the specify vessel, internal jugular vein, before any manipulation of the primary tumor (TV1), and from the same tumor vessel 2 h after resection (TV2). At each timepoint, 7.5 ml of blood was collected into EDTA tubes (S-Monovette; Sarstedt) using a standardized protocol. Samples were centrifuged at $800 \times g$ for 10 min at room temperature to sediment cells and cellular debris, and the resulting platelet-rich plasma (PRP) was transferred to fresh tubes. Platelet-poor plasma (PPP) was then obtained by a second centrifugation of the PRP at $10,000 \times g$ for 30 min at 4°C. PPP was aliquoted and stored at -80°C until EV isolation, avoiding repeated freeze-thaw cycles³³.

Table 1: Patient characteristics (n = 11).

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Characteristic	N = 11
Age, years, Median (Min-Max)	68 (55-83)
Sex, n (%)	
Male	8 (73%)
Female	3 (27%)
Primary site, n (%)	
Oral cavity	4 (36%)
Oropharynx	3 (27%)
Hypopharynx	1 (9.1%)
Larynx	1 (9.1%)
Major salivary gland	1 (9.1%)
Unknown primary	1 (9.1%)
Histology, n (%)	
Squamous cell carcinoma	8 (73%)
Polymorphous adenocarcinoma	1 (9.1%)
Carcinoma, NOS	2 (18%)
T category, n (%)	
T1	1 (9.1%)
T2	3 (27%)
T3	2 (18%)
T4	5 (45%)
N category, n (%)	
N0	6 (55%)
N1	1 (9.1%)
N2	2 (18%)
N3	2 (18%)
M category, n (%)	
M0	10 (91%)
M1	1 (9.1%)
UICC stage (8th ed.), n (%)	
I	1 (9.1%)
II	1 (9.1%)
III	1 (9.1%)
IVA	5 (45%)
IVB	3 (27%)
IVC	0 (0%)
Grade, n (%)	
G1	0 (0%)
G2	2 (33%)
G3	4 (67%)
Not available	5
p16 status, n (%)	
Negative	6 (100%)
Not available	5
PD-L1 TPS, %, Median (Min-Max)	70 (0-70)
Not available	8
Margin, n (%)	
R0	8 (100%)
Not available	3

EV isolation and protein extraction:

EVs were captured with in-house prepared EXÖBead, magnetic microparticles coated with galectins that bind N-linked glycans on the vesicle surface^{33,34}. The approach exploits EV surface glycoproteins such as galectin-3-binding protein (LGALS3BP)³⁵. EXÖBead has since been applied in experimental atherosclerosis³⁶ and amyotrophic lateral sclerosis³⁷, and in our previous HNSCC work EXÖBead-

captured plasma EVs showed strong EV marker enrichment with minimal lipoprotein contamination or protein aggregate carryover and retained immunosuppressive activity^{33,34}.

Platelet-poor plasma (1 ml) was combined with³⁴ 500 µl PBS and incubated with 100 µl EXÖBead (1 µm, 1×10^9 particles/ml) for 1 h at 37 °C. The resulting EV–EXÖBead complexes were retained on a magnetic separation rack and washed twice with 1 ml 0.1 M glycine (Merck KGaA, Darmstadt, Germany) in PBS. Protein extraction followed our previously published procedure^{34,38}. Washed complexes were resuspended directly in 1× RIPA buffer (Abcam plc, Cambridge, United Kingdom; ab156034) containing 1× ProteaseArrest™ Protease Inhibitor Cocktail (G-BIOSCIENCES, St. Louis, MO, United States) and held at –80 °C until processing. Lysates were subsequently boiled at 70 °C for 10 min, sonicated on ice for 5 min, and cleared by centrifugation at $10,000 \times g$ for 30 min at 4 °C. Protein concentration was quantified by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc., Waltham, United States). For each sample, at least 5 µg of protein was combined with 1× Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, United States) supplemented with 2-mercaptoethanol (Merck KGaA, Darmstadt, Germany) to a final volume of 35 µl before submission to the proteomics core facility at the Bavarian Center for Biomolecular Mass Spectrometry.

Label-Free Quantitative Proteomics and Integrated Statistical Analysis:

Protein identification and quantification were executed in accordance with our previous study³⁹, utilizing in-gel protein digestion, data-dependent acquisition (DDA) mass spectrometry on a Q-Exactive HF-X mass spectrometer (ThermoFisher Scientific, Bremen) and the MaxQuant computational platform (version 1.6.3.4) for data analysis. Tandem mass spectrometry (MS/MS) spectra were searched against the UniProt reference proteome for *Homo sapiens* (Taxon ID: 9606; UP000005640; July 2020), with the search space augmented by the standard MaxQuant contaminant database⁴⁰. Search parameters were configured with Trypsin/P specificity; carbamidomethylation of cysteine was defined as a fixed modification, while methionine oxidation and N-terminal acetylation were treated as variable modifications. To ensure statistical reliability, a target-decoy strategy utilizing reversed sequences was applied, restricting the false discovery rate (FDR) to 1% at both the peptide spectrum match (PSM) and protein levels.

The resulting proteinGroups.txt output file was further processed in RStudio (version: 2026.07.1+147)⁴¹ with R (version: 4.5.1)⁴². Protein groups flagged by MaxQuant⁴⁰ as "only identified by site", "reverse" or potential contaminants were removed, and only proteins quantified with at least two peptides were retained. LFQ intensities were log2-transformed and proteins were kept if they showed valid values in at least 75 % of the replicates of at least one experimental group (PP1, PP2, TV1, TV2). The retained matrix was quantile-normalized across samples (rank-based, using the mean of the sorted intensity distributions as reference). Remaining missing values were treated as missing not at random (MNAR) and imputed by a left-shifted Gaussian approach analogous to that implemented in Perseus⁴³, drawing random values from a normal distribution shifted into the low-intensity range of the total matrix (width $0.3 \times SD$, down-shift $1.8 \times SD$). A fixed random seed was used throughout to ensure reproducibility.

Pairwise differential abundance between all group combinations was assessed by two-sided paired Student's t-tests on the log2 intensities, with the difference of group means taken as the log2 fold change; p-values were corrected for multiple testing within each comparison by the Benjamini–Hochberg (BH) procedure, and proteins with BH-adjusted $p < 0.05$ and fold change > 2 were considered significantly regulated and visualised in volcano plots. In parallel, unsupervised structure in the normalised and imputed matrix was resolved by self-organising tree algorithm (SOTA) clustering⁴⁴ (clValid version 0.7 & cogen version 1.6.2; Euclidean distance, maxCycles = 8 yielding 9 clusters, maxEpochs = 1000, wcell = 0.6, pcell = 0.005, scell = 0.001, $\delta = 1 \times 10^{-4}$, unrestricted growth), with the number of splits guided by the elbow of the within-cluster sum of squares from k-means solutions ($k = 2-50$). For each cluster and for each significant protein set, per-sample means were compared between groups by paired t-tests with BH correction within the respective family of comparisons. Functional interpretation was performed by over-representation analysis using Fisher's exact test (one-sided, over-representation) on 2×2 contingency tables constructed against a background of all quantified protein groups, using the leading protein symbol of each protein group as the counting unit. Annotations were obtained from Gene Ontology Biological Process (org.Hs.eg.db v3.22.0, GO.db v3.22.0), Reactome (reactome.db v1.95.0) and KEGG⁴⁵ (KEGGREST v1.50.0); terms were tested only if they contained ≥ 3 and $\leq [1.5 \times N^{0.75}]$ background units (floor 50) and were hit by ≥ 3 units of the query set, and p-values were BH-adjusted separately within each annotation database, with adjusted $p < 0.05$ considered significant. Overlaps between differentially abundant proteins and SOTA clusters were visualised with ComplexUpset (v1.3.3); figures were produced with ggplot2 (v4.0.2), ggrepel (v0.9.8) and ggsignif (v0.6.4), and tables exported with openxlsx (v4.2.8.1). Finally, the prognostic relevance of the identified target leading proteins was assessed using publicly available pan-cancer RNA-seq data from 499 HNC patient tissues in the Kaplan–Meier Plotter resource (<https://kmplot.com>; Pan-Cancer RNA-seq)⁴⁶, with patients stratified into high- and low-expression groups and survival differences evaluated by log-rank test ($p < 0.05$ considered significant).

EV characterization:

EVs were characterized by nanoparticle tracking analysis (NTA) and cryo-transmission electron microscopy (cryo-TEM) as described in our previous publication³³. EV concentration and size distribution were determined using a ZetaView® PMX 110 Nanoparticle Tracking Analyzer (Particle Metrix GmbH, Germany). Samples were diluted in PBS to a final volume of 1 mL, and each measurement consisted of two cycles scanning 11 positions at 60 frames per second. Acquisition was performed at a sensitivity of 80, a shutter setting of 70, a cell temperature of 25 °C and a trace length of 15. Videos were analyzed using ZetaView software version 8.05.11 SP1 with a minimum particle brightness of 20, a particle size range of 5–1,000 pixels, and 10 nm per class and 10 classes per decade for the particle size distribution.

For cryo-TEM, 4 μL of the EV sample was applied to a holey carbon-coated grid (Ted Pella, Inc., USA), blotted with Whatman No. 1 filter paper and vitrified in liquid ethane at -178°C using a Leica GP plunger (Leica Microsystems GmbH, Germany). The frozen grids were transferred to a Talos electron microscope

(FEI, USA) using a Gatan 626 cryo-holder and imaged at an accelerating voltage of 200 kV under low-dose conditions ($20 \text{ e}^-/\text{\AA}^2$) while maintaining the sample at cryogenic temperature. Micrographs were acquired with a CETA camera (Thermo Fisher Scientific, Inc., USA).

Cell proliferation assay:

Cell viability was assessed using the EZMTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay (Merck KGaA, Darmstadt, Germany; CBA410) according to the manufacturer's instructions. HaCaT, SCC-016 and SCC-125 cells were seeded at 1×10^4 cells per well in 96-well plates and treated with 1×10^6 EVs per well (100 EVs per cell) for 24 h. EZMTT reagent was then added, and absorbance was measured after 5 h of incubation.

Results

Based on the filtering criteria applied in the proteomic analysis, the average number of detected proteins was 314 ± 15 in peripheral plasma (PP) extracellular vesicles (EVs) before tumor resection (PP1) and 306 ± 18 after tumor resection (PP2) ($n = 11$). Similarly, tumor venous (TV) plasma EVs contained 308 ± 16 proteins before resection (TV1) and 310 ± 18 proteins after resection (TV2) ($n = 11$). Through pairwise comparison, we identified nine proteins by proteomics that were significantly elevated (adjusted $p < 0.05$) with a fold-change greater than 2 in PP1 compared with PP2 (Fig. 1A). These proteins included platelet-derived growth factor subunit B (PDGFB) and subunit C (PDGFC), pro-platelet basic protein (PPBP), tissue inhibitor of metalloproteinase 3 (TIMP3), heparanase (HPSE), collagen alpha-1(VI) chain (COL6A1), latent-transforming growth factor beta-binding protein 1 (LTBP1), platelet factor 4 (PF4/CXCL4), and platelet factor 4 variant 1 (PF4V1). In contrast, the equivalent comparison in tumor venous plasma EVs yielded only a single differentially abundant protein, enolase 3 (ENO3), which was more abundant in TV2 than in TV1 (Supplemental Fig. 1A). Although ENO3 also appeared higher in PP2 than in PP1 at the group level, this difference was not significant in the pairwise PP1 vs PP2 comparison across all quantified proteins (BH-adjusted) and was therefore not considered further. As expected from their selection, the mean $\log_2$ LFQ intensity of the nine proteins was higher in PP1 than in PP2; notably, it was also significantly higher in TV1 than in PP2 (paired t-test, BH-adjusted $p < 0.05$; Fig. 1B).

The high abundance of these nine proteins in PP1 pointed to the coordinated activation of processes involved in positive regulation of cell division, antiviral and antimicrobial immune responses, chemotaxis, and PI3K-Akt signaling (adjusted p -value < 0.05) (Fig. 1C). To assess clinical relevance, the target EV proteins were examined at the transcript level in tissue-derived pan-cancer RNA-seq data (KM Plotter). In the head and neck squamous cell carcinoma cohort (TCGA, $n = 499$; overall survival, auto-selected best cut-off across percentiles, follow-up censored at the respective threshold), patients with high mean expression of the nine-protein signature showed significantly poorer 3-year overall survival than those with low expression (HR 1.40, 95 % CI 1.05–1.88; log-rank $p = 0.023$; Fig. 1D). This association was no longer significant at 5 years (HR 1.24, 95 % CI 0.94–1.65; log-rank $p = 0.12$; Supplemental Fig. 1B).

Pairwise analysis identified only nine differentially abundant proteins in peripheral plasma EVs and one in tumor venous plasma EVs. To capture modest but coordinated changes that single-protein testing may miss, we applied unsupervised Self-Organizing Tree Algorithm (SOTA) clustering, which groups proteins by shared abundance patterns without requiring a predefined number of clusters and is robust to noise. This analysis yielded nine clusters (Supplemental Fig. 2). Only Cluster 8 showed a significant difference between PP1 and both PP2 and TV2 (adjusted $p < 0.05$; Fig. 2A, B). Cluster 8 comprised 24 leading proteins: ADH4, ANGPTL6, B2M, C3orf58, CAT, CD36, CD9, COL6A2, EMILIN1, FCN2, GP1BA, GP1BB, GP9, HLA-B, HSP90B1, HSPA5, PDGFA, PDGFC, PRDX2, RAB11B, SLC2A1, TIMP3, TPM4, and TUBB1. Two of these, PDGFC and TIMP3, overlapped with the nine-protein signature identified by pairwise comparison. Pathway analysis of Cluster 8 showed enrichment of platelet-related pathways, collagen-associated pathways, extracellular matrix (ECM)–receptor interaction, prostate cancer, and human T-cell leukemia virus 1 infection (Fisher's exact test, adjusted $p < 0.05$; Fig. 2C).

Unlike the pairwise signature, high expression of the 24 Cluster 8 genes was associated with poor 3-year overall survival in the KMplot HNSCC cohort (HR 1.72, 95% CI 1.18–2.51; logrank $p = 0.0043$) (Fig. 2D) and 5-year overall survival (HR 1.47, 95% CI 1.04–2.06; logrank $p = 0.026$) (Supplemental Fig. 3A). UpSet analysis of the two protein sets identified two proteins in common, PDGFC and TIMP3 (Fig. 2E). High average expression of PDGFC and TIMP3 was likewise associated with reduced 3-year overall survival (HR 1.43, 95% CI 1.06–1.92; logrank $p = 0.018$) (Fig. 2F) and 5-year overall survival (HR 1.39, 95% CI 1.05–1.84; logrank $p = 0.021$) (Supplemental Fig. 3B), suggesting that these two proteins may represent a compact, clinically tractable core of the broader EV-associated signature, although this exploratory association requires validation in an independent cohort.

We next investigated whether these compositional changes in plasma EVs differentially influence tumor cell proliferation. Nanoparticle tracking analysis (NTA) and cryo-EM revealed no differences in EV size or particle number among the groups (Fig. 3A–C). Treatment with plasma EVs from PP1 and TV1 significantly increased the growth of UPCI-SCC-016 cells compared with medium and lactose elution buffer controls ($p = 0.0091$ and $p = 0.0364$, respectively) (Fig. 3D). When comparing PP1 and PP2 plasma EVs, the PP2 group showed a significantly reduced ability to stimulate cell proliferation compared with the PP1 group ($p = 0.0078$) (Fig. 3D); PP2 EVs were likewise less stimulatory than TV1 EVs ($p = 0.0091$). Conversely, no differences in cell proliferation were observed in HaCaT cells treated with PP1, PP2, TV1, or TV2 plasma EVs compared with medium or lactose elution buffer controls (Fig. 3E). These results indicate that the growth-promoting activity of circulating EVs declines after tumor resection, consistent with the compositional changes observed in the proteomic analysis.

Discussion

A major challenge in head and neck cancer (HNC) is that the tumor microenvironment simultaneously restrains and promotes angiogenesis, so that small EVs (sEVs) circulating in patients' plasma carry a mixed cargo whose net effect on tumor progression remains difficult to predict^{47,48}. In our cohort, PDGFB, PDGFC, PPBP, TIMP3, HPSE, COL6A1, LTBP1, PF4, and PF4V1 in peripheral plasma EVs were

consistently elevated before tumor resection (PP1) and declined afterward (PP2), and were enriched for platelet activation and ECM-remodeling pathways (Fig. 1) - a platelet-associated pattern whose likely cellular source we consider below. Tengler et al. also identified pro-angiogenic proteins, such as MMP-9, and anti-angiogenic proteins, such as PF4, thrombospondin-1, and serpin F1, as well as dual-function proteins such as pentraxin-3 and dipeptidyl peptidase IV (DPPIV), in plasma sEVs from HNC patients⁴⁷. Notably, PF4 was identified as anti-angiogenic in both studies, consistent with our result (Fig. 1). Beyond the previously documented anti-angiogenic mediators, our analysis revealed a complex repertoire of EV-associated proteins with opposing vascular functions: pro-angiogenic factors including PDGFB⁴⁹, PDGFC⁵⁰, PPBP⁵¹, LTBP1⁵², and HPSE⁵³ coexisted with anti-angiogenic mediators PF4V1⁵⁴ and TIMP3⁵⁵, suggesting a dynamic equilibrium of competing signals within the HNC microenvironment. Although anti-angiogenic factors such as TIMP3 and PF4 were present, the net effect of pre-resection plasma EVs (PP1 and TV1) was to promote proliferation of UPCI-SCC-016 cells, an effect not seen in non-malignant HaCaT keratinocytes (Fig. 3D, 3E). Crucially, the post-resection decline in these EV-associated signals (PP2) significantly attenuated this growth-stimulating effect (Fig. 3D), consistent with removal of the tumor reducing the growth-promoting activity of circulating EVs. Notably, TV1 and TV2 were highly similar in composition, with only a single differentially abundant protein (Supp. Figure 1A), so that although no difference was detectable locally, the peripheral compartment still registered a clear change. At least two, non-exclusive explanations are possible. First, technical factors may have masked a local change: the short two-hour sampling interval, or intraoperative manipulation of the tumor-draining vessel, may mean that TV2 no longer samples the same vascular bed as TV1. Second, the peripheral signature may reflect a systemic response to tumor burden rather than direct locoregional EV release. These explanations are not mutually exclusive: local sampling artifact and genuine systemic integration of tumor-derived signal could operate concurrently, with technical dilution attenuating a locoregional change while the peripheral compartment simultaneously captures an integrated systemic readout of tumor burden. Future studies are required to determine whether loss- or gain-of-function of these proteins alters EV activity, and to clarify how pre- versus post-operative shifts in EV cargo balance opposing angiogenic signals to drive vascular remodeling and tumor progression in vivo.

SOTA clustering yielded nine clusters. Only Cluster 8 (24 proteins) was associated with poor 5-year overall survival (Fig. 2), unlike the pairwise signature (Fig. 1). This dissociation is informative. The proteins that respond most strongly to tumor removal are not necessarily those that track long term outcome, so the two analyses capture distinct features: dynamic response and prognostic burden. Proteins shared by both are therefore of particular interest. Because SOTA groups proteins by abundance pattern rather than by annotation, the Cluster 8 members changed in parallel before and after resection. This coordinated behavior may reflect a shared cellular source or a common regulatory stimulus. Several of these proteins have been reported previously in HNC. These survival associations come from bulk tissue transcript data (KM Plotter), not from EV protein measurements in our cohort. They therefore offer indirect support rather than direct validation of the vesicular signature. Because the survival cut-off was selected automatically as the best performing percentile, these results should be considered exploratory. Bai et al. revealed that Rab11a coordinates with the exocyst complex to promote

multivesicular body trafficking and exosome biogenesis in HNC⁵⁶. CD9, a canonical tetraspanin, serves here as a vesicle-identity marker confirming EV enrichment rather than as an independent prognostic contributor; its presence in Cluster 8 should therefore be read as validation of vesicular origin. In HNC tissue, CD9 has separately been reported mainly as a favorable marker, with reduced expression linked to nodal metastasis and shorter survival, although the direction differs between subsites⁵⁷. The largest group in Cluster 8 is platelet-related. GP1BA (CD42b), GP1BB (CD42c) and GP9 (GPIX & CD42a) encode the platelet GPIb-IX subunits, and GP1BA and GP9 have been identified directly in the proteome of platelet-derived EVs^{31,58,59}. TUBB1 encodes a β -tubulin isoform expressed only in platelets and megakaryocytes⁶⁰. It has not been examined in platelet-derived EVs or HNC EVs specifically, but was quantified in plasma EVs and formed part of an inflammatory network in newly diagnosed rheumatoid arthritis⁶¹. TPM4 is required non-redundantly for thrombopoiesis⁶², although it is ubiquitously expressed and has also been reported as upregulated in the saliva of OSCC patients⁶³. CD36 corresponds to platelet glycoprotein IV and also marks metastasis-initiating cells in human oral carcinoma, where CD36⁺ CD44^{bright} cells uniquely seeded lymph node and lung metastases and were suppressed by neutralizing antibody⁶⁴. PDGFC and TIMP3 also fall in this group, both being α -granule cargo released upon platelet activation^{65,66}, although to our knowledge neither has previously been reported as EV cargo in HNC. UpSet analysis showed that TIMP3 and PDGFC were the only proteins shared between the pairwise signature (Fig. 1A) and SOTA Cluster 8, and high combined expression was associated with reduced 5-year overall survival again at the transcript level, pending confirmation with protein-based outcome data (Fig. 2E, F).

PDGF-C is a stroma-derived mitogen and angiogenic factor that sustains vascularization when VEGF is blocked, and its neutralization slows anti-VEGF-refractory tumors⁵⁰. It is also stored in platelet α -granules and released on activation⁶⁵. Both sources would plausibly contribute to the proliferative effect we observed in UPCI-SCC-016 cells (Fig. 3D), and both would diminish once the tumor is removed. TIMP3, by contrast, is more difficult to reconcile. In HNSCC the gene is characteristically silenced by promoter hypermethylation, and TIMP3 methylation in post-treatment salivary rinse independently predicts local recurrence^{67,68}. Tumor-cell TIMP3 would therefore be expected to be low, yet TIMP3 protein was enriched before resection. This apparent paradox is resolved by the platelet source proposed above: because TIMP3 is stored in and released from platelet α -granules, all four TIMPs are expressed by megakaryocytes and platelets, TIMP3 is released from activated platelets, and megakaryocytes actively sort metalloproteinase-inhibitor transcripts into platelets, consistent with regulated α -granule storage rather than passive plasma contamination^{66,69–71}. Taking together, these observations suggest that PDGFC and TIMP3 may serve as a compact prognostic readout in HNSCC. Future work will need to establish their cellular origin, determine whether either retains activity in the vesicular compartment, or validate the pair in an independent cohort.

Our study is limited by cohort size, transcript-level basis of the survival associations noted above, and using a single HNC cell line. The platelet origin we propose remains an inference from co-abundance;

without tumor- and platelet-specific EV markers, the cellular source of the pre-resection signature is not established. Post-resection samples were collected two hours after surgery, so effects of intraoperative fluids or blood loss cannot be excluded; surgical trauma would nonetheless be expected to increase rather than decrease platelet α -granule release, so perioperative platelet activation alone does not readily account for the observed decline. Even so, PDGFC and TIMP3 declined after resection with no change in EV number or size, indicating altered cargo composition rather than reduced vesicle output. Larger cohorts with protein-level survival data, tumor- and platelet-specific EV markers, and standardized perioperative sampling will be needed to confirm the pair and define its cellular source. In conclusion, pre-resection plasma EVs in HNC carry a platelet- and ECM-associated cargo that promotes tumor cell proliferation and resolves after surgery, with PDGFC and TIMP3 emerging as a compact candidate prognostic pair.

Declarations

Compliance with Guidelines and Regulations:

All methods were performed in accordance with the relevant guidelines and regulations.

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Author Contribution

Author Contributions: DMC, MWP, and LM conceived and designed the study. LM provided the patient samples. DMC, MWP, MR, and LM acquired funding and developed the methodology. DMC, CL, and AS performed proteomic measurements and acquired the raw data. DMC developed the R scripts, performed the data analysis, and wrote the original draft of the manuscript. All authors critically reviewed and revised the manuscript and approved the final version for submission.

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Data Availability

Data availability: The mass spectrometric raw files as well as the MaxQuant output files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository and can be accessed using the identifier: PXD084238. Code availability: The R scripts used for NTA assay, MTT cell proliferation assay, the patient characteristics table, and proteomics data analysis, together with the imputed and normalized proteinGroups files generated in R and RStudio, have been uploaded to GitHub. (<https://doi.org/10.5281/zenodo.22877761>)

Code availability:

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Figures

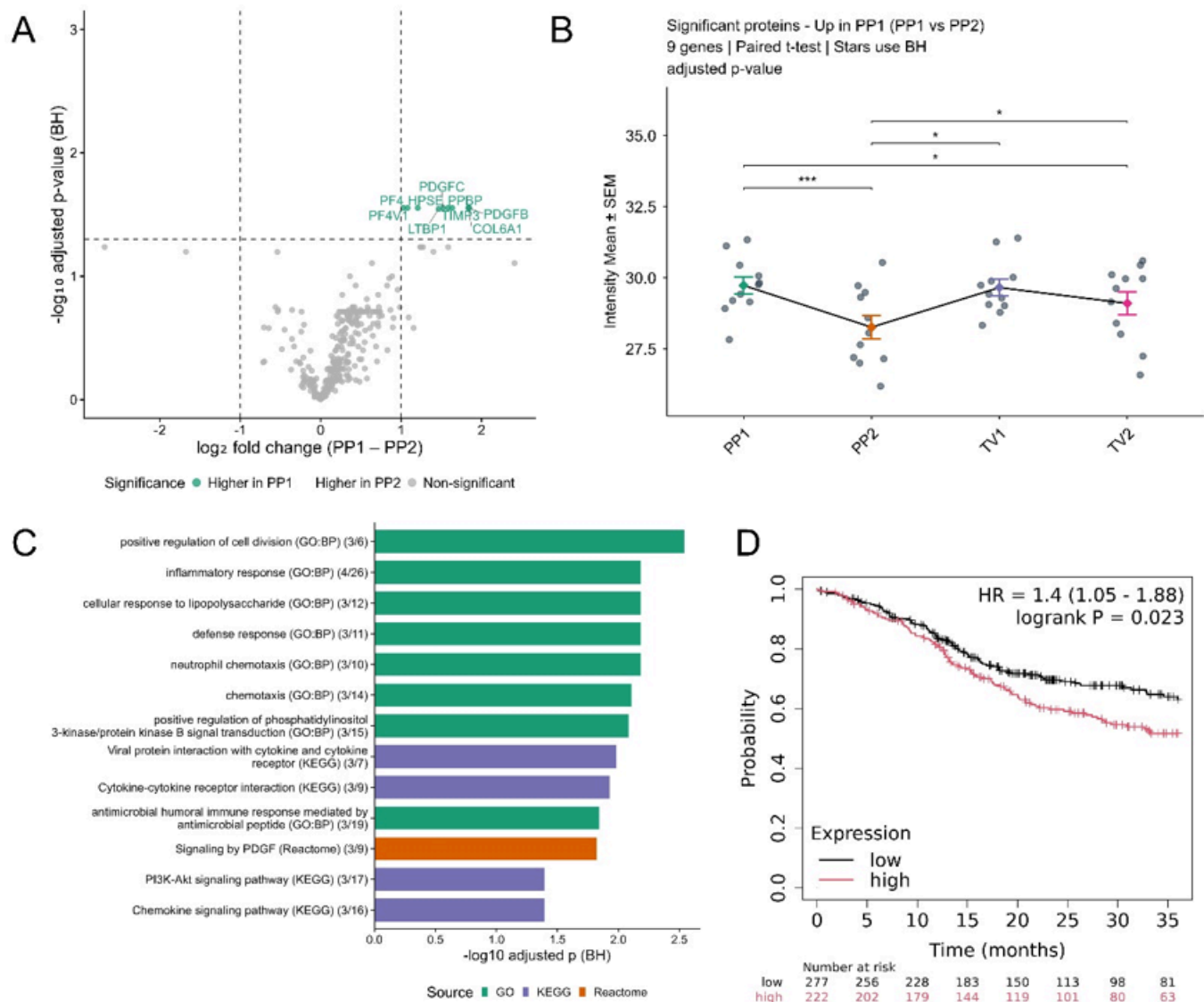


Figure 1

Proteomic profiling of plasma EVs before and after tumor resection. **(A)** Volcano plot of peripheral plasma EVs before (PP1) versus after (PP2) resection (n = 11); nine proteins elevated in PP1 (adjusted p < 0.05, fold change > 2). **(B)** Mean log₂ LFQ intensity of the nine-protein signature in PP1, PP2, TV1, and TV2; each dot represents one patient, mean ± SEM. Paired t-test, BH-adjusted: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. **(C)** GOBP, KEGG, and Reactome enrichment of the nine proteins (adjusted p < 0.05); numbers in parentheses, significant/detected proteins per term. **(D)** Kaplan–Meier 3-year overall survival of HNSCC patients stratified by nine-protein signature expression (KMplot.com, Pan-cancer RNA-seq, n = 499; best cutoff).

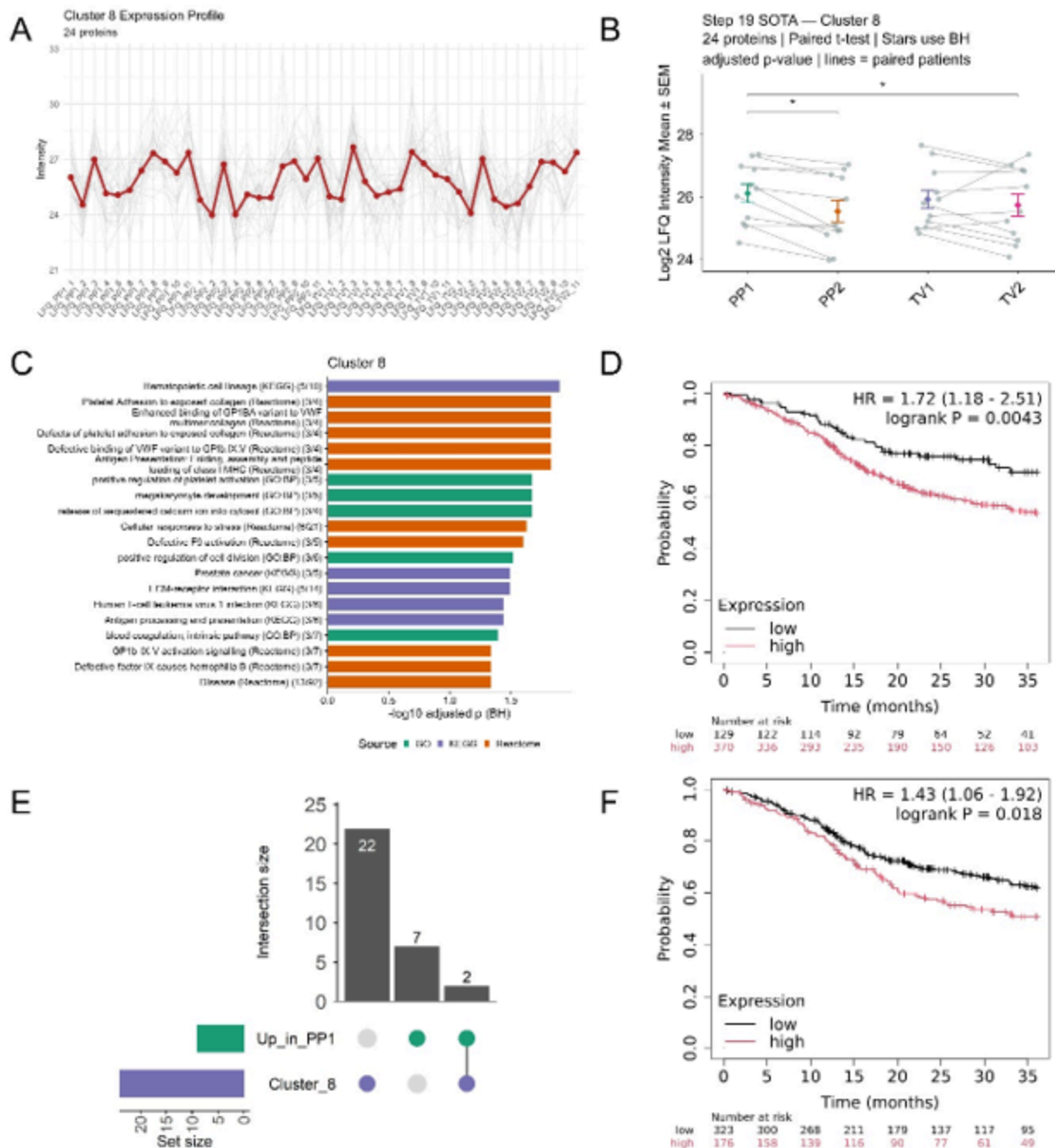


Figure 2

Unsupervised clustering reveals a survival-associated EV protein signature. **(A, B)** Unsupervised SOTA (self-organizing tree algorithm) clustering of protein abundance profiles yielded nine co-varying clusters; Cluster 8 (24 proteins) differed significantly between PP1 and both PP2 and TV2 (adjusted $p < 0.05$). **(C)** Pathway over-representation analysis of Cluster 8 proteins (Fisher's exact test, adjusted $p < 0.05$). **(D)** Kaplan–Meier 3-year overall survival curves for HNSCC patients stratified by average expression of the 24-protein signature identified in Cluster 8 (KMplot.com, Pan-cancer RNA-seq, $n = 499$; auto-selected best cutoff). **(E)** UpSet plot comparing the Cluster 8 and pairwise differential protein sets, identifying two

shared proteins. **(F)**Kaplan–Meier 3-year overall survival curves for HNSCC patients stratified by average expression of the two-protein signature co-identified in Cluster 8 and the pairwise differential protein sets (KMplot.com, Pan-cancer RNA-seq, n = 499; auto-selected best cutoff).

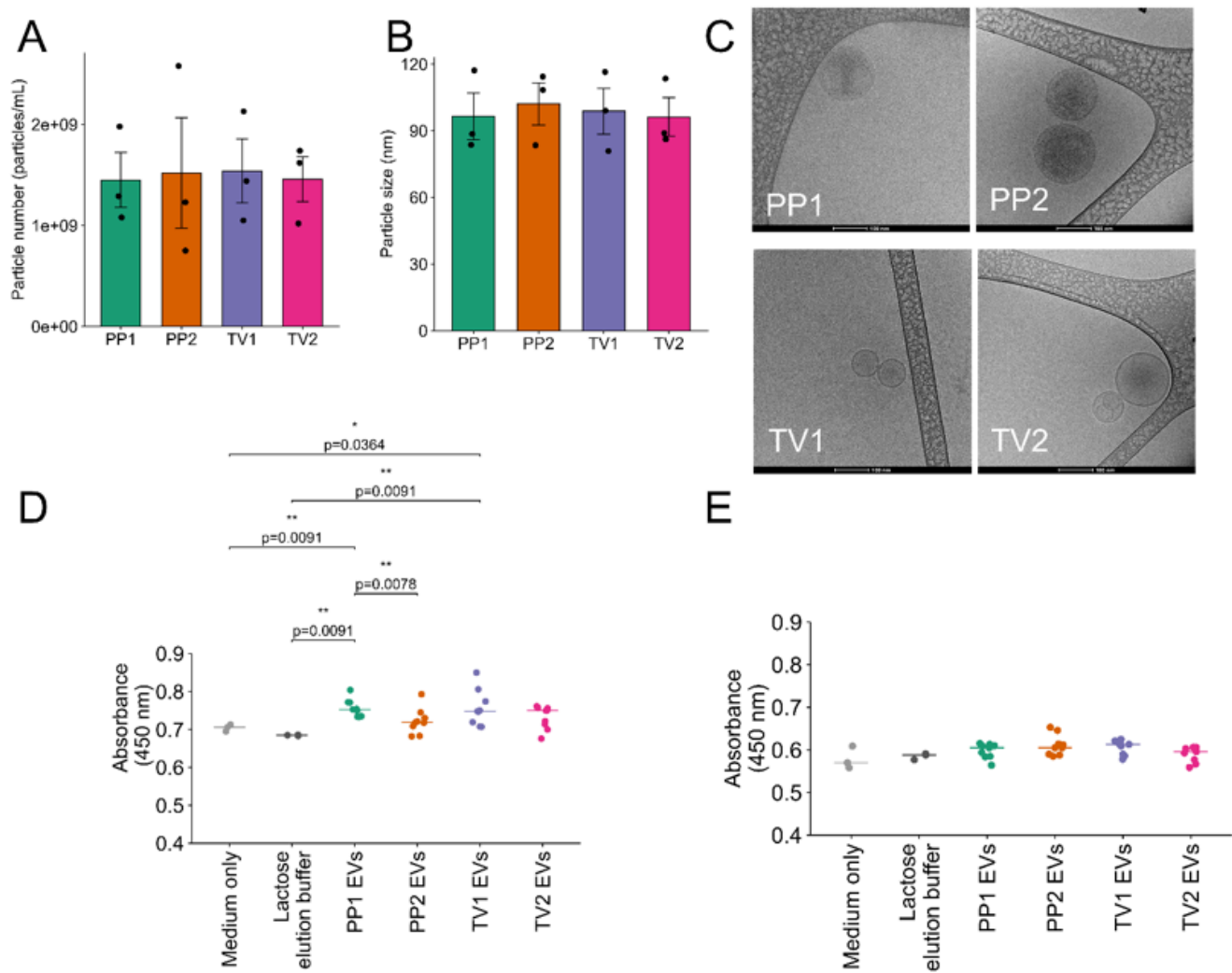


Figure 3

Growth-promoting activity of plasma EVs declines after tumor resection. **(A, B)** Nanoparticle tracking analysis of plasma EV size distribution and particle concentration across groups (no significant differences). **(C)** Representative cryo-EM images of plasma EVs. **(D)**Proliferation of UPCI-SCC-016 cells treated with plasma EVs from PP1, PP2, TV1, and TV2 versus medium and lactose elution buffer controls. **(E)** Proliferation of HaCaT cells under the same conditions.

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