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1 **Main Manuscript for**

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3 **Gene signature of circulating platelet-bound neutrophils is associated with poor prognosis**
4 **in cancer patients**

5

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20 **The full manuscript includes:**

21 Main text

22 Figures 1 to 5

23 Supplemental Figures 1 to 5

24 Supplemental Tables 1 to 6

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26

27

28 **Novelty and Impact**

29

30 Platelets physically interact with peripheral blood neutrophils to form neutrophil-platelet aggregates
31 (NPAs), known to promote disease worsening in various inflammatory diseases. However, characterization
32 of NPAs in cancer remains totally unexplored. We showed that NPAs-associated neutrophils were a yet-
33 unreported unique subset of circulating neutrophils associated with a worse patient prognosis in several
34 cancer types. NPAs may hold clinical utility as novel non-invasive blood prognostic biomarker in cancer
35 patients with solid tumors.

36

37 **Abstract**

38

39 Beyond their critical role in hemostasis, platelets physically interact with neutrophils to form
40 neutrophil-platelet aggregates (NPAs), enhancing neutrophil effector functions during inflammation. NPAs
41 may also promote disease worsening in various inflammatory diseases. However, characterization of NPAs
42 in cancer remains totally unexplored. Using ImageStream®X (ISX) imaging flow cytometer, we were not
43 only allowed able to detect CD15⁺ CD14⁻ CD36⁺ ITGA2B⁺ NPAs in both healthy donors' (HDs) and cancer
44 patients' bloods, but we also showed that NPAs result from the binding of platelets preferentially to low-
45 density neutrophils (LDNs) as opposed to normal-density neutrophils (NDNs). By re-analyzing two
46 independent public scRNAseq data of whole blood leukocytes from cancer patients and HDs, we could
47 identify a subset of neutrophils with high platelet gene expression that may correspond to NPAs. Moreover,
48 we showed that cancer patients' derived NPAs possessed a distinct molecular signature compared with the
49 other neutrophil subsets, independently of platelet genes. Gene ontology (GO) term enrichment analysis of
50 this NPAs-associated neutrophil transcriptomic signature revealed a significant enrichment of neutrophil
51 degranulation, chemotaxis and trans-endothelial migration GO terms. Lastly, using The Cancer Genome
52 Atlas (TCGA), we could show by multivariate Cox analysis that the NPAs-associated neutrophil
53 transcriptomic signature was associated with a worse patient prognosis in several cancer types. These
54 results suggest that neutrophils from NPAs are systemically primed by platelets empowering them with
55 cancer progression capacities once at tumor site. NPAs may therefore hold clinical utility as novel non-
56 invasive blood prognostic biomarker in cancer patients with solid tumors.

57

58 **Keywords**

59 Neutrophils – Low-density-neutrophils – Platelets – Neutrophil-platelet aggregates – Cancer.

60

61 **Article Category**

62 Research Article

63

64

65 **List of abbreviations**

66	ANC	Absolute neutrophil count
67	CPM	Count per million
68	GO	Gene ontology
69	HDs	Healthy donors
70	HNC	Head and neck cancer
71	HR	Hazard Ratio
72	ISX	ImageStream®X
73	LDNs	Low-density neutrophils
74	LIHC	Hepatocellular carcinoma cancer patients
75	LUAD	Lung adenocarcinoma
76	LUSC	Lung squamous cell carcinoma
77	NDNs	Normal-density neutrophils
78	NETs	Neutrophil extracellular traps
79	NLR	Neutrophils-to-lymphocytes ratio
80	NPAs	Neutrophil-platelet aggregates
81	NSCLC	Non-small-cell lung cancer
82	OS	Overall survival
83	PAAD	Pancreatic adenocarcinoma cancer patients
84	PCA	Principal component analysis
85	PCs	Principal components
86	PFI	Progression-free interval
87	RBC	Red blood cells
88	RT	Room temperature
89	ssGSEA	Single sample Gene Set Enrichment Analysis
90	TANs	Tumor-associated neutrophils
91	TCGA	The Cancer Genome Atlas
92	UMAP	Uniform manifold approximation and projection

93 **Background**

94

95 Although, some recent evidences suggested that tumor-associated neutrophils (TANs) could
96 display anti-tumor properties [1–3], a large body of evidence demonstrated that TANs could be pro-tumoral
97 and promote metastasis [4–6]. Pro-tumor TANs can assist metastasis by blunting anti-tumor T-cell
98 responses [4], and stimulating proliferation and invasiveness of metastatic tumor cells [5,6]. Accumulating
99 evidence in mice and humans showed that blood neutrophils may also contribute to cancer progression.
100 Numerous retrospective analysis across multiple different cancer types showed that an elevation in the
101 absolute count of circulating neutrophils together with a decrease in the absolute count of circulating
102 lymphocytes, both accounting for a high neutrophils-to-lymphocytes ratio (NLR) on blood, was associated
103 with a worse prognosis in patients [7–10]. More recently, studies have shown that a subset of circulating
104 neutrophils, named low-density neutrophils (LDN) expanded in cancer patient's blood and was associated
105 with worse prognosis, especially in non-small-cell lung cancer (NSCLC) and head and neck cancer (HNC)
106 [11,12]. Moreover, LDN was found to display features of pro-tumor neutrophils, such as increased T-cell
107 suppressive functions, as opposed to normal-density neutrophils (NDN) [11,13]. Nevertheless, LDN
108 remains a heterogeneous population of cells including mature and immature neutrophils [13,14]. A subset
109 of circulating LDN expressing the LOX-1 scavenger receptor was reported to display higher T-cell
110 suppressive functions as compared to LOX-1⁻ LDN [13]. LOX-1⁺ T-cell suppressive neutrophils were
111 recently reported to infiltrate lung tumors and were associated with a worse patient survival [15]. Besides
112 studies in humans, recent evidence in mice demonstrated that the pre-programming of neutrophils in the
113 periphery was required for acquisition of their tumor-promoting functions once at tumor site [16,17]
114 supporting the idea that tumor-promoting TANs derived from a distinct subset of neutrophils pre-existing in
115 the periphery.

116

117 Beyond their critical role in hemostasis, platelets can bind to circulating leukocytes forming
118 leukocyte-platelet aggregates, enhancing their effector functions. For instance, platelets have been recently
119 shown to interact with peripheral blood monocytes to activate antigen cross-presentation [18]. In sepsis, in
120 contrast to free neutrophils, NPAs (neutrophil-platelet aggregates) produce higher amount of neutrophil

121 extracellular traps (NETs) [19–21] and display increased phagocytic functions [22–24]. Furthermore, NPAs
122 display enhanced trans-endothelial migration capacity [25,26] therefore promoting neutrophil infiltration to
123 site of injury and fueling pathological inflammation in various diseases [21,27–29]. However, to our
124 knowledge, the characterization of NPAs in cancer patients remains unexplored.

125

126 In this study, we showed that neutrophils from NPAs represent a unique subset of activated
127 neutrophils in cancer patients by combining ImageStream®X (ISX) imaging flow cytometry and analysis of
128 public single cell transcriptomic data of human circulating neutrophils. In particular, we showed that LDN
129 as opposed to NDN were preferentially involved in NPA formation. Finally, we could generate a specific
130 gene signature of neutrophils in NPAs that we found associated with a worse prognosis in pancreatic
131 adenocarcinoma patients and liver hepatocellular carcinoma patients.

132

133 **Materials and Methods**

134

135 **Human cohorts**

136 Whole blood from a cohort of 6 metastatic NSCLC patients (Table S1) who gave informed consent at the
137 Léon Bérard Hospital (France), was collected in EDTA-coated tubes. As an age and sex-matched control
138 cohort, whole blood from healthy donors (HD) was collected via the Etablissement Français du Sang (Table
139 S1).

140

141 **Preparation of whole blood, NDNs and LDNs-enriched fractions for multi-spectral Imaging Flow 142 Cytometry (ImageStream®X, ISX)**

143 Blood from NSCLC patients or HD was withdrawn in EDTA-coated vacutainers 1 hour prior to the
144 processing. Two tubes of 3 ml of blood were centrifuged at 800 rpm (no brake) for 15 min at room
145 temperature (RT) to separate plasma from leukocyte-enriched red pellet. Platelet-rich plasma was
146 discarded for both tubes. One out of the two tubes was used for whole blood staining while the other served
147 for the separation of LDNs from NDNs. For such separation, leukocyte-enriched red pellet was re-
148 suspended in 6 ml of PBS_{1x} (5 mM EDTA) and then layered on the top of 3 ml of Ficoll (CMSMSL01-01,

149 EUROBIO), before being centrifuged at 1800 rpm for 20 min at RT (acceleration 5 and brake 1). LDNs-
150 enriched PBMC ring and NDNs-enriched red pellet were washed with PBS_{1x}, and pellets were re-
151 suspended in BD Pharm Lyse™ (555899) according to manufacturer's protocol to eliminate red blood cells.
152 In parallel, whole blood leukocyte-enriched red pellet was also re-suspended in BD Pharm Lyse™ for the
153 same purpose. After incubation, red blood cells (RBC)-lysed leukocytes were washed twice with PBS_{1x} (5
154 mM EDTA) were re-suspended at a final concentration of 20.10⁶ cells/ml prior to be stained. Samples were
155 acquired on ImageStream®X (ISX) no more than 5 days following cell staining and fixation.
156 Two millions cells per sample were stained for 30 min in the dark at 4°C in 100 µl staining buffer (PBS_{1x}, 2
157 % SVF, 5 mM EDTA) with antibodies directed against CD15 (561584, Clone HI98), CD14 (562335, MφP9),
158 CD41a (559777, HIP8) (all from BD), and CD36 (130-110-739, REA760) and CD62P (130-105-714,
159 REA389) from Miltenyi Biotec. Cells were washed twice with PBS_{1x} (5 mM EDTA, 2% fetal bovine serum
160 (FBS)) before being fixed in 2% formaldehyde solution (252549, Sigma-Aldrich). After incubation at 4°C,
161 cells were washed twice with PBS_{1x} (5 mM EDTA, 2% FBS) and re-suspended in a final volume of 180 µl
162 PBS_{1x} (5 mM EDTA, 2% FBS).
163 NPAs were imaged with ImageStream®X (ISX) imaging flow cytometer (Amnis Corporation-Luminex,
164 Seattle, WA) using 405, 488, 561, and 642 lasers and the 40X objective. Brightfield provided morphological
165 and structural details of the cell. At least 300 000 RBC-lysed blood cells from NSCLC patients or HDs
166 separately, excluding debris and free-platelet with low area, were collected for each sample. Since flow-
167 speed could influence the proportion of NPAs [30], RBC-lysed blood cells were acquired with a flow-speed
168 between 500 to 1000 events/second for each sample. Data were analyzed using IDEAS image analysis
169 software (Amnis Corporation, Seattle, WA). A specific gating strategy, with multiple filtering steps was set
170 up to identify non-coincidental viable NPAs. Such strategy aimed first at retaining only focused cells based
171 on Gradient RMS values for each event. Remaining debris and free platelets with low area were discarded.
172 Dead cells positive for Zombie NIR (Biolegend) viability marker were then eliminated. Neutrophils were
173 selected as cells with a high expression of CD15 and a low expression of CD14 markers. Low area event
174 on CD15 object mask were eliminated to exclude CD15⁺ debris or CD15⁺ insufficient quality staining for an
175 accurate estimation of true NPAs. Platelet-related events were then selected by gating on events positive
176 for both ITGA2B and CD36 cell-surface markers. A second filter was applied to narrow down the selection

177 of platelet-related events by only considering the ones with high ITGA2B intensity on ITGA2B-related
178 component with the largest area to remove residual platelet debris. To only retain non-coincidental NPAs,
179 two distinct masks delimiting neutrophil-related CD15 stained-area and platelet-related ITGA2B stained-
180 area were created. Non-coincidental NPAs were events for which neutrophil and platelet-associated masks
181 were overlapping with at least one pixel, and confirmed by image examination of random samples. Only
182 the most-focused platelet-related events were considered by selecting events with high gradient RMS of
183 ITGA2B marker on platelet area. Frequencies of NPAs were calculated on at least 100 non-coincidental
184 NPAs for each NSCLC patients and HDs.

185

186 **May-Grünwald-Giemsa staining**

187 A blood smear from a drop of NSCLC patients' whole blood was performed on a glass slide. Blood smear
188 was dried up for 10/15 min at RT. May-Grünwald staining was layered on dried blood smear using Hema-
189 Tek Stain Pak Kits designed for the Bayer Hema-Tek 2000 Slide Stainer. Pumps deliver fresh reagents
190 (Stain, Buffer and Rinse solution) in precise volume. Slide was then taken out and completely dried at RT
191 before being covered glasses and liquid mounting media using Tissue-Tek Coverslipping Film. Slides are
192 immediately available for being digitized on Panoramic scan II slide scanner by 3DHistech (x40, Z Stack,
193 Multilayer Mode 5*0,2um) and being visualized using case viewer software.

194

195 **Bioinformatic analysis of public scRNAseq data of human whole blood leukocytes**

196 *ScRNAseq data of NSCLC patients' whole blood leukocytes (GSE127465)*

197 ScRNAseq data of 6 NSCLC patients' whole blood leukocytes were downloaded from GEO as normalized
198 count per million (CPM) gene expression matrix. Briefly, pre-processing of scRNAseq data performed by
199 the authors [31] including the removal of: i) dead cells identified by a mitochondrial gene content > 20%
200 among total gene transcribed ; ii) cells with poor quality transcriptome, as being cells with less than 300
201 counts ; iii) doublet cells performed with Scrublet [32]. We next performed analysis of CPM gene expression
202 matrix from 6 NSCLC patients' whole blood leukocytes by using Seurat R package (version 3.1.1). We first
203 removed genes that were not expressed in at least 3 cells and filtered-out cells with less than 200 different
204 transcribed-genes (Seurat default parameters). Matrix was log-transformed and scaled prior to principal

205 component analysis (PCA) with ScaleData Seurat function. PCA was performed on the 2000 most-variable
206 genes, using RunPCA function. We used JackStraw function to determine the statistical significance of
207 PCA scores that led us to retain the first 9 principal components (PCs). We next define the number of
208 clusters by using FindNeighbors and FindClusters functions that take into account the first 9 PCAs and a
209 resolution of 0.66, leading to 11 clusters. We ran the uniform manifold approximation and projection (UMAP)
210 dimensional reduction technique, using RunUMAP function, and then display 2D UMAP projections. We
211 used the authors major cell type annotations [31] based on the validated leukocyte gene signature matrix
212 (LM22) [33].

213

214 *ScRNAseq data of healthy donors' whole blood leukocytes (GSE145230)*

215 ScRNAseq data of 3 human males' and 4 human females' whole blood leukocytes were downloaded from
216 GEO as normalized count per million (CPM) gene expression matrix. Pre-processing of scRNAseq data
217 was performed identically as described above for GSE127465, except that doublet cells were removed
218 based on UMI number and % of mitochondrial gene expression [34]. We next define the number of clusters
219 by using FindNeighbors and FindClusters functions that take into account the first 19 PCAs and a resolution
220 of 0.6, leading to 22 clusters. Cells were annotated by combining Clustifyr (version 1.0.0) [35] (using
221 reference matrices from Seurat CBMC, Zilionis Blood datasets [31], and MCP-counter genes list [36]) with
222 SingleR (version 1.2.4) [37] annotation tools (Human Primary Cell Atlas used as reference).

223

224 *Differential gene expression analysis*

225 From Seurat object generated from GSE127465 CPM gene expression matrix, differential gene expression
226 analysis between 2 distinct clusters was performed using FindAllMarkers function from Seurat R package.
227 Only genes detected in at least 25% of cells and with a positive average log₂ fold change of 0.25 were
228 retained, but no cutoff value for the adjusted p-value was used. Union function from dplyr R package
229 (version 1.0.2) was used to get the union of upregulated genes (Figure 4.B ; Table S4) from multiple
230 differential gene expression analysis. Setdiff function was used to get the exclusive list of upregulated genes
231 of a given cluster by opposition to another one.

232

233 *Single sample Gene Set Enrichment Analysis (ssGSEA) on bulk transcriptomic data*

234 SsGSEA from GSVA R package (version 1.32.0) was performed on public microarray data of *in vitro*
235 stimulated and unstimulated neutrophils (GSE15139 and GSE49757). For both data sets, control probes,
236 and probes with ambiguous gene symbol were removed. For probes matching to identical genes, mean
237 expression value per gene was calculated on probes matching to that gene to retain only one gene
238 expression value per gene. SsGSEA of the Raghavachari platelet signature and the Ponzetta neutrophil
239 signature (Table S2) was performed on each replicate of each group using GSVA R package. All ssGSEA
240 scores were bar-plotted with ggplot2 R package (version 3.2.1).

241

242 *Single sample Gene Set Enrichment Analysis (ssGSEA) on scRNAseq data*

243 ssGSEA from R escape package (version 0.99.9) was used for calculating an enrichment score for
244 Raghavachari platelet signature and Ponzetta neutrophils signature (Table S2) of all individual cells. Only
245 cells with at least 1 count per gene present in Raghavachari or Ponzetta signatures were considered for
246 calculating the enrichment score. Resulting ssGSEA scores obtained for each cluster was plotted using
247 ggviolin function from ggpubr R package (version 0.4.0).

248

249 *Venn diagrams*

250 Venn diagrams were generated by using the Venn and compute.Venn functions from the Vennerable
251 package (version 3.1.0.9000).

252

253 *MCP counter on scRNAseq data*

254 Normalized CPM expression matrix for all cells was generated from Seurat object. MCP counter scores
255 (mean expression of all genes for a given signature (in \log_2 TPM +1)) of the “16_gene_NPA_signature”
256 (Table S5) were calculated for each individual cells using MCPcounter.estimate function from MCPcounter
257 package (version 1.2.0). Comparison of mean MCP score between NPA cluster (Neu 5 PPBP^{high} PF4^{high}
258 NRGN^{high} cells) and each individual neutrophil clusters was performed with the Stat_compare_means
259 function from the ggpubr package (version 0.4.0) choosing non-parametric Wilcoxon rank-sum (Mann–

260 Whitney) statistical test. Results were presented through violin plots that were generated with ggplot2 R
261 package (version 3.3.2).

262

263 **Survival analysis**

264 TCGA pan-cancer RSEM normalized log2 transformed gene expression data were downloaded from UCSC
265 Xena Browser and log2 +1 transformed. Updated clinical data with survival endpoints were retrieved from
266 TCGA-CDR paper (Cell 2018, <https://doi.org/10.1016/j.cell.2018.02.052>).

267 Immune signatures (including the “NPA_12”) were calculated as the mean expression of each individual
268 gene. Patients were then stratified into three groups based on each signature’s tercile values.

269 Survival analysis was performed using the survminer and survival R packages. For each cancer type,
270 survival curves were estimated with Kaplan Meier and compared with the log-rank test. Multivariate
271 analyses considering age and tumor stage as cofounding factors were then performed using a cox model
272 with p-value correction using the Benjamini-Hochberg method.

273

274 **Results**

275

276 **Identification of NPAs in peripheral blood of cancer patients and healthy donors (HDs).**

277

278 NPAs from NSCLC patients and HDs’ whole blood were identified as CD15⁺ CD14⁻ neutrophils that
279 were double positive for platelet glycoprotein 4 (CD36) and integrin alpha-IIb (ITGA2B, also known as
280 CD41a) by using ImageStream®X (ISX) imaging flow cytometer (Fig. 1A-B). We could also easily identify
281 NPAs in unprocessed NSCLC patients’ whole blood smear through May–Grünwald–Giemsa staining (Fig.
282 1C). Because the frequency of NPAs was shown to be increased in the blood of patients with various
283 pathologies as compared to HDs [38–40], we next quantified by ISX the frequency of NPAs among
284 neutrophils between NSCLC patients and HDs with the same gating strategy. Although the frequency of
285 NPA between NSCLC patients (mean frequency = 1.48%) and HDs (mean frequency = 1.16%), was roughly
286 the same, with no statistically significant difference (p value = 0.64), one NSCLC patient displayed more
287 than a three-fold increase of NPA proportion (mean frequency = 4.59%) compared to the mean frequency

288 of NPA in NSCLC patients and HDs (Fig. 1D). Interestingly, among all 6 NSCLC patients analyzed, this
289 patient with high percentage of NPAs also displayed by far the highest NLR value (34.56 as opposed to
290 7.21 for the others), mostly explained by a higher absolute neutrophil count (Table S1), and the highest
291 tumor burden (7 vs 2 metastatic sites in the rest of the cohort) (Table S1).

292

293 **Platelets preferentially bind to low-density neutrophils (LDNs), rather than normal-density**
294 **neutrophils (NDNs) in cancer patients' blood.**

295

296 Given the well-known detrimental role of LDNs in cancer [11–14,41,42], we next analyzed a
297 potential link between LDNs and NPAs.

298 We first performed a single sample Gene Set Enrichment Analysis (ssGSEA) on publicly available
299 transcriptomic data of bulk LDNs and bulk NDNs from NSCLC and Head and Neck Cancer (HNC) patients'
300 blood (GSE79404). We showed that the published platelet-specific gene signature “Raghavachari” [43]
301 (Table S2) was significantly (p-value = 0.0022) more enriched in LDNs than in NDNs (Fig. 2A).

302 We next isolated LDNs and NDNs (Fig. 2B). Using ISX, we could visualize CD15⁺ CD14⁻ CD36⁺
303 ITGA2B⁺ NPAs from LDNs but not from NDNs (Fig. 2B-C). NPAs represented around 2% of LDNs but were
304 negligible in the NDN population (Fig. 2B-D). Collectively, these results demonstrate that platelet are
305 preferentially bound to LDNs rather than NDNs.

306

307 **Identification of NPAs in public scRNAseq datasets from whole blood leukocytes in both cancer**
308 **patients and HDs.**

309

310 We next aimed at characterizing NPAs from cancer patients at the transcriptomic level. Indeed,
311 given the small size of platelets, we hypothesized that NPAs could be isolated as a single cell (Fig. 1B),
312 just like free neutrophil, and therefore be present in scRNAseq data. For this purpose, we took advantage
313 of a recently published scRNAseq dataset of NSCLC patients' whole blood leukocytes (GSE127465) [31].
314 Therefore, for our analysis we used normalized counts tables and kept author's major cell type annotations
315 to identify blood cell populations. We could re-cluster all leukocytes (Fig. 3A) and distinguished 6 clusters

316 of neutrophils (named Neu 1, Neu 2, Neu 3, Neu 4, Neu 5 and Neu 6 (Fig. 3B). Interestingly, we observed
317 that Neu 5 cluster seemed to be distinct from the other neutrophil clusters, through its close proximity with
318 the platelet cluster (Fig. 3B). Using a published neutrophil-specific gene signature “Ponzetta” (Table S2),
319 we showed that cells from Neu 5 had a ssGSEA enrichment score (median ssGSEA score = 0.21) similar
320 to the other neutrophil clusters (median ssGSEA score = 0.24), and greater than cells from the other cell
321 types clusters (median ssGSEA score = -0.15) (Fig. 3C), validating that cells from Neu 5 are neutrophils.
322 This was supported by the highest expression level of neutrophil-specific genes (*CSF3R*, *CXCR2* and
323 *FCGR3B*) in all six clusters of neutrophils, including Neu 5 cluster (Fig. 3E). We then evaluated the ssGSEA
324 enrichment score of the published platelet-specific gene signature “Raghavachari” (Table S2) (33) across
325 all clusters identified. Platelet cluster was by far the most enriched cell type for the platelet-specific gene
326 signature (median ssGSEA score = 0.25 versus -0.28 in the other clusters), validating the specificity of this
327 signature (Fig. 3D). We found that a fraction of the Neu 5 neutrophils as well as of monocytes were more
328 enriched for the platelet-specific gene signature than any other of the non-platelet cell types (Fig. 3D).
329 Looking at the gene level, *PF4*, *PPBP* and *NRGN* (Table S3) platelet genes were in the 5 most-differentially
330 upregulated genes (Table S4) able to discriminate the Neu 5 cluster from the other neutrophils and immune
331 cell clusters (Fig. 3F). In conclusion, the Neu 5 cluster of platelet gene-expressing neutrophils seems to
332 correspond to NPAs.

333 To validate that this Neu 5 cluster of platelet gene-expressing neutrophils containing NPAs was not
334 exclusive to this particular scRNAseq dataset, we next investigated for the presence of platelet gene-
335 expressing neutrophils in a public scRNAseq dataset of HDs’ whole blood leukocytes (GSE145230). By
336 performing the same analysis as for the NSCLC patients scRNAseq dataset (GSE127465), we were able
337 to identify a cluster of neutrophils (Neu 17) located in between the platelet cluster and the remaining
338 neutrophil clusters (Fig. S1A-B). Consistent with what we previously found in the NSCLC patients
339 scRNAseq dataset, a fraction of Neu 17 cells was expressing high level of neutrophil (Fig. S1C-E) and
340 platelet-specific genes (Fig. S1D-F).

341

342 In order to rule out that upregulation of platelet genes in neutrophils is not a consequence of
343 neutrophil activation, we next tested whether the enrichment of the platelet-specific genes signature

344 “Raghavachari” was increased upon neutrophil activation. We used public transcriptomic data of *in vitro*
345 activated neutrophils by either GM-CSF [45] or plasma from septic patients [46] and we show that the
346 enrichment score of the platelet signature in neutrophils did not significantly differ upon activation as
347 compared to controls (Fig. S2). The expression of platelet genes in Neu 5 (in GSE127465) or Neu 17
348 (GSE145230) clusters seems therefore not to result from neutrophil activation. Collectively, all these results
349 strongly suggest that Neu 5 and or Neu 17 neutrophil cluster displaying a high expression of platelet genes
350 most likely contained NPAs.

351

352 **Gene signature of neutrophils from NPAs reveals enhanced degranulation, chemotaxis and trans-**
353 **endothelial migration capacities.**

354

355 To go deeper into the characterization of NPAs from NSCLC patients’ whole blood leukocytes at
356 the mRNA level, we focused our analysis on NSCLC patients’ scRNAseq dataset (GSE127465). We noticed
357 that only a fraction of cells of the Neu 5 cluster expressed platelet specific genes (*PF4*, *PPBP* and *NRGN*)
358 (Fig. 3F). When we re-projected cells for each individual neutrophil cluster, keeping the same UMAP
359 parameters used to discriminate each individual subset of leukocytes, we could show that the Neu 5 cluster
360 was more heterogeneous than the other neutrophil clusters based on visual cell dispersion (Fig. 4A and
361 Fig. S3). We selected the 48 cells co-expressing the 3 platelet specific genes (*PF4*, *PPBP* and *NRGN*) at
362 a level above 1 log₂ CPM (Fig. 4A) (named “Neu 5 PPBP^{high} PF4^{high} NRG^{high}”) out of the 437 of the whole
363 Neu 5 cluster. 48 cells out of the 8873 neutrophils analyzed represents 0,54% which is consistent with the
364 median frequency of NPAs among all neutrophils (0.85%) found in our cohort of NSCLC patients (Fig. 1D
365 and Table S1).

366

367 To next determine if neutrophils from the NPA cluster were distinct from any other subset of
368 neutrophils, we first performed a differential gene expression analysis of the NPA cluster (Neu 5 PPBP^{high}
369 PF4^{high} NRG^{high} cells) in comparison to each individual neutrophil clusters (Fig. 4B). We then took the
370 union of upregulated genes in NPA cluster from each pairwise comparison, therefore yielding 191 genes.

371 We then removed the platelet genes (Fig. 4C) and identified 120 non-platelet genes discriminating NPAs-
372 derived neutrophils from any other subset of neutrophils.

373

374 We next performed a pathway analysis and found a significant enrichment of neutrophil
375 degranulation/chemotaxis-related GO biological process, secretory granules-related GO cellular
376 component and trans-endothelial migration-related KEGG gene set (Fig. 4D). Collectively, these data
377 demonstrate that NPAs-derived neutrophils display enhanced degranulation, chemotaxis and trans-
378 endothelial migration functionalities.

379

380 **Specific NPAs-associated neutrophil signature is associated with a worse prognosis in pancreatic**
381 **adenocarcinoma and liver hepatocellular carcinoma patients.**

382

383 Next we addressed the prognostic significance of neutrophils from NPAs across the different tumor
384 types available in The Cancer Genome Atlas (TCGA). The first step was to generate a specific NPAs-
385 associated neutrophil signature excluding any other genes expressed in the other non-neutrophil blood
386 immune cell type and platelets (Fig. 5A) yielding 16 non-platelet NPAs-associated neutrophils specific
387 genes (Fig. 5B). Using scRNAseq data from human NSCLC tumor samples (discovery dataset) matching
388 previously-used patients' whole bloods [31], we then eliminated *RBPJ*, *IDH2*, *HELLPAR* and *CCNI* that
389 were highly expressed by multiple other tumor-associated cell types (Fig. S4), thus yielding a final 12-genes
390 NPAs-associated neutrophil signature (Fig. 5B). We next validated the specificity of this new NPAs-
391 associated neutrophil signature in the discovery dataset (scRNAseq dataset from NSCLC tumors) by
392 showing that cells coexpressing at least 3 out of the 12-genes NPA-associated neutrophil signature were
393 significantly more enriched in tumor-associated neutrophils than any other major cell types present in the
394 tumor microenvironment (Fig. 5C).

395

396 We next evaluated the prognostic value of the 12-genes NPAs-associated neutrophil signature
397 (Fig. 5B) and the canonical neutrophil signature "Ponzetta" (Table S2) with a pan-cancer approach in the
398 TCGA database. By performing a multivariate Cox analysis, we did not find any association between the

399 NPAs-associated neutrophil signature and the prognosis of either types of NSCLC cancer patients, lung
400 squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD) (Fig. 5D). Nevertheless, we could find
401 that the signature was statistically associated with a worse progression-free interval (PFI) (p-value < 0.001,
402 Hazard Ratio (HR) = 2.9) and overall survival (OS) (p-value < 0.001, HR = 2.6) in pancreatic
403 adenocarcinoma cancer patients (PAAD) (Fig. 5D and Fig. S5A), as well as in liver hepatocellular
404 carcinoma cancer patients (LIHC) (PFI: p-value = 0.063, HR = 1.45; OS: p-value = 0.004, HR = 2.1) (Fig.
405 5D and Fig. S5B). The canonical neutrophil signature “Ponzetta” could not recapitulate such significant
406 prognostic values in PAAD and LIHC reinforcing the idea that NPAs-associated neutrophils is a unique
407 population of neutrophils with different outcomes (Fig. 5D). Kaplan Meier univariate analysis confirmed the
408 poorer prognostic values of the 12-genes NPAs-associated neutrophil signature in PAAD (Fig. 5E) and
409 LIHC cancer patients (Fig. 5F). Whereas the canonical neutrophil signature “Ponzetta” did not show
410 statistically significant association with prognosis in PAAD cancer patients (Fig. 5E), it was associated with
411 an opposite outcome in LIHC cancer patients (Fig. 5F). This implies again that NPAs-associated neutrophils
412 are distinct from canonical neutrophils. Collectively, our results therefore suggest that a subset of
413 neutrophils, corresponding to NPAs-associated neutrophils, when present in tumors may be detrimental in
414 PAAD and LIHC cancer patients.

415

416 **Discussion**

417

418 To our knowledge, our study is the first to report the identification and characterization of NPAs at
419 the transcriptomic level, based on scRNAseq data from human whole blood leukocytes. Based on their
420 transcriptomic profile, we found that neutrophils bound to NPAs displayed enhanced degranulation,
421 chemotaxis and trans-endothelial migration features. Our results collectively showed that neutrophils from
422 NPAs represent a distinct subset of neutrophils, related to LDNs. Lastly, using TCGA database, we showed
423 that our specific NPAs-associated neutrophil signature was associated with a worse prognosis in PAAD
424 and LIHC cancer patients. Hence, NPAs may hold clinical utility as novel non-invasive blood prognostic
425 biomarker in cancer patients with solid tumors.

426

427 Using ISX, we were able to distinguish and quantify accurately NPAs in our cohort of NSCLC cancer
428 patients and HDs. Although we did not find a significant difference in the percentage of NPAs between
429 NSCLC cancer patients and HD, one NSCLC patient displayed more than a three-fold increase of NPA
430 proportion compared to the mean frequency of NPAs in NSCLC patients and HDs (Fig. 1D). Interestingly,
431 this patient also displayed the highest number of different metastatic sites (Table S1), as well as an
432 extremely high NLR value of 34.56 (Table S1). This is consistent with the poorer clinical outcome of patients
433 with elevated NLR values, including NSCLC patients [47]. This high NLR value was mostly explained by a
434 massive increase in the absolute neutrophil count (ANC), rather than a decrease in the absolute lymphocyte
435 count. Mounting evidence have shown that high ANC alone, was also significantly associated with poorer
436 prognosis in NSCLC cancer patients [48,49], including in response to immune checkpoint inhibitors (ICIs)
437 [50–52]. It would be of great interest in future studies to determine if the percentage of circulating NPAs, in
438 comparison to the other cost-effective and non-invasive NLR and ANC, is more accurately associated with
439 response to immunotherapy.

440

441 By performing a Ficoll density gradient, we showed that NPAs define a subset of LDNs but are
442 absent in NDN fraction, illustrating that platelets preferentially bind to LDN in contrast to NDN (Fig. 3B-C-
443 D). Several studies have reported a link between LDNs and cancer progression [47]. Many research groups
444 indeed reported that LDNs displayed enhanced T-cell suppressive properties, expanded in cancer patients'
445 blood as opposed to healthy donors [11–14,53]. Recent evidence have shown that high LDN expansion in
446 cancer patients' blood was associated with worse prognosis [11,12], including NSCLC patients [12].
447 Nevertheless, it remains to be known why platelets bind preferentially to LDNs. The lower density of
448 neutrophil bound to platelet may be a consequence of platelet binding. Since NDN activation is sufficient to
449 convert NDNs into LDNs [41], one can hypothesize that platelets interacting with NDN activate and convert
450 them into LDNs. It is also likely that platelets may behave as floats changing NDNs' density and retaining
451 them at the surface of the Ficoll gradient, explaining their recovery within peripheral blood mononuclear
452 cells. Although we did not document a significant increase of NPAs frequency in patient, likely as a
453 consequence of the small size of the cohort, the fact that LDNs increase in cancer patients strongly
454 suggests that the frequency of NPAs also increases in cancer patients.

455

456 Although unexpected, this study identified a yet unreported new subset of neutrophils in scRNAseq
457 data with high platelet gene expression that most likely correspond to NPAs. We ruled out the possibility
458 that platelet genes could be expressed by a subset of activated neutrophils as we did not find enrichment
459 of platelet signature in stimulated neutrophils (Fig. S2). Moreover, it is also unlikely that neutrophils
460 expressing platelet genes correspond to a common progenitor of megakaryocyte (platelet-producing cell)
461 and neutrophils, as the closest common progenitor between the two is the common myeloid progenitor
462 (CMP), which gives rise to all myeloid cells [54]. High expression of neutrophil-specific genes at this stage
463 of differentiation is therefore unlikely to occur. Taken together, platelet genes are more likely to be
464 expressed exogenously by neutrophils through aggregated-platelets. It is however plausible that platelets
465 could have been isolated in the same droplet than neutrophils, without any interaction. The potential random
466 contamination of platelets during single cell isolation of immune cells would have yielded cluster of cells
467 expressing platelet-specific genes in all immune cell types. However, expression of platelet specific genes
468 was mostly observed in neutrophils as opposed to other major immune cell types (Fig. 3F). To note that
469 some monocytes also displayed expression of platelet specific genes which is consistent with the fact that
470 platelets may also aggregate with circulating monocytes [18]. Interestingly, we found that the frequency of
471 neutrophil co-expressing the 3 platelet specific genes (*PF4*, *PPBP* and *NRGN*) was very similar to the one
472 we accurately determined by ISX in our cohort of NSCLC patients. This further supports that the cluster of
473 neutrophils with high expression of platelet genes identified in Zilionis' scRNAseq dataset (GSE127465)
474 corresponds to NPAs.

475

476 In contrast to the other subsets of circulating neutrophils, we found that neutrophils from NPAs
477 displayed enhanced degranulation, chemotaxis and trans-endothelial migration features, based on the
478 specific transcriptomic signature of NPAs. This is consistent with previous reports showing that platelet
479 binding to neutrophils boosted their potential to release granules [22], increased their capacity to
480 transmigrate through endothelial cells [25,26] to exacerbate inflammation thereby promoting disease
481 progression [21,27–29]. One can speculate that if neutrophils from NPAs display higher transmigration
482 potential, they would infiltrate tumors more efficiently and then fuel tumor growth and metastasis.

483

484 We found that NPA signature was highly detected in few tumor types in the TCGA data base and
485 was strongly associated with a poorer prognosis in PAAD and LIHC cancer patients. This is consistent with
486 the tumor-promoting role of TAN documented in pancreatic [55,56] and liver [57] cancer. Interestingly, we
487 found that the canonical neutrophil signature “Ponzetta” could not recapitulate such significant poorer
488 prognostic values in PAAD and LIHC, reinforcing the idea that neutrophil from NPA would account for most
489 of tumor-promoting role of TAN in such cancer types. Moreover, we provided evidence that NPAs-
490 associated neutrophils more likely resemble LDNs (Fig. 2) that are described to possess tumor-promoting
491 functions [11,13], supporting the idea that NPAs-associated neutrophils once becoming TANs after tumor
492 infiltration would fuel tumor growth.

493

494 Beyond their potential role in cancer, recent studies have reported an increase in the percentage
495 of circulating NPAs in COVID-19 patients in contrast to HDs [58,59]. Interestingly, patients who had severe
496 forms of COVID-19 displayed a significant higher level of NPAs in contrast to patients who had moderate
497 forms [59]. We showed that NPAs displayed enhanced degranulation/activation, based on their specific
498 transcriptomic signature (Fig. 4D). This is in accordance with a recent report showing in bulk granulocyte
499 RNAseq data from COVID-19 patients an increase expression of granulocyte activation-associated factors
500 in severe COVID-19 patients in contrast to mild ones [60]. Whether NPAs may not just be a consequence
501 but also a cause of COVID-19 severity remains to be investigated in future studies.

502

503 **Declarations**

504

505 ***Ethics approval and consent to participate***

506 Metastatic NSCLC patients (Table S1) included in the LIBIL clinical trial (NCT02511288) at the Léon Bérard
507 Hospital as well as healthy donors from Etablissement Français du Sang provided informed consent
508 regarding the use of their blood for research purpose.

509

510 ***Consent for publication***

511 Not applicable

512

513 ***Availability of data and materials***

514 The publicly available datasets analyzed during the current study are available from the *GEO repository*

515 GSE15139 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15139>)

516

517 GSE49757 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49757>)

518

519 GSE79404 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE79404>)

520

521 GSE127465 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE127465>)

522

523 GSE145230 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145230>)

524

525

526 ***Competing interests***

527 The remaining authors declare that they have no competing interests.

528

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540

541 ***Authors' contributions***

542

543 Conceptualization: PL, CC and M-CM; methodology: PL, MA, SD, VA, LM, MH, HH-V, AV, CC and M-CM;
544 formal analysis: PL, MA, SD, VA, LM and MH; investigation: PL, MA, SD, VA, LM, MH, AS, HH-V, AV, CC
545 and M-CM; resources: AS, CC and M-CM; writing—original draft: PL; writing—review and editing: MA, SD,
546 VA, LM, CC and M-CM; visualization: PL, MA, SD, VA, LM and MH; supervision: CC and M-CM; funding
547 acquisition: PL, AV, CC and M-CM. All authors read and approved the final manuscript.

548

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550

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560

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713

714 **Figures**

715

716

717

718 **Figure 1. Identification of NPAs in both NSCLC patients and healthy donors' peripheral blood.**

719 **(A)** Gating strategy on ISX to identify CD36⁺ ITGA2B⁺ NPAs from CD15⁺ CD14⁻ neutrophils in NSCLC
720 patients' peripheral whole blood (one representative data of n= 6 NSCLC patients' blood). **(B)** Visualization
721 of CD15⁺ CD14⁻ CD36⁺ ITGA2B⁺ NPAs by ISX in NSCLC patients and HDs' peripheral blood. Images were
722 taken with X40 objective. CD15: Neutrophil marker; CD14: Monocyte marker; ITGA2B, CD36, CD62P:
723 Platelet markers. **(C)** May-Grünwald-Giemsa coloration on NSCLC patient's whole blood smear. X100
724 magnification. Dark arrows show the presence of platelets aggregated to neutrophil (NPAs). Neutrophil is
725 evidenced by segmented nucleus in dark purple. Free neutrophil refers to neutrophil free of platelets. **(D)**
726 Box plot representing the frequency of non-coincidental CD15⁺ CD14⁻ CD36⁺ ITGA2B⁺ NPAs (among all
727 neutrophils) in whole blood of NSCLC (n=6) patients and HDs (n=10), determined by ISX. Difference in
728 terms of frequency of NPAs between NSCLC patients and HDs was assessed by the Wilcoxon rank-sum
729 (Mann–Whitney) statistical test.

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735 **Figure 2. NPAs define a subset of LDNs not present in NDNs.**

736 **(A)** Box plot representing ssGSEA enrichment scores of the Raghavachari platelet signature (Table S2)
737 across LDNs and patients' matched NDNs from four HNC (blue dots) and two NSCLC (red dots) cancer
738 patients (GSE79404). Differential enrichment of platelet signature between LDN and NDN groups was
739 assessed by the Wilcoxon rank-sum (Mann–Whitney) statistical test. P-value was displayed on graphs, **
740 $p \leq 0.01$. **(B)** Schematic representation of Ficoll density gradient centrifugation of human whole blood
741 showing the separation of the low-density fraction (containing PBMCs) from the normal-density fraction
742 (containing mostly granulocytes). Both fractions from NSCLC patients' blood were analyzed by ISX, to
743 identify CD15⁺ CD14⁻ LDNs (in the low-density fraction) and CD15⁺ CD14⁻ NDNs (in the normal-density
744 fraction). From either LDNs or NDNs gates, all CD36⁺ ITGA2B⁺ events were gated as "NPAs". **(C)**
745 Visualization by ISX in NSCLC patients of non-coincidental CD36⁺ ITGA2B⁺ NPAs from LDN gate in the
746 PBMC fraction and NDN gate in the granulocyte fraction. Images were taken with X40 objective. CD15:
747 Neutrophil marker; CD14: Monocyte marker; ITGA2B, CD36, CD62P: Platelet markers. **(D)** Dot plot
748 reflecting the frequencies of non-coincidental CD15⁺ CD14⁻ CD36⁺ ITGA2B⁺ NPAs among NDNs and LDNs
749 determined by ISX, across n=4 NSCLC patients.

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751

752 **Figure 3. Identification of a cluster of neutrophils expressing platelet genes in public scRNAseq**
753 **data from NSCLC patients' peripheral blood leukocytes.**

754 **(A-B)** Two dimensional-UMAP representation of re-clustered NSCLC patients' whole blood leukocytes from
755 scRNAseq data (GSE127465). **(A)** Major immune cell types were labeled using Zilionis et al. annotation.
756 **(B)** Each of the 11 clusters defined by the analysis were labeled based on the type of immune cell. The 6
757 clusters of neutrophils were annotated as follow: Neu 1, Neu 2, Neu 3, Neu 4, Neu 5 and Neu 6. Dark
758 arrows show Neu 5 neutrophil cluster of interest displaying high expression of platelet genes (see
759 differential gene expression analysis Table S3). **(C)** Violin plot representing ssGSEA enrichment score per
760 cell of the Ponzetta neutrophil signature (Table S2) across all clusters of blood immune cells. **(D)** Violin plot
761 representing ssGSEA enrichment score per cell of the Raghavachari platelet signature (Table S2) across
762 all clusters of blood immune cells. **(E)** Violin plots representing the log₂ gene expression (count per million,
763 CPM) of neutrophil-specific genes (*CXCR2*, *CSF3R* and *FCGR3B*) (Table S2) across all clusters of blood
764 immune cells. **(F)** Violin plots representing the log₂ gene expression (CPM) of platelet-specific genes (*PF4*,
765 *PPBP* and *NRGN*) (Table S3) across clusters of blood immune cells.

766 In **(C)**, **(D)**, **(E)** and **(F)** P values were calculated with Wilcoxon test, taking Neu 5 cluster as the population
767 of reference for each pairwise comparison with other clusters. P values were adjusted with Bonferroni test.
768 Adjusted p-values (Adj P) were displayed on graphs, **** Adj P ≤ 0.0001, *** Adj P ≤ 0.001, ** Adj P ≤ 0.01
769 , * Adj P ≤ 0.05 , ns = Adj P > 0.05.

770

771 **Figure 4. NPA gene signature is enriched for neutrophil degranulation, chemotaxis and trans-**
772 **endothelial migration GO terms, as opposed to the other neutrophil subsets.**

773 **(A)** Strategy to make the Neu 5 population more homogeneous (public scRNAseq data from NSCLC
774 patients' whole blood leukocytes - GSE127465). Left panel represents the two dimensional-UMAP
775 projection plot of cells from Neu 5 cluster (comprising 437 cells), based on the UMAP parameters used to
776 discriminate the major subsets of leukocytes. Middle panel shows the selection of Neu 5 cells based on the
777 co-expressed high level of the three platelet specific genes (*PPBP*, *PF4* and *NRGN*) above 1 log₂ gene
778 expression (CPM). Right panel represents the two dimensional-UMAP projection plot of Neu 5 cells co-
779 expressing *PPBP*, *PF4* and *NRGN* genes (comprising 48 cells annotated Neu 5 *PPBP*^{high} *PF4*^{high} and
780 *NRGN*^{high}). Cells were plotted based on the UMAP parameters used to discriminate the major subsets of
781 leukocytes. **(B)** Differential gene expression approach to get the union of upregulated genes in Neu 5
782 *PPBP*^{high} *PF4*^{high} *NRGN*^{high} (annotated NPA cluster) as opposed to each of the remaining neutrophil clusters
783 (Neu 1, Neu 2, Neu 3, Neu 4 and Neu 6). The statistical test used in the differential gene expression analysis
784 was the Wilcoxon rank-sum (Mann–Whitney) test. **(C)** Venn diagram representing mutually and exclusive
785 upregulated genes between the NPA cluster (union of upregulated genes in NPA cluster compared to each
786 of the remaining neutrophil clusters – see Table S4) and the platelet cluster (Table S3). **(D)** Top 10 most-
787 enriched GO Biological Processes, Cellular Components and KEGG gene sets calculated based on the
788 120 specific upregulated genes in NPA compared to platelet cluster (Table S5).

789

790

791 **Figure 5. Specific NPA gene signature is associated with a worse prognosis in pancreatic**
792 **adenocarcinoma and liver hepatocellular carcinoma patients.**

793 **(A)** Differential gene expression approach was used to get the list of mutually upregulated genes in Neu 5
794 PPBP^{high} PF4^{high} NRG1^{high} (annotated NPA cluster) across all pairwise comparison with the remaining
795 neutrophil clusters (Neu 1, Neu 2, Neu 3, Neu 4 and Neu 6) and non-neutrophil clusters (Monocytes, T
796 cells, B cells/pDC and NK cells) based on public scRNAseq data from NSCLC patients' whole blood
797 leukocytes - GSE127465. The statistical test used in the differential gene expression analysis was the
798 Wilcoxon rank-sum (Mann–Whitney) test. **(B)** Venn diagram representing mutually and exclusive
799 upregulated genes between the NPA cluster (mutually upregulated genes in NPA cluster across all pairwise
800 comparison with the remaining neutrophil clusters and non-neutrophil clusters – see Table S6) and the
801 platelet cluster (Table S3), yielding 16 upregulated genes specific to NPAs as compared to free platelet
802 cluster. 4 genes out of the 16, were removed as they were highly expressed by lung tumor stromal cells
803 (based on public scRNAseq data from human NSCLC tumors (GSE127465)), giving rise to the 12 gene
804 NPA signature (listed in a table). **(C)** Violin plot representing the MCP counter score per cell for each stromal
805 non-immune cell types (Ciliated cells, Club cells, Endothelial cells, Fibroblasts, Smooth muscle cells, Type
806 I pneumocytes and Type II pneumocytes), stromal immune cell types (B cells, Mast cells,
807 Monocytes/Macrophages/DC (Dendritic Cells), Neutrophils, NK (Natural Killer) cells, pDC (Plasmacytoid
808 Dendritic cells), Plasma cells and T cells) and patient-specific tumor cell clusters, based on public
809 scRNAseq data from human NSCLC tumors (GSE127465). All cell types were labeled based on author's
810 annotations (GSE127465). **(D)** Heatmap summarizing results of the multivariate Cox analysis of prognostic
811 values of various immune signatures across all available TCGA cohorts. Y axis correspond to TCGA
812 cohorts. Each line corresponds to a particular cancer type annotated with the TCGA short name. X axis
813 corresponds to the canonical neutrophil signature (annotated "Neutrophil_Ponzetta" – see Table S2) and
814 the 12-genes NPAs-associated neutrophil signature (annotated "NPA_12" – see Figure 5.B). HR
815 corresponds to the hazard ratio expressed in log₁₀. Positive HR values (in red) correspond to a worse
816 prognosis and negative HR values (in blue) correspond to a better prognosis. * refers to an adjusted p-
817 value < 0.05. ** refers to an adjusted p-value < 0.01. **(E-F)** Univariate survival analysis in pancreatic
818 adenocarcinoma (PAAD) and Liver Hepatocellular Carcinoma (LIHC) cancer patients (all stages) using

819 Kaplan Meier method. Cohort of PAAD and LIHC cancer patients were cut at tercile values based on mean
820 expression of “Neutrophil_Ponzetta” or “NPA_12” signature, yielding two groups (highest tercile or lowest
821 tercile for expression of the signature of interest).

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826 **Supplemental Figures**

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829 **Figure S1. Identification of a cluster of neutrophils expressing platelet genes in public scRNAseq**
830 **data from healthy donors' peripheral blood leukocytes.**

831 **(A-B)** Two dimensional-UMAP representation of re-clustered NSCLC patients' whole blood leukocytes from
832 scRNAseq data (GSE145230). **(A)** Major immune cell types were labeled using Clustifyr and SingleR
833 annotation tools from pre-selected immune cell signatures. **(B)** Each of the 22 clusters defined by the
834 analysis were labeled based on the type of immune cell. The 6 clusters of neutrophils were annotated as
835 follow: Neu 0, Neu1, Neu 6, Neu 15, Neu 17 and Neu 18. Dark arrows show Neu 17 neutrophil cluster of
836 interest displaying high expression of platelet genes. **(C)** Violin plot representing ssGSEA enrichment score
837 per cell of the Ponzetta neutrophil signature (Table S2) across all clusters of blood immune cells. **(D)** Violin
838 plot representing ssGSEA enrichment score per cell of the Raghavachari platelet signature (Table S2)
839 across all clusters of blood immune cells. **(E)** Violin plots representing the \log_2 gene expression (count per
840 million, CPM) of neutrophil-specific genes (*CXCR2*, *CSF3R* and *FCGR3B*) (Table S2) across all clusters of
841 blood immune cells. **(F)** Violin plots representing the \log_2 gene expression (CPM) of platelet-specific genes
842 (*PF4*, *PPBP* and *NRGN*) (Table S3) across clusters of blood immune cells.

843 In **(C)**, **(D)**, **(E)** and **(F)** P values were calculated with Wilcoxon test, taking Neu 5 cluster as the population
844 of reference for each pairwise comparison with other clusters. P values were adjusted with Bonferroni test.
845 Adjusted p-values (Adj P) were displayed on graphs, **** Adj P \leq 0.0001, *** Adj P \leq 0.001, ** Adj P \leq 0.01.

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847

848 **Figure S2. Enrichment analysis of platelet signatures in stimulated versus unstimulated**
849 **neutrophils.**

850 **(A)** Box plot representing ssGSEA enrichment scores of two independent platelet signatures Raghavachari
851 platelet signature (Table S2) in GM-CSF-treated neutrophils (“treated”) versus untreated neutrophils
852 (“Control”). Blood from 3 HDs were used for each group (GSE15139). Differential enrichment of platelet
853 signature between “treated” and “control” groups was assessed by the Wilcoxon rank-sum (Mann–Whitney)
854 statistical test (p-value = 0.4). **(B)** Box plot representing ssGSEA enrichment scores of the Raghavachari
855 platelet signature (Table S2) in neutrophils treated either with plasma from septic patients (“Septic plasma”,
856 n = 35) or from HDs (“Uninfected plasma”, n=19) (GSE49757). Differential enrichment of platelet signature
857 between “Septic plasma” and “Uninfected plasma” groups was assessed by the Wilcoxon rank-sum (Mann–
858 Whitney) statistical test (p-value = 0.05202).

859 **Figure S3. Two dimensional-UMAP representation of each neutrophil cluster.**

860 Two dimensional-UMAP representation of individual neutrophil clusters (from NSCLC patients' whole blood
861 leukocytes from scRNAseq data (GSE127465)) whose projection was based on UMAP parameters used
862 to discriminate the major subsets of leukocytes.

863 **Figure S4. Two dimensional-UMAP representation of NSCLC patients' tumor scRNAseq data.**

864 Expression of each of the 16 genes of the NPA signature (Table S5) plotted on the two dimensional-UMAP

865 representation of NSCLC patients' stromal and patient-specific tumors from scRNAseq data (GSE127465)).

866 **Figure S5. Multivariate analysis (Forest plot) of the prognostic impact of the 12-genes NPAs-**
867 **associated neutrophil signature.**

868 Multivariate analysis taking into account age and stage as cofounding factors. Cohort of PAAD (**A**) and
869 LIHC (**B**) cancer patients were cut at tercile values based on mean expression of “NPA_12” signature. For
870 both cohorts, progression free-interval (panel on the left) and overall survival (panel on the right) were
871 assessed.

872

873

874 **Supplemental Tables**

875

876 **Table S1. Blood-related biological information and clinical features of NSCLC patients and healthy**
877 **donors.**

878 **Table S2. Published platelet and neutrophil-specific signatures.**

879

880 **Table S3. List of upregulated genes in free platelet cluster as compared to all other leukocytes.**

881 Based on public scRNAseq data from NSCLC patients' whole blood leukocytes - GSE127465.

882 **Table S4. List of upregulated genes in Neu 5 as compared to all other leukocytes.**

883 Based on public scRNAseq data from NSCLC patients' whole blood leukocytes - GSE127465.

884

885 **Table S5. Platelet gene-free specific NPA signature as opposed to the remaining neutrophils**
886 **clusters.**

887 List of 120 genes of the NPA signature generated based on public scRNAseq data from NSCLC patients'
888 whole blood leukocytes - GSE127465.

889 **Table S6. NPAs-associated neutrophil signature as opposed to the remaining neutrophils clusters**
890 **and all the other leukocytes.**

891 List of 16 genes of the NPA signature generated based on the public scRNAseq data from NSCLC patients'
892 whole blood leukocytes - GSE127465.

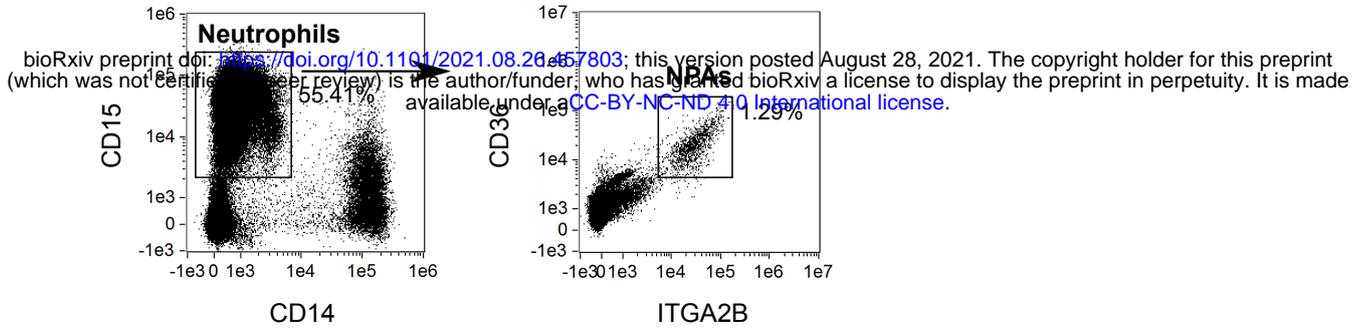
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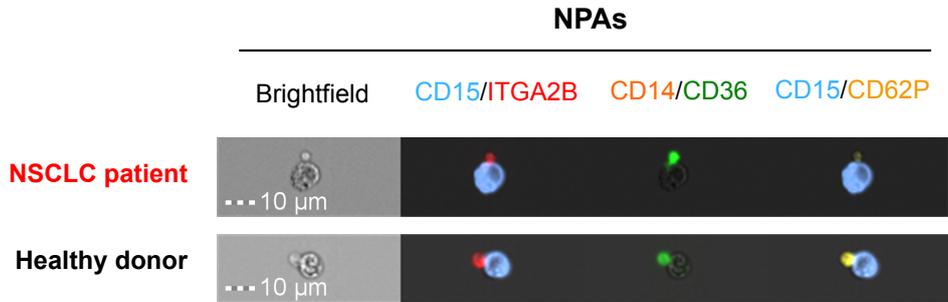
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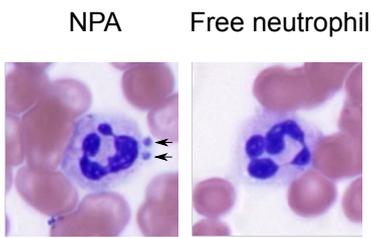
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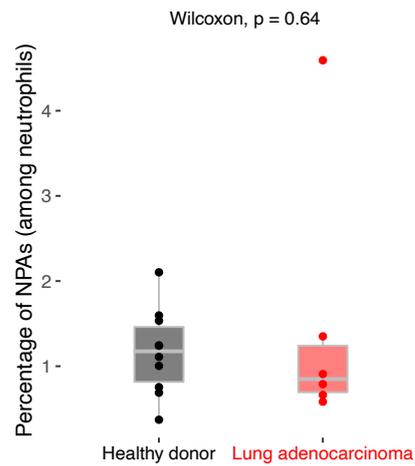
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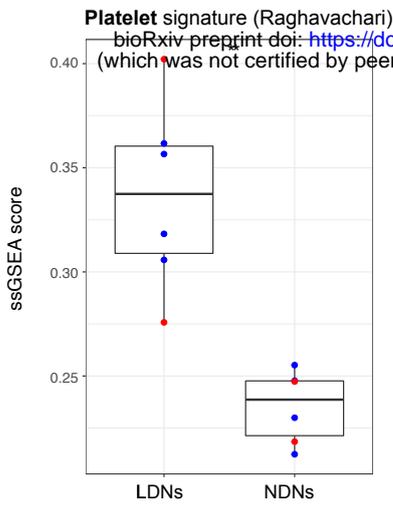
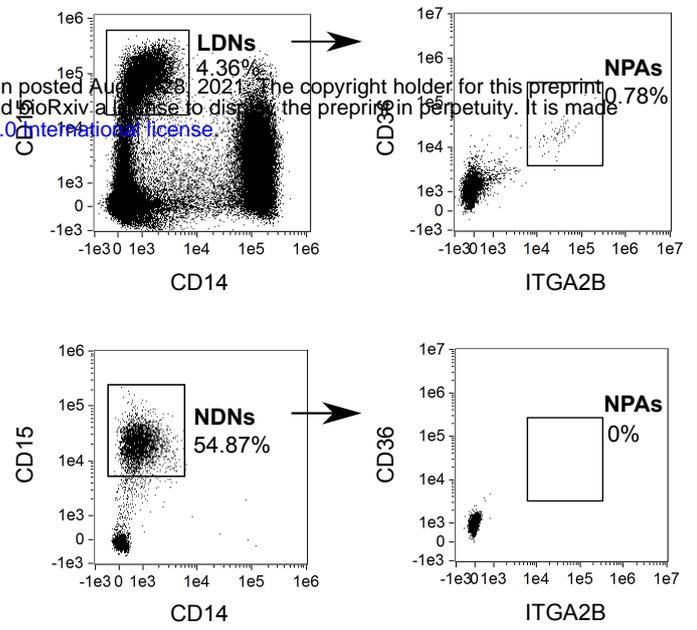
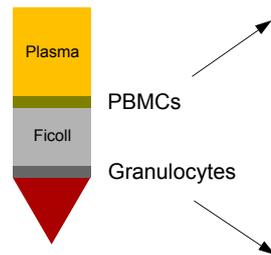
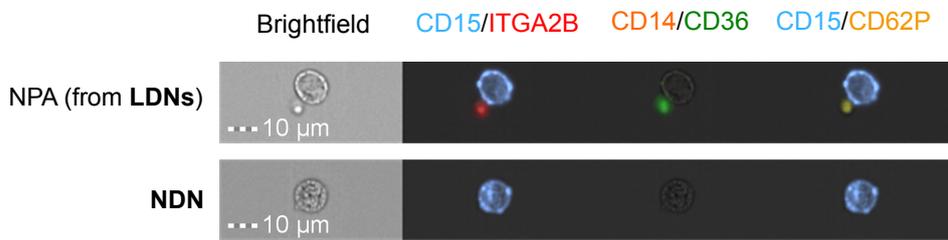
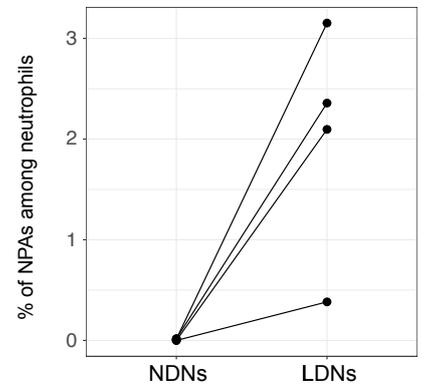


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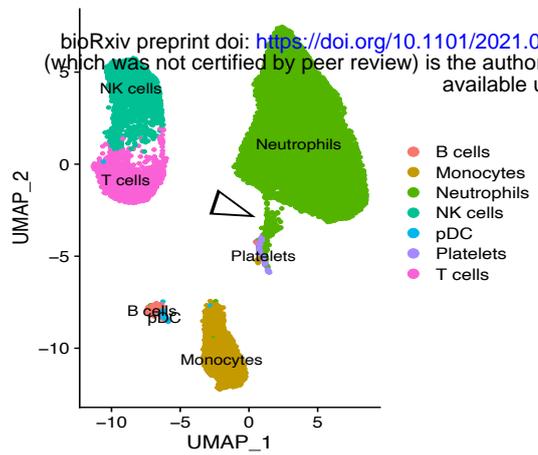


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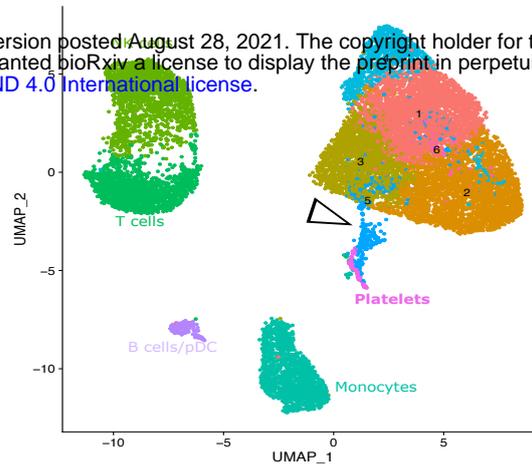


A**B****C****D****Figure 2**

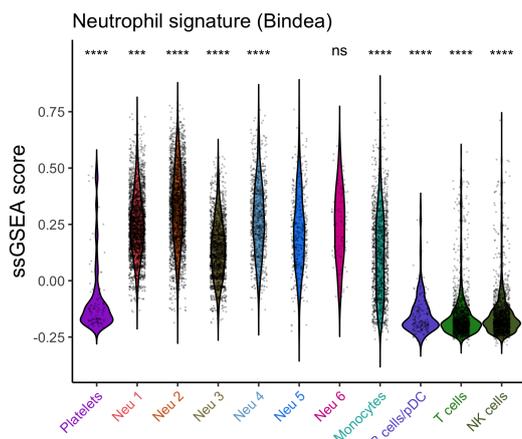
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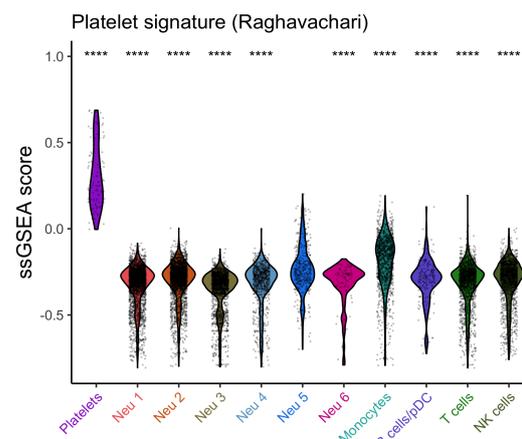
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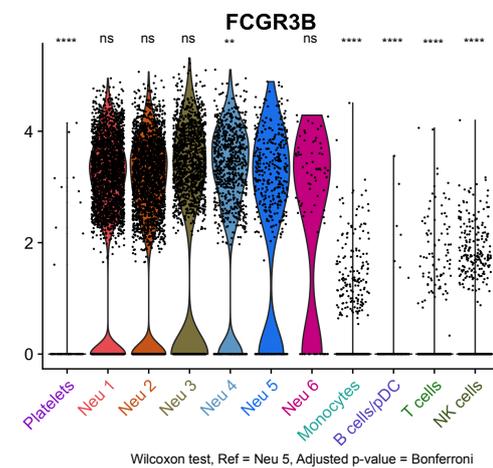
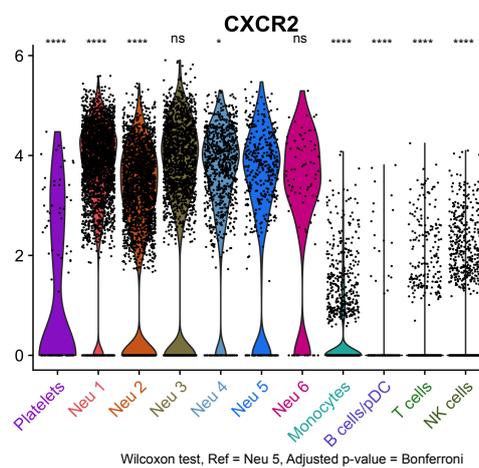
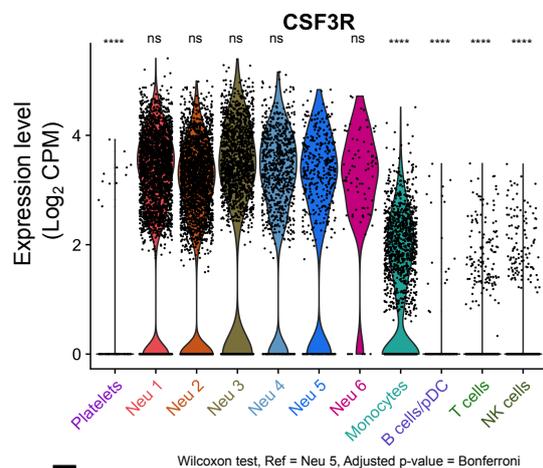
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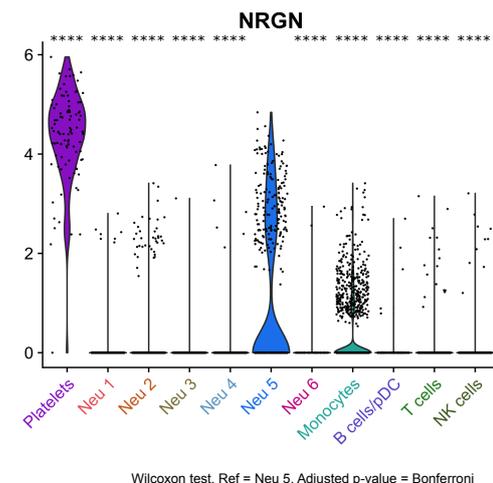
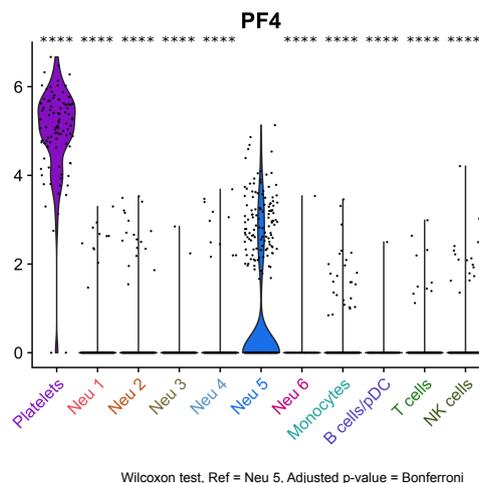
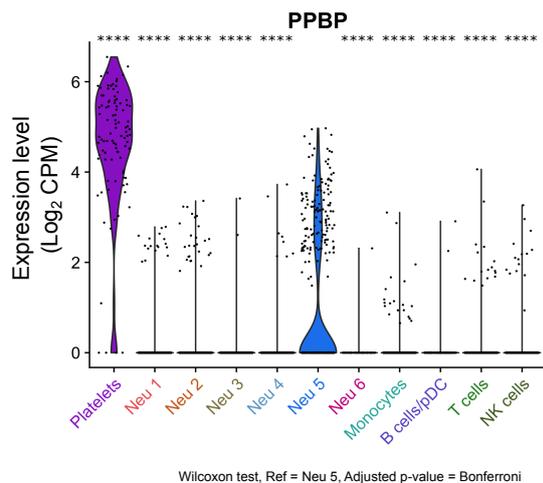
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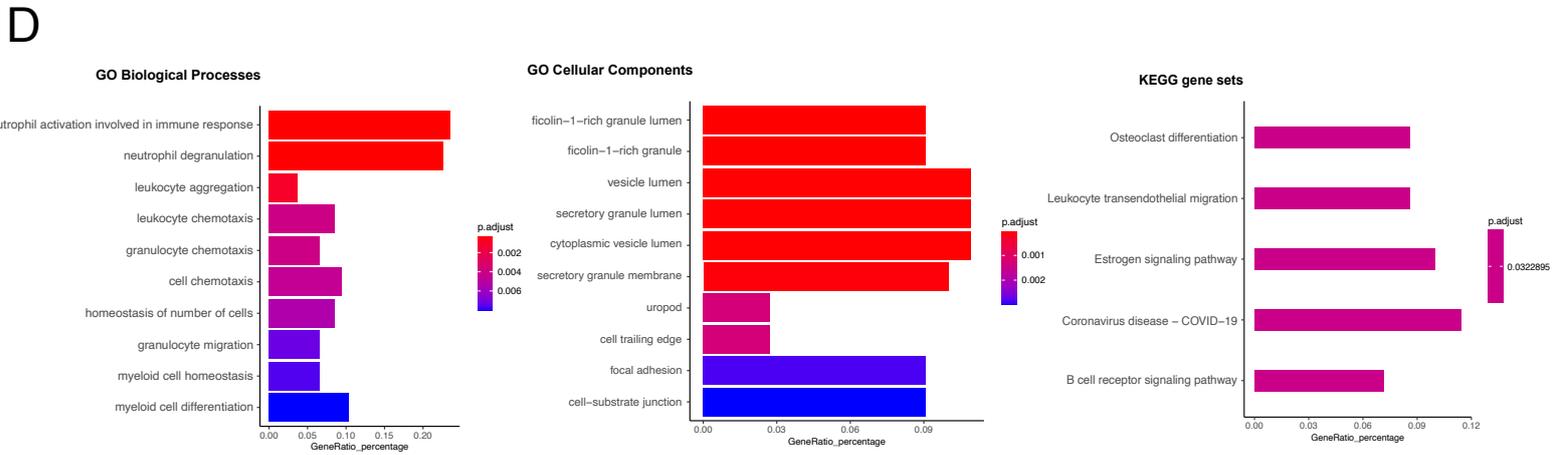
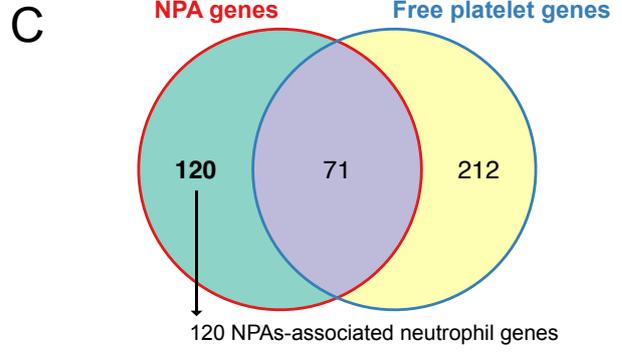
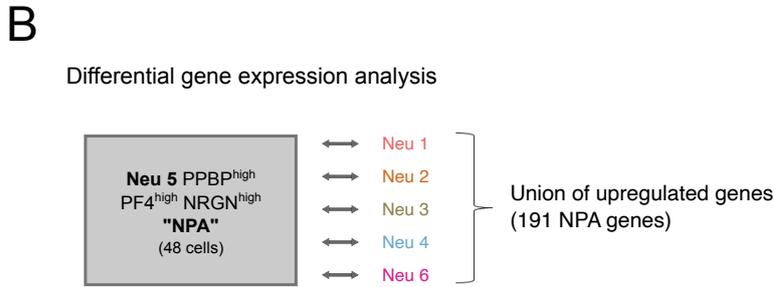
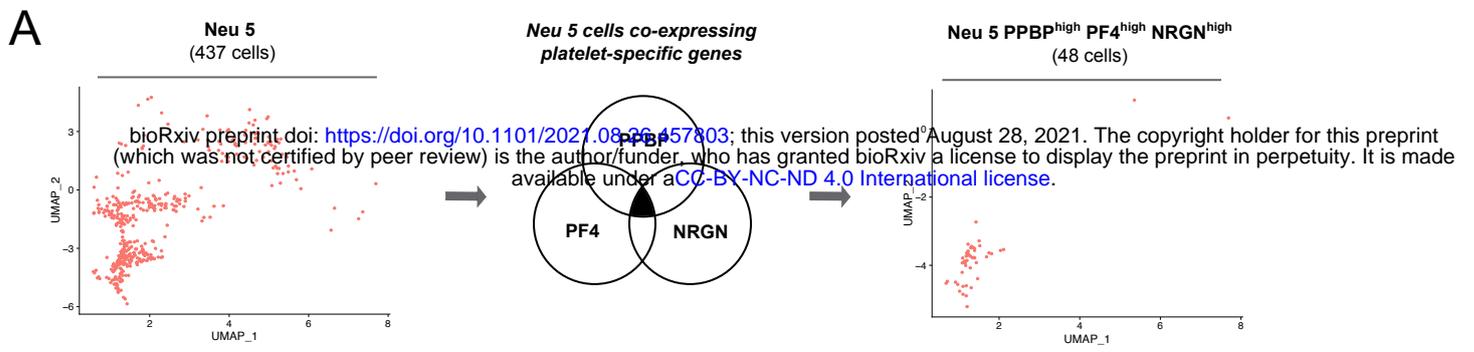
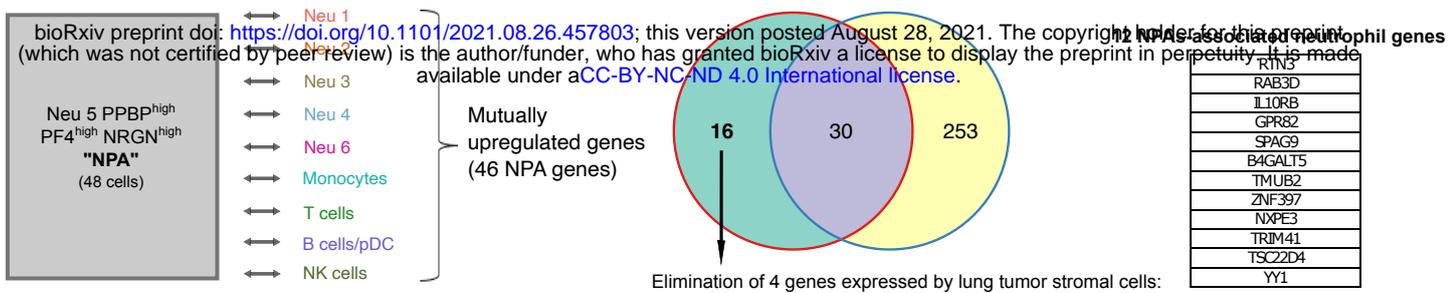


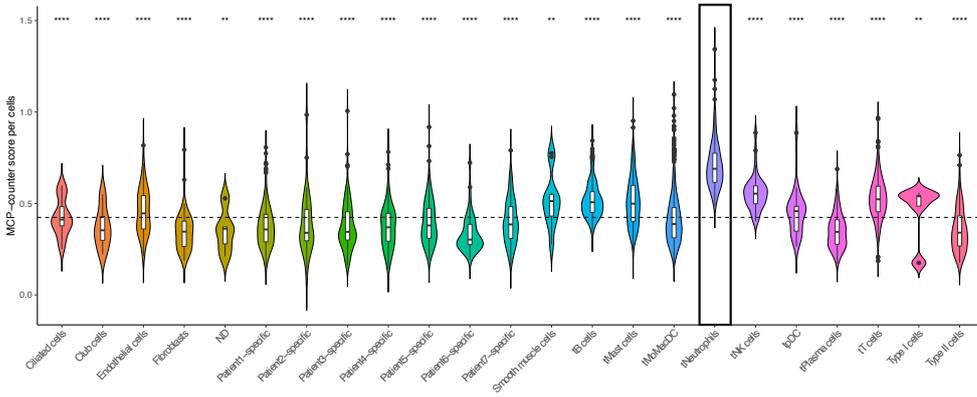
Figure 4

A

Differential gene expression analysis

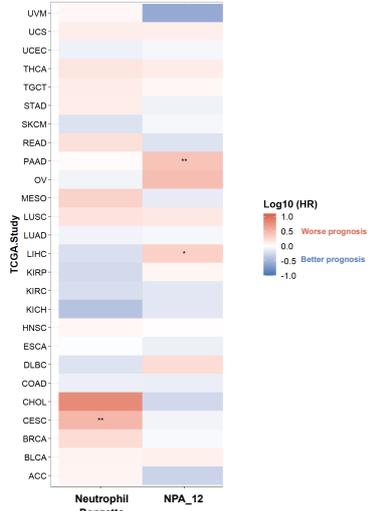


C

Cells coexpressing at least 3 genes from the 12-genes NPAs-associated neutrophil signature
Tumor subset

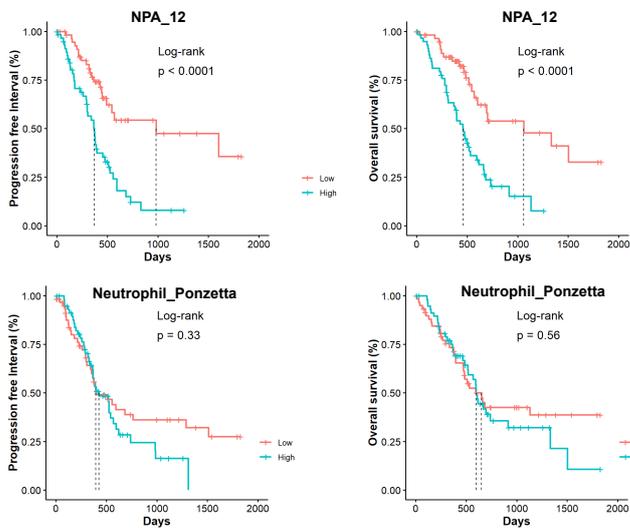
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Multivariate analysis



E

Pancreatic Adenocarcinoma (all stages)



F

Liver Hepatocellular Carcinoma (all stages)

