Original Research

A comprehensive analysis of immune landscape of Indian triple negative breast cancer

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Abstract

Background: Triple-negative breast cancer (TNBC) is a heterogeneous disease with a significant clinical challenge. TNBC alarmingly comprises 25-30% of breast cancers in India compared with only 10-15% in the West. However, immunotherapy was approved for high-risk early-stage TNBCs in the West. Hence, a long-standing question is whether Indian TNBCs immunologically and clinically resemble Western TNBCs such that they respond similarly to immunotherapies. Here we sought to elucidate the immune landscape of Indian TNBCs for the first time, compare them to Western disease and associate them with clinical parameters, cellular types/signaling, and immunotherapy response.

Methods: We profiled 730 immune genes in 88 retrospective Indian TNBC samples using NanoString platform, clustered them into subtypes using a machine-learning approach, and compared them with Western TNBCs (n=422; public datasets). Subtype-specific gene signatures

were identified, followed by clinicopathological, immune cell type, and pathway (multiomics) analyses. We also assessed responses to (cross-cancer) immunotherapy. Tumor-infiltrating lymphocytes (TILs) and pan-macrophage marker were evaluated using hematoxilin-eosin staining and immunohistochemistry, respectively.

Results: We identified three robust and similarly distributed TNBC immune transcriptome subtypes (Subtypes-1-3) in Indian women, and they are represented correspondingly in Western TNBCs and associated with well-known TNBC subtypes. Irrespective of the ethnicity, Subtype-1 harbored tumor microenvironmental and anti-tumor immune events associated with smaller tumors, younger age, and a better prognosis. Subtype-1 mainly represented basal-like/claudin-low breast cancer and immunomodulatory TNBC subtypes. Subtype-1 showed an increase in a cascade of events, including damage-associated molecular patterns, acute inflammation, Th1 responses, T-cell receptor-related and chemokine-specific signaling, antigen presentation, and viral-mimicry pathways. Subtype-1 was significantly ($p<0.05$) associated with pre-menopausal women, dense TILs and responses and/or improved prognosis to anti-PD-L1 and MAGEA3 immunotherapies. Subtype-2 was enriched for Th2/Th17 responses, $CD4^+$ regulatory cells, basal-like/mesenchymal subtypes, and an intermediate prognosis. Subtype-3 patients expressed innate immune genes/proteins, including those representing macrophages and neutrophils, and had poor survival.

Conclusion: We identified three immune-specific TNBC subtypes in Indian patients with differential clinical and immune behaviors, which largely overlapped with the Western TNBC cohorts. This study suggests cancer immunotherapy in TNBC may work similarly in both populations. Hence, this may expedite pharmaceutical adoption of immunotherapy to Indian TNBC patients.

Keywords: Immunity; immunotherapy; India; molecular stratification; triple-negative breast cancer; low-middle income countries; global oncology; DAMP; TCR signaling; immune landscape; Western countries; health disparity

INTRODUCTION

Breast cancer is the foremost cause of cancer-related deaths in women worldwide.[1] Ten to fifteen percent of breast cancers in Western women are triple-negative breast cancers (TNBCs), which do not express the estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor 2 (HER2) and have a poor overall prognosis and high recurrence risk.[2] TNBCs remain a clinical management challenge due to their aggressive characteristics and lack of targeted therapies.[3] Chemotherapy is used to treat primary and metastatic diseases, and while targeted therapies, including immunotherapy, are slowly emerging to treat TNBCs in the West.[3]

The incidence of TNBC is 20-31% in India, which is classified as a middle-income country by The World Bank based on its per capita income, than in Western populations (high-income-countries (HIC); 10-15%).[4 5] In India, TNBC affects younger, pre-menopausal women; and has a five-year survival rate of <30% when metastatic.[4-6] Approximately 100,000 women are diagnosed with TNBC each year in India, with a case fatality rate of 40%, contributing to India having one of the highest breast cancer-related death rates worldwide.[4-6] One of the proposed hypotheses for these increased death rates in India is that these TNBC patients have more aggressive disease with different immune biology than those from the West.[4 5] However, to our knowledge, no study has explored to address this hypothesis of immune infiltration differences between Indian and Western populations. If geographically/ethnically these diseases are different, it warrants distinct treatment opportunities for TNBC in India compared to the West. Therefore, understanding the molecular heterogeneity of TNBC and the biology of its response to existing therapies in this population are essential for improving outcomes both in low-middle-income countries (LMIC) and HIC.

To characterize TNBC biology, multiple groups have defined TNBC subtypes in the West using gene expression or integrated molecular profiles.[7-9] Immune cell infiltration into tumors is now recognized as intrinsic to tumor biology and immunotherapy responses, so a more granular analysis of immunity in TNBC is required for prediction and prognostication, especially given recent data showing that a proportion of patients respond well to immunotherapy - atezolizumab in combination with nab-paclitaxel for advanced PD-L1^{+} TNBCs (IMPassion130 study).[10] This combination therapy has been approved in India in 2020 based on IMPassion130 study,[11] which is

89 no longer an approved drug in the United States (US) after the outcomes from the follow-up trial
90 – IMPassion131 study.[12 13] Moreover, pembrolizumab (anti-PD1 immunotherapy), combined with
91 neoadjuvant chemotherapy, has been approved in the US for treating high-risk early-stage TNBCs
92 (based on KEYNOTE-522 study).[14] However, the unaddressed question is whether Indian and
93 Western TNBCs have similar biology and clinical outcomes; therefore, combination
94 immunotherapies from the West may benefit Indian TNBC women.

95 Hence, we sought to understand India-specific TNBC heterogeneity and functionally distinct
96 immune cell types infiltrating tumors in these patients using gene expression profiling and
97 compare this to Western TNBC patients and well-known TNBC subtypes[7-9]. The study goals were
98 to determine: (i) whether the immune composition and their clinical associations in Indian (LMIC)
99 TNBC women differed to that of TNBCs in Western populations (HIC); and (ii) whether differences
100 in the cellular composition of the immune infiltrate in TNBCs were associated with pathways,
101 immune signaling, patient survival and responses to therapy. We identified three robust immune
102 subtypes based on consensus clustering of the expression of immune-related genes in Indian TNBC
103 samples and found them to be represented similarly in two independent Western TNBC meta-
104 cohorts and associated with well-known TNBC subtypes from the West. Overall, our analysis
105 compared Indian TNBCs with those of the West at immune subtype levels (bi-directional) and
106 suggests avenues to rationally understand the immune and clinical landscapes to potentially
107 translate and personalize successful immunotherapy opportunities (from the West) to TNBC
108 patients in India.

109

110 ## METHODS

111 ### Patient samples, clinical characteristics and RNA isolation

112 TNBC patient samples were retrospectively collected as two independent cohorts from different
113 tertiary cancer care hospitals in Bangalore, India. Fifty out of 92 TNBC samples from one hospital,
114 St. John's Research Institute, were identified to be qualified for the study (**Figure 1A**). Similarly, 38
115 out of 48 TNBC samples were qualified for the study from Shri Shankara Cancer Hospital and
116 Research Centre. Both the studies were approved by the respective institutional ethics review
117 board and informed consent was obtained from the subjects.

118 Tumor tissue samples were collected at the time of surgery (before treatment), fixed in buffered
119 neutral formalin and processed as paraffin embedded blocks. Sections were cut from these blocks,
120 stained with hematoxylin and eosin (H&E) and examined by a pathologist to confirm the presence
121 of tumor. Immunohistochemistry for ER, PR and HER2 were done for determining receptor status
122 by standard procedures[15]. Only formalin-fixed paraffin embedded (FFPE) blocks containing pre-
123 treatment tissue with greater than 50% cancer epithelial cells were used for molecular analysis.
124 All clinical, histopathological and demographic details were collected from the hospital medical
125 records (**Figure 1B**). Radiologically recorded distant metastases or histologically confirmed local
126 recurrence and death related to disease had been documented.
127
128 RNA from all FFPE specimens was extracted by the Trizol method according to the manufacturer's
129 instructions and as described.[16] Briefly, 2x20 um sections were deparaffinized using xylene,
130 washed in absolute ethanol and overnight digested with Proteinase K. Lysate was taken for
131 extraction and RNA recovered from the aqueous phase.
132
133 nCounter profiling and subtype analysis
134 nCounter Immune profiling (NanoString Technologies)[17], subtype analysis[18] and the PPCCA
135 machine learning (ML) method[19] were performed as described. A detailed description has been
136 provided in Supplementary Methods.
137
138 TIL Scoring
139 Assessment of the TILs was done according to guidelines established by the International TIL
140 working group.[20] Large areas of central necrosis or fibrosis are not included for evaluation, tumor
141 was focused only on tumor-stroma at low magnification. Only mononuclear infiltrate of
142 lymphocytes and plasma cells were included and granulocytic infiltrate in areas of tumor necrosis
143 was not included. Based on the percentage of stromal TILs present, they were grouped into mild
144 (no or minimal immune cells), moderate (tumor with intermediate/heterogenous infiltrate) and
145 dense (tumor with high immune infiltrate) infiltrates.
146

147 Immunohistochemistry and scoring

148 IHC was done for CD68 according to standard procedures as described in Supplementary Methods.

149

150

151 RESULTS

152 Clinical characteristics of the Indian TNBC cohort

153 To our knowledge, this is the first comprehensive immune gene, cell type, signaling and
154 therapeutic analysis of retrospectively collected Indian TNBCs (n=88). A study flow chart and
155 collection source are shown in **Figure 1A**. The median age of the study population was 51 years,
156 with half ≤50 years of age and 44% pre-menopausal (**Figure 1B**). Most patients (77.3%) had grade-
157 3 tumors, the median tumor size was 2-5 cm (77.2%), 67% had stage II disease, and 60% were
158 lymph-node (LN) negative. Forty-two percent of samples had mild, 25% moderate, and 28% dense
159 TILs.

160 Indian TNBCs are heterogenous but can be divided into distinct subtypes with different prognoses

161 To characterize immune-based gene expression profiles of TNBCs in Indian women, 88 TNBC
162 samples were profiled for 730 immune genes using the PanCancer Immune gene panel
163 (NanoString Technologies). Batch effects in gene expression profiles between cohorts were
164 diagnosed and corrected using our exploBATCH[19] machine-learning (ML) tool (see Methods;
165 **Supplementary Figure 1A-D**). After selecting 392 variable genes (standard deviation ≥1), three
166 distinct TNBC immune gene expression subtypes were defined using unsupervised nonnegative
167 matrix factorization (NMF)-based consensus clustering (**Figure 2A).** The robustness of the clusters
168 and samples (or the likelihood of samples staying in the same cluster with re-iterative/consensus
169 clustering analysis) were defined by cophenetic coefficient and silhouette statistical metrics, as
170 previously described[18] (**Supplementary Methods; Supplementary Figure 1E-G and Supplementary**
171 **Table 1A**). The three subtypes were present in approximately equal proportions (27-38%; **Figure**
172 **2B**). 281 genes were significantly differentially expressed across the three subtypes according to
173 supervised significance analysis of microarrays (SAM) analysis (**Supplementary Table 1B**; see
174 Methods). Furthermore, prediction analysis for microarrays (PAM) statistical tool was used to

175 define summary statistics for each gene per subtype (centroids; **Supplementary Table 1C; see**
176 **Supplementary Methods**). Based on PAM centroids, we derived subtype-specific genes and
177 derived scores (mean expression of genes across samples; **Figures 2C-E**). Among the 281 SAM
178 genes, 204 were subtype-specific (non-zero) highest PAM centroid scores (**Supplementary Table**
179 **1D; Figure 2F**). A majority (79% and 19%) of 204 subtype-specific genes belonged to Subtype-1
180 and -2, respectively, whereas only 2% genes belonged to Subtype-3 (**Figure 2C**). Accordingly,
181 Subtype-1/-2 showed significantly ($p<0.001$) higher gene diversity as measured using the Shannon
182 Entropy method, as previously described[21]; **Figure 2G**), an index assessing immune gene
183 expression patterns. Hence, Subtype-1 shows immune-high gene expression patterns, whereas
184 Subtype-3 shows an immune-dormant or -exclusion pattern.

185 Subtype-3 patients had significantly ($p<0.05$; log-rank test) poorer overall survival (OS) and
186 disease-free survival (DFS; **Figures 2H-I**) than Subtype-1 (favorable prognosis) and Subtype-2
187 (intermediate prognosis) patients. In a multivariate analysis of tumor-infiltrating-lymphocyte (TIL)
188 groups, tumor size, menopausal status, stage, and molecular subtypes, Subtype-3 and stage-3
189 were independently associated with DFS (**Figure 2J**).

190 Next, we investigated whether these subtypes were present in TNBC samples from two Western
191 cohorts - The Cancer Genome Atlas (TCGA, n=123)[22] and Molecular Taxonomy of Breast Cancer
192 International Consortium (METABRIC; n=299)[23], which mainly represent Caucasian ethnic group
193 from HIC. There was no significant difference in the distribution of subtypes in the Indian TNBC
194 cohort compared with the Western cohorts, suggesting that the Indian and Western TNBCs are
195 similar (**Figure 2K, Supplementary Table 1E-F**). There was also a similar trend in OS according to
196 subtype in the METABRIC data as in the Indian cohort (**Figure 2L**).

197 Since the incidence of TNBC is reported to be higher in pre-menopausal women world-wide,
198 including in India[2 6], we sought to further understand the association between TNBC subtypes and
199 menopausal status. Subtype-1 TNBCs were more common in pre-menopausal patients and
200 Subtype-3 TNBCs were more common in post-menopausal patients (**Figure 2M**; $p<0.16$). A similar,
201 but not-significant, trend of menopausal status was observed in the TCGA ($p<0.16$) and METABRIC
202 ($p<0.28$) data (**Supplementary Figures 2A-B**).

203 Related to these data and as expected, Subtype-1 patients were younger (median age 44) than
204 Subtype-2 (median age 50) and Subtype-3 (median age 54) patients (**Supplementary Figures 2C;**
205 $p<0.05$, Chi-square test), and this trend (not statistically significant) was similar in TCGA and
206 METABRIC data (**Supplementary Figures 2D-E)**. Interestingly, Subtype-1 tumors were smaller than
207 Subtype-2 and -3 tumors (**Figure 2N**), and Subtypes-1 and -3 were associated with a greater
208 number of involved LNs (from METABRIC data) than Subtype-2 tumors (**Figure 2O**). With respect
209 to intrinsic subtypes, Subtype-1 shows a similar distribution of claudin-low and basal-like subtypes,
210 whereas Subtypes-2 and -3 predominantly show basal-like. Among the subtypes, Subtype-3
211 reveals increased HER2-like subtype, as assessed using METABRIC data (**Figure 2P**). There was no
212 significant association between METABRIC[23] and our subtypes (**Supplementary Figure 2F**).
213 Interestingly, a majority of Subtype-1 is associated with Vanderbilt's immune TNBC subtype[7] and
214 Subtype-2 with mesenchymal. In contrast, Subtype-3 was a mixture of all Vanderbilt's subtypes
215 (**Figure 2Q**). Although TCGA immune subtypes[24] show a significant association with our immune
216 subtypes, there is no specific association of each subtype with TCGA subtypes. Intriguingly,
217 Subtype-2 has more than 50% samples representing wounding healing C1 TCGA immune subtypes
218 (**Supplementary Figure 2F**). Overall, we identified three immune-specific TNBC subtypes in the
219 Indian cohort with different immune gene expression, prognoses, and menopausal status, which
220 largely overlapped with Western TNBC and certain molecular classifications.

221 **Subtype-1 is enriched for TILs, Th1 cellular immunity and a cascade of immune changes**

222 We next investigated whether our subtypes from the Indian cohort, representing the overall TNBC
223 immune profiles, were associated with histological assessment of TILs. In our cohort, 44% had
224 mild, 30% had dense, and 26% had moderate TILs. Most tumors with dense TILs were Subtype-1
225 tumors (68%; n=25), and those with moderate (82%; n=22) and mild (81%; n=37) TIL infiltrates
226 were mainly Subtype-2 and -3 tumors (**Figure 3A**). With respect to immune gene and cellular
227 composition, Subtype-1 had higher expression of Th1-specific genes (*IFNG* and *IL12A*; **Figure 3B-**
228 **C**), reflecting increased cellular immunity. Interestingly, most chemokines represented in the 204
229 subtype-specific genes were only highly expressed in Subtype-1 (**Figures 3D**). These patterns
230 apparent in the Indian cohort were also seen in TCGA data, with increased Th1 cell, leukocyte,
231 stromal, and TIL regional fractions and lymphocyte infiltration signature scores in Subtype-1

232 (**Figure 3E**). Hence, Subtype-1 appears to have a gene expression profile balanced in favor of Th1
233 responses and chemokine expression (**Figures 3F**).

234 We next explored the proportion and types of different immune cells within the three subtypes
235 using single-sample geneset enrichment analysis (ssGSEA)[25] and the entire 730 genes. The geneset
236 distribution in the three subtypes and the inferred cell populations significantly associated (FDR
237 <0.2) with the three subtypes are shown in **Figure 3G-K**. Subtype-1 showed increased $CD8^+$
238 effector T-cells (and their genes *CD8A*/*CD8B*) and the cytolytic activity genes *GZMA and PRF1*, as
239 assessed using cytolytic scores and ssGSEA (see Methods; **Figures 3G-I**). This increase in $CD8^+$
240 effector T-cell activity was also associated with patterns representing increased plasmacytoid
241 dendritic cells, major histocompatibility class (MHC) class-I, and co-stimulation of APC and T-cells
242 and associated antigen processing and presentation (APP) genes, including genes representing
243 transporters (TAP), immunoproteases, MHC class-II, and human leukocyte antigen (HLA) (**Figure**
244 **3I-K**). Subtype-1 also showed increased expression of immune checkpoint and T-cell exhaustion
245 genes such as PD-1 (*PDCD1*), PD-L1 (*CD274*), *CTLA4*, and *LAG3* (**Figure 3L**), suggesting that
246 persistent stimulation of T-cells potentially promoted T-cell exhaustion through increased
247 expression of these genes in Subtype-1 tumors, as described[26]. Further interrogation of TCGA data
248 showed increased INF-γ response genes and macrophage regulation, specifically anti-tumor M1
249 macrophages, in Subtype-1 samples (**Figure 3M**). We also examined T-cell receptor (TCR) and B
250 cell receptor (BCR) repertoires in our subtypes using TCGA data. As expected, TCR and BCR
251 Shannon indices and richness scores were significantly higher in Subtype-1 tumors, which might
252 represent a highly reactive immune-associated stroma in the tumor (**Figure 3E and M**). Our
253 subtypes capture an immune landscape and heterogeneity in TNBC beyond those represented by
254 TIL grouping alone.

255 **Subtype-1 immune changes are associated with DAMPs, acute inflammation, and viral-mimicry**

256 The cascade of adaptive immune responses and activation of dendritic cells is known to be
257 associated with damage-associated molecular patterns (DAMPs), which are molecules released by
258 acute inflammatory, hypoxic, or stressed cells linked to tumor progression or treatment.[27] Hence,
259 we examined a score derived from the average of 15 genes representing various processes
260 involved in DAMPs.[28] Interestingly, the DAMP gene score was significantly ($p<0.05$) higher in

261 Subtype-1 tumors compared with other subtypes (**Figure 4A**). Unlike DAMPs linked to hypoxia and
262 adaptive immunity in our previous study of pancreatic neuroendocrine tumors,[21] DAMPs were not
263 linked to hypoxia in Subtype-1 TNBCs, which had significantly lower hypoxia scores than Subtypes-
264 2 and -3 as assessed using ssGSEA and the hypoxia signature from MSigDB (**Figure 4B**). Hence, we
265 hypothesized that these significant increases in DAMP scores and Th1 responses are associated
266 with acute inflammation[27] in Subtype-1 tumors. As predicted, the acute inflammation gene score
267 (mean expression of *IL15*, *IL21*, and *LTA* in each sample) was significantly ($p<0.05$) higher in
268 Subtype-1 tumors compared with the other subtypes (**Figure 4C**).

269 To further explore if enrichment of DAMP genes is associated with (statistically dependent on)
270 acute inflammation and Subtype-1, we applied our previously described ML approach,
271 probabilistic principal component and covariate analysis (PPCCA),[19] to the Indian TNBC cohort. This
272 method combines probabilistic principal component and multivariate regression analyses to infer
273 statistical associations or dependencies between three parameters (subtypes, acute
274 inflammation, and DAMP gene expression), which cannot be assessed by Pearson or other
275 correlation analyses. For this purpose, we categorized the severity of acute inflammation into high
276 and low based on the median acute inflammation score as a cut-off. Then, we removed the
277 expression differences in 15 DAMP genes between the high and low acute inflammation samples
278 by statistically adjusting or normalizing the expression data using PPCCA. This adjusted data led to
279 the loss of association or dependency of Subtype-1 on DAMP expression in TNBCs ($p<0.05$;
280 modified negative $\log_{10}$ p-value > 0 in **Figure 4D**), as assessed using the first probabilistic principal
281 component (pPC1; with higher proportion of variability) and linear regression analysis with the
282 PPCCA model. Before adjustment of acute inflammation by group, both acute inflammation
283 groups and subtypes were significantly (modified negative $\log_{10}$ p-value > 0 in **Figure 4D**)
284 associated with DAMP expression. This was also reflected as a partial change in DAMP gene
285 expression between Subtype-1 vs. Subtypes-2 and -3 after adjusting the acute inflammation
286 dependency in DAMP genes (**Figure 4F**). Conversely, there was no loss of a significant association
287 between acute inflammation and DAMP gene expression in pPC1 in the reverse analysis of
288 adjusting subtype dependency on DAMP gene expression, followed by regression analysis of acute
289 inflammation groups in pPC1 (**Figure 4E and G**). Moreover, we observed enrichment of host viral-

290 infection mimicry pathways and genes in Subtype-1 (**Figure 4H**), in a similar way to our rectal
291 cancer study[29]. These results demonstrate partial dependency of Subtype-1 on acute
292 inflammation for DAMP enrichment and provide clues that the progression of Subtype-1 is related
293 to viral-mimicry during infection.

294 **Subtype-1-specific pathways and associated immunotherapies**

295 Pathway analysis showed enrichment of Th1, IL2, IL12, T-cell receptor (TCR), B cell receptor (BCR),
296 cytolytic activity, PD-1, NFκB, chemokine/cytokine signaling and other related immune pathways
297 in Subtype-1 tumors using Indian samples (**Figure 5A-B; Supplementary Table 1G-H**). These
298 analyses allowed us to postulate a Subtype-1-specific pathway in $CD4^+$ Th1 lymphocytes (based on
299 **Figures 5A-B** and published studies[30 31]), where MEF2D pathway genes are activated through the
300 TCR and LCK/FYN signaling (**Figure 5C**). In this pathway, $CD4^+$ T-cell co-stimulation, potentially via
301 increased chemokines/cytokines and IL2, IL12/STAT4, and LCK/FYN signaling, further triggers
302 TNFR2-specific NFκB signaling to increase interferon-γ signaling, connecting multiple studies[32-36].
303 To further validate this $CD4^+$ Th1 lymphocyte signaling independently, we used TCGA multi-omics
304 profiles, including RNA-seq-based gene expression and mass spectrometry- and reverse phase
305 protein array (RPPA)-based protein expression, and associated them with our subtypes. These
306 independent analyses showed significant enrichment of all genes and majority of (total and/or
307 phosphorylated) proteins from these representative pathways, suggesting activation of this
308 pathway in Subtype-1 samples (**Figure 5D**).

309 Based on the above data and immune checkpoint gene expression (**Figure 3L**), we hypothesized
310 that Subtype-1 tumors might be amenable to targeting with immune checkpoint inhibitors. Hence,
311 we applied the expanded interferon-γ response gene signature from Ayers, et al. to our data,
312 which has been shown to be associated with responses to anti-PD-1 therapy in multiple cancer
313 types.[37] We detected a significant association between the Ayers, et al., signature and Subtype-1,
314 suggesting that Subtype-1 may respond to anti-PD-1 immunotherapy (**Figure 5E**). Moreover, we
315 applied Subtype-1 scores to Mariathasan *et al.*, gene expression profiles, where melanoma
316 patients were treated with anti-PD-L1 therapy.[38] Despite the difference in cancer types, we
317 observed that melanoma patients with high Subtype-1 scores had a better prognosis and enriched

318 significantly ($p<0.05$) for compete response and stable disease than patients with low scores
319 (**Figure 5F-G**).

320 Similarly, a signature derived from MAGE-A3 vaccination therapy[39] was significantly associated
321 with Subtype-1 tumors (**Figure 5H-I**) and, correspondingly, a majority of samples from melanoma
322 patients responding to MAGE-A3 vaccination were significantly enriched for the Subtype-1
323 signature as assessed using our PAM centroids. These results suggest mechanisms of immune
324 evasion in Subtype-1 breast tumors that may be targeted by anti-checkpoint therapy or MAGE-A3
325 vaccination, which warrants further assessment in TNBCs.

326 **Subtype-2 is associated with Th2-based humoral and Th-17 immunity**

327 In contrast to Subtype-1, the characteristics of Subtype-2 included Th2 and Th17 gene expression,
328 representing a shift towards humoral and Th17 immunity (**Figures 6A-E**). As expected, this Th2
329 response was associated with an increased proportion of $CD4^+$ regulatory cells in Subtype-2
330 tumors (**Figure 6F**). When the tumor cell fraction was evaluated using TCGA data, Subtype-2
331 showed increased tumor purity (enriched for cancer cells rather than stroma) compared with the
332 other two subtypes, opposite to Subtype-1 (**Figure 6G** and **Figure 3E**).

333 Next, we evaluated pathway regulation in Subtype-2 in the Indian cohort. Subtype-2 was enriched
334 for cytokine signaling, specifically via IL17 (Th17) and lectin via the ficolin-based complement and
335 coagulation cascade pathways (**Figure 6H-J, Supplementary Table 1I**). Also, the data suggests a
336 balance tipping towards GATA3-induced Th2 cytokines in this subtype (**Figure 6J**). Overall, the Th2
337 response in Subtype-2 differs from Subtype-1.

338 **Subtype-3 is an immune desert subtype but enriched for innate immune cells**

339 Subtype-3 samples significantly expressed only five subtype-specific immune genes and therefore,
340 represents an immune desert subtype. However, there was high expression of five innate immune
341 genes and cell types, specifically macrophage and neutrophil genes (**Figure 7A-B**). Interestingly,
342 Subtype-3, along with Subtype-2, was relatively hypoxic compared with Subtype-1 (**Figure 4B**). We
343 confirmed the presence of macrophages using the pan-macrophage marker CD68 in subtype-
344 specific samples (n=39), which were indeed significantly more numerous in Subtype-3 samples
345 (**Figure 7C-D**). CD68 positivity was confined to the tumor stroma and not epithelial nests. There

346 was also a strong correlation between Subtype-3 and CD68-positive macrophages, with 31% of
347 the >50% positively stained tumors belonging to Subtype-3 ($p<0.05$). As opposed to Subtype-1,
348 there is an increased chronic inflammation signature in Subtype-3 patients (**Figure 7E**). The
349 Subtype-3 signature was associated with a worse prognosis (borderline significance) in melanoma
350 patients in Mariathasan et al. dataset[38] (**Figure 7F**). The overall summary of all the subtypes is
351 provided in **Figures 7G-H**.

352

353 DISCUSSION

354 Approximately 20-31% of all breast cancers in Indian women are TNBCs[4 5] compared with
355 approximately 15% in Western populations,[2] and the incidence of TNBC is higher in pre-
356 menopausal and young women in India.[5] Ethnic differences in cancer prevalence and type are
357 known to exist.[40] For instance, about 40% of breast cancers in African American women in the US
358 are TNBCs, a much higher prevalence than in the White ethnic groups.[41] A better understanding
359 of ethnicity-related disparities in cancer between Indian and Western populations would be useful
360 for developing diagnosis and management guidelines tailored to specific populations. Given the
361 increased prevalence of TNBC in India, there is a particular need to better understand the
362 molecular characteristics of TNBCs in the Indian context to improve clinical outcomes by
363 identifying potential population-specific biomarkers (if they exist) and personalizing affordable
364 treatment regimens. Suppose the differences between Indian and Western TNBCs are minor,
365 which is the case here, it will make it easy to adapt existing therapies, including potential
366 immunotherapy options tailored towards TNBC, in India.

367 In this study, we investigated the immune landscapes of 88 breast cancer samples from South
368 Indian women. To our knowledge, this is the first comprehensive analysis of immune
369 transcriptome analysis of Indian TNBCs. To avoid bias, we retrospectively analyzed two distinct
370 cohorts of retrospective samples from two different hospitals. Although these samples were
371 collected from one major metropolitan city in South India (Bangalore), the clinical parameters
372 were comparable to other Indian breast cancer studies, in that half of patients were aged 50 or
373 under and over two-fifths were premenopausal.[5 6] The age and menopausal symptom

374 characteristics more closely resembled an African and African-American populations but were
375 distinct from those usually seen in Western populations.[41 42]

376 TNBC is increasingly recognized as a heterogeneous disease with high recurrence and mortality
377 rates that more often displays aggressive clinical features such as high grade and lymph node
378 positivity.[2] Despite international efforts to identify effective therapeutic targets in TNBCs by
379 employing multi-omics strategies including proteomics, genomics, transcriptomics, and
380 methylomics, only a handful of targeted therapies exist or under consideration for this subgroup.[3]
381 Breast cancer was one of the first cancer types to undergo molecular classification.[43] Multiple
382 groups have defined subtypes in Western TNBCs,[7-9] and here, using Indian samples, we define
383 three immune TNBC subtypes with distinct gene and cell type profiles and prognoses (**Figures 7G-**
384 **H**). These immune subtypes defined in an Indian population were also present in Western TNBC
385 samples and in approximately the same proportions. There is an association between our's and
386 Vanderbilt's subtypes, however, our subtypes are specifically defined based on gene expression
387 representing immune cell infiltrations that display different characteristics from Vanderbilt's
388 subtypes. Nevertheless, the observed geographic differences are probably less to do with the
389 intrinsic immune biology of TNBC.

390 Although the incidence of TNBC is higher in young, premenopausal women,[5] most of these women
391 with TNBC belonged to the good prognosis Subtype-1 with active Th1 responses. In contrast, most
392 post-menopausal women with TNBC had poor prognosis, immune desert Subtype-3 tumors. This
393 increased risk of poor prognostic Subtype-3 in this age group may be attributable to compromised
394 immune function, specifically dysregulated adaptive Th1 responses, with aging; the capacity to
395 develop memory T-cells against cancer antigens is reduced in older people, compromising the
396 ability of the immune system to reject cancer cells[44]. Therefore, subsets of pre-menopausal and
397 post-menopausal women with TNBC may require distinct immunotherapies depending on their
398 subtype.

399

400 Here, we have also attempted to understand the mechanistic insights of these immune subtypes
401 associated with different immune infiltrations. The cytolytic activity of $CD8^+$ T-cells seen in

402 Subtype-1 depends on dedicated APCs, i.e., dendritic cells and, consistent with this, there was
403 increased MHC-I expression and antigen processing and HLA genes in these tumors, which leads
404 to co-stimulation of T-cell genes via TCR signaling in CD4 T-helper cells. Using pathway and
405 literature[30-36] analysis, we found that Subtype-1 was enriched for DAMP and CD4 T-helper TCR
406 signaling, and we further validated TCR and related signaling pathways using multi-omic gene and
407 protein data. This pathway may be associated with increased cytolytic activity in $CD8^+$ T-cells,
408 potentially reducing tumor progression and contributing to the better prognosis seen in patients
409 with Subtype-1 TNBCs. There was also increased expression of MHC-II-associated HLA genes
410 representing potentially activation of CD4, dendritic cells and M1 macrophages in Subtype-1
411 tumors, which are known to be associated with a good prognosis in several tumor types.[45] In
412 contrast, Subtype-3, with its low proportion of M0 and M1 macrophages and higher proportion of
413 M2 macrophages (validated using CD68 IHC) and neutrophils, was associated with poorer
414 outcomes. This is consistent with a study by Gentles et al.[46], who found that macrophages,
415 neutrophils, and plasma cells are poor prognostic markers in both breast and lung cancer.

416 Collectively, our findings show that there is considerable immune infiltrate variability in TNBCs,
417 which is partly determined by the molecular characteristics of the primary tumor and that
418 influences clinical outcomes. For example, we found that Subtype-1 may be primarily associated
419 with an acute immune response, as described previously in other cancers[47]. Remarkably, using our
420 PPCCA ML approach[19], we demonstrated a significant multivariate association between acute
421 immunity and subtypes with DAMP gene expression, suggesting increased acute immunity may be
422 associated with or driving Th1-enriched Subtype-1 TNBCs, a hypothesis that requires validation.
423 On the other hand, Subtype-3 may be associated with chronic inflammation and hypoxia, leading
424 to a poorer prognosis. While the roles of Th1 responses in acute inflammation and Th2 and M2
425 macrophages in chronic inflammation during cancer development are well described,[47] here we
426 relate this to differential stratification, prognosis, signaling, and therapeutic responses. Our
427 findings show that there is a complex relationship between acute/chronic immunity, intratumoral
428 immune cell heterogeneity, molecular subtype, and disease progression in TNBC. Treatments that
429 aim to enhance Th1-based cellular immunity against tumors are only effective in a subset of

430 patients[48], and our findings may help to identify this population using our Indian TNBC signature
431 for specific targeting and further therapeutic development.

432 The success of immunotherapy in a subset of TNBC patients has raised hope of efficacy in TNBCs,[10]
433 [14] and TNBCs with dense TILs are expected to have a better prognosis.[49] Nevertheless, the
434 objective response rate (ORR) for immune checkpoint inhibitors (ICI) monotherapy in metastatic
435 TNBC is only modest, and the IMpassion130 trial for locally advanced and metastatic TNBC
436 demonstrated that combined atezolizumab and nab-paclitaxel was more effective than
437 monotherapy with nab-paclitaxel alone.[10] In contrast, a follow-up trial, IMPassion131 showed no
438 improvement in patient survival with the same therapy.[13] Furthermore, several small studies have
439 reported only marginal ORRs with ICIs, with poor responses likely to be multifactorial and include
440 molecular heterogeneity, the use of ICIs as monotherapy, and their use in non-first-line settings.[3]
441 This is likely to be due, at least in part, to overall low PD-L1 expression (biomarker) in TNBCs,[50] so
442 it is essential to develop robust biomarkers to select patients who respond to immunotherapy.

443 Our data suggest Subtype-1 TNBC is potentially targetable with immunotherapy based on cross-
444 cancer analysis using melanoma data. Specifically, Subtype-1 was associated with immune
445 checkpoint inhibitor (ICI) responses and prognosis, as assessed using the anti-PD-1 treatment
446 prediction signature from Ayers et al.[37] and prognosis analysis using an anti-PD-L1 treated
447 melanoma dataset.[39] Our study also suggests an association between MAGEA3 immunotherapy
448 and Subtype-1 tumors. Recently, a phase-III trial assessing MAGEA3 immunotherapy in stage III
449 melanoma patients in the adjuvant setting failed.[51] Although MAGEA3-positive cutaneous
450 melanoma patients were selected for this trial, they were not further selected based on immune
451 landscape such as those defined here. Similarly, a recent study has implicated the role of MAGEA3
452 in hepatocellular carcinoma (HCC) and suggested as a novel therapeutic avenue of targeting
453 MAGEA3 for HCC.[52] In contrast, It is currently uncertain what therapy would best suit patients with
454 Subtype-2 tumors characterized by Th2/Th17 responses, infrequent LN involvement, an
455 intermediate prognosis, and increased tumor purity; further investigation is required to identify
456 potential immunotherapy targets associated with the immune complement cascade and GATA3.
457 The increased wound healing response in Subtype-2 is congruent with the association of Th2
458 response with wound healing[53], which might qualify them for antiangiogenic immunotherapies as

459 described[54]. However, this subtype may benefit from bioengineered immunotherapy using
460 collagen-binding domain (CBD)-IL12 to reactivate Th1 pathways,[55] followed by treatment with an
461 ICI. Subtype-3 was also associated with increased poor prognostic basal-like and HER2-like intrinsic
462 subtypes compared with Subtype-1. Based on our data, Subtype-3 may not respond to first-line
463 ICI-based immunotherapy.

464 In conclusion, here we characterized the immune heterogeneity in TNBCs in Indian women. In
465 doing so, we identified three subtypes with distinct immune cell infiltrates, immune cell signaling,
466 and gene signatures associated with prognosis and responses to immunotherapy. Overall, immune
467 gene expression in Indian and Western TNBCs appears to be largely similar. This immune-
468 transcriptome study suggests that therapies targeting immune microenvironment in Western
469 populations may be as effective in Indian populations, however, genetic changes may need to be
470 considered for geography-specific personalized therapy. This may accelerate pharmaceutical
471 adoption to Indian TNBC patients.

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CONTRIBUTIONS

AS conceived and developed the idea; AS and AK further developed this idea; AK, JP and SS provided patient samples and clinical follow up data; AS, AK, HPS, CR, YP, and KD performed the experiments; AK and JP performed histopathology and related data analysis; AS, MC and SS supervised the study.

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COMPETING INTERESTS

None

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Figure Legends

Figure 1. Consolidated Standards of Reporting Trials (CONSORT) diagram and clinical characteristics of Indian TNBC samples. A. Consort diagram showing workflow of samples selected for the study. **B.** A table of clinical characteristics of samples.

Figure 2. Identification and clinical characterization of immune subtypes using the Indian TNBC samples and comparison with Western TNBC cohorts. A. Heatmap of immune subtypes from Indian TNBC (n=88) identified by NMF clustering method. Subtypes are shown on the top bar. The scales are shown at the bottom. **B.** Proportion of immune subtypes from 88 Indian TNBCs. **C-E.** Subtype-specific scores. **F.** Proportion of subtype-specific genes out of 204 genes. **G.** Shannon diversity index of three immune subtypes. **H-I.** Kaplan-Meier curves representing H) OS (n=83) and

515 I) DFS (n=79) from the Indian TNBC cohort. **J.** Multivariate analysis of DFS and other clinical
516 parameters. **K.** Proportion of three immune subtypes in different cohorts of samples from India
517 (n=88) and Western populations – TCGA (n=123) and METABRIC (n=299). **L.** Kaplan-Meier curve
518 representing OS from the METABRIC TNBC cohort (n=299). **M.** Proportion of pre- and post-
519 menopausal samples represented in three immune subtypes from the Indian TNBC cohort (n=81).
520 **N.** Tumor size differences in immune subtypes from the Indian TNBC cohort (n=86). **O.** Number
521 of LNs involved in different immune subtypes from the METABRIC cohort (n=299). **P-Q.** Proportion
522 of P) intrinsic and Q) Vanderbilt subtypes represented in immune subtypes from the METABRIC
523 cohort (n=299). Kruskal-Wallis statistical test was performed for most of the analysis, except for
524 survival analysis and proportion analysis, where the log-rank test was used for survival anlaysis and
525 chi-squared test was used for the proportions. p-value of <0.05 was considered significant.

526 **Figure 3. Immune and clinical characteristics specific to Subtype-1. A.** Proportion of dense,
527 moderate, and mild TILS represented in three immune subtypes from the Indian TNBC cohort
528 (n=84). Chi-squared test was used for p-value calculation. **B-C.** Gene expression of Th1 response
529 genes – B) *IFNG* and C) *IL12A* in immune subtypes from the Indian TNBC cohort (n=88). **D.** Heatmap
530 showing chemokine expression in immune subtypes. **E.** Heatmap validation of specific immune
531 characteristics in immune subtypes from TCGA data. **F.** Schematic representing balance in Th1
532 response and chemokine gene expression in subtypes. **G-L.** Heatmaps and boxplot showing
533 changes in G) CD8 T-cell-specific genes, H) cytolytic activity, I) T and B cell types and activities
534 based on ssGSEA analysis, J) antigen presenting and processing genes, K) MHC-I &II HLA genes, L)
535 T-cell exhaustion genes. **M.** Heatmap showing macrophages, IFNG response, TCR diversity and
536 intratumoral heterogeneity from TCGA data. All the figures used the Indian TNBC cohort (n=88),
537 except figures E) and M), where TCGA data was used. Kruskal-Wallis statistical test was performed
538 for p-value significance for those in boxplots.

539 **Figure 4. Machine-learning based association analysis of acute inflammation, subtypes and DAMP**
540 **gene expression in the Indian cohort. A-C.** Boxplots showing differential changes in DAMP, hypoxia
541 and acute inflammation scores from the Indian TNBC cohort (n=88). Kruskal-Wallis statistical test
542 was performed for p-value significance. **D-E.** Barplots showing significant (p<0.01; linear
543 regression) association of 15 DAMP genes with D) acute inflammation (high vs. low) and E)

544 subtypes (Subtype-1 vs. others) in principal component (PC1) as assessed by PPCCA method using
545 the Indian TNBC cohort (n=88). **F.** Heatmap showing change in 15 DAMP pathway genes (median
546 expression across samples from each subtype) in immune subtypes before and after statistically
547 adjusting acute inflammation using the *PPCCA* method using the Indian TNBC cohort (n=88). **G.**
548 Heatmap showing change in 15 DAMP pathway genes (median expression across samples from
549 each subtype) in acute inflammation low and high groups before and after statistically adjusting
550 subtypes using the *PPCCA* method using the Indian TNBC cohort (n=88). **H.** Dotplot showing
551 enrichment of anti-viral-mimicry pathways in Subtype-1 from hallmarks gene sets from the
552 Molecular Signature DataBase (MSigDB) database using Subtype-1-specific genes. False discovery
553 rate (FDR) was calculated from p-values from hypergeometric test using hypeR R package (see
554 methods).
555
556 **Figure 5. Association of gene enrichment and therapy response to immune TNBC subtypes. A-B.**
557 Barplot showing gene enrichment of A) BIOCARTA and B) Reactome pathways using Enrichr tool
558 (see Methods) in Subtype-1. **C.** Schematic showing TCR and downstream signalling that effects
559 immune cell types based on data from the Indian TNBC gene expression, pathway analysis in A-B)
560 and literature. **D.** Multiomics enrichment analysis validation specific pathways from C) using TCGA
561 TNBC samples (n=18). Kruskal-Wallis statistical test was performed for p-value significance. **E.**
562 Boxplot showing differential T-cell-inflammed GEP in subtypes in the Indian cohort (n=88). **F.**
563 Kaplan-Meier curve and median survival data showing differential OS in melanoma samples (pre-
564 treatment; Mariathasan et al.[38]; n=348) with high and low enrichment of Subtype-1 genes and
565 their association with immunotherapy response. Log-rank test was performed for p-value
566 significance. **G.** Barplot showing the association of immune subtypes with clinical RECIST response
567 to immunotherapy in melanoma (Mariathasan et al.[38]; n=298). * - represents a significant ($p<0.05$;
568 Kruskal-Wallis test) enrichment in samples with high Subtype-1 score compared to those with low
569 score. **H.** Boxplot showing differential MAGEA3 therapy response signature in subtypes in the
570 Indian cohort (n=88). **I.** Proportion of melanoma samples (n=56) showing immune TNBC subtypes
571 with differential MAGEA3 therapy response, as a cross-cancer comparison analysis. Kruskal-Wallis

572 statistical test was performed for p-value significance for E) and H). Chi-squared test was
573 performed for p-value calculations for G) and I).
574
575 **Figure 6. Immune characteristics specific to Subtype-2. A-D.** Gene expression of Th2 and Th17
576 response genes – A) *IL4,* B) *IL5*, C) *IL17B* and D) *IL17A* in immune subtypes using the Indian TNBC
577 cohort (n=88). **E.** Schematic representing balance in Th2 and Th17 response and chemokine gene
578 expression in immune subtypes. **F.** Boxplot showing changes in CD4+ T regulatory cells in immune
579 subtypes as assessed by ssGSEA analysis using the Indian TNBC cohort (n=88). **G.** Boxplot showing
580 changes in tumor purity in immune subtypes from the TCGA data (n=101). **H-I.** Barplot showing
581 enrichment of A) KEGG and B) Reactome pathways using Enrichr tool (see Methods) in subtype-2.
582 **J.** A table showing BIOCARTA enrichment analysis in Subtype-2. Kruskal-Wallis statistical test was
583 performed for p-value significance for A-G).
584
585 **Figure 7. Immune characteristics and validation of macrophage markers in Subtype-3. A.** Heatmap
586 showing Subtype-3 specific genes associated with macrophages and neutrophils. **B.** Boxplot
587 showing changes in macrophages in immune subtypes as assessed by ssGSEA analysis using the
588 Indian TNBC cohort (n=88). **C-D.** IHC and quantitation (n=39) of pan-macrophage marker – CD68
589 in immune subtype samples using the Indian cohort. **E.** Boxplot showing differential changes in
590 chronic inflammation scores in immune subtypes using the Indian cohort (n=88). **F.** Kaplan-Meier
591 curve and median survival data showing differential OS in samples with high and low enrichment
592 of immune TNBC Subtype-3 genes in melanoma samples (pre-treatment; Mariathasan et al.[38];
593 n=348). **G.** Molecular characteristics of subtypes. **H.** Clinical characteristics of subtypes. Kruskal-
594 Wallis statistical test was performed for p-value significance for A), D) and E). Log-rank test was
595 performed for p-value significance for F).
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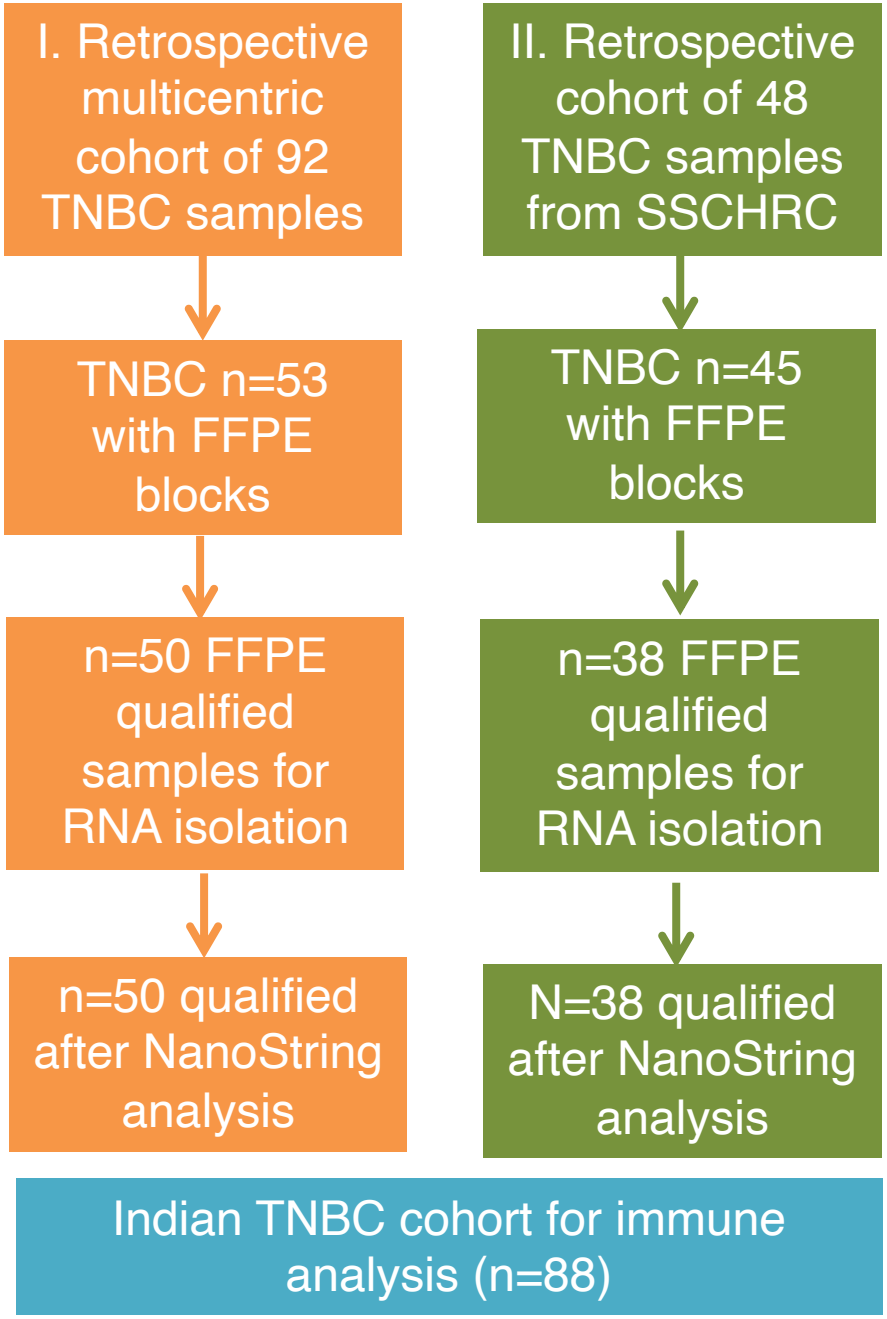
Figure 1

A



B

Clinical Table(N=88)		n(%)
Age(yrs)	Mean	50
	Median	51
	≤50	44(50)
	>50	44(50)
Menopausal status	Pre	39(44)
	Post	49(56)
Grade	2	17(19.3)
	3	68(77.3)
	NA	3(3.4)
Tumor Size	<2 cm	7(8)
	2-5 cm	68(77.2)
	>5 cm	11(12.5)
	NA	2(2.3)
Stage	I	9(10)
	II	59(67)
	III	20(23)
Lymph node status	Positive	33(38)
	Negative	53(60)
	NA	2(2)
Lymphocyte infiltrate groups	Dense	25(28)
	Mild	37(42)
	Moderate	22(25)
	NA	4(5)

Figure 2

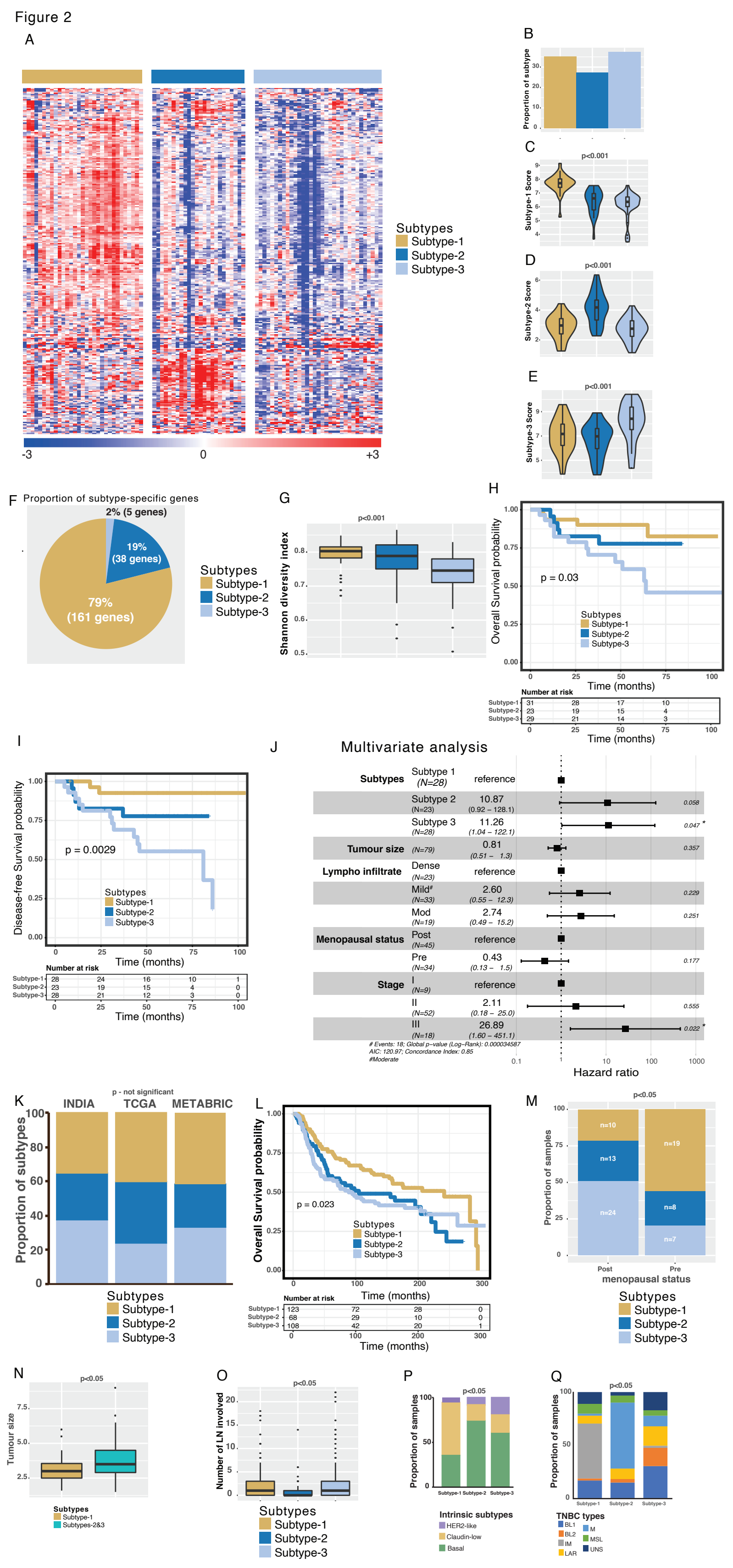

A
-3
0
+3
Subtypes
Subtype-1
Subtype-2
Subtype-3
B
Proportion of subtype
C
p<0.001
Subtype-1 Score
D
p<0.001
Subtype-2 Score
E
p<0.001
Subtype-3 Score
F
Proportion of subtype-specific genes
2% (5 genes)
19% (38 genes)
79% (161 genes)
Subtypes
Subtype-1
Subtype-2
Subtype-3
G
p<0.001
Shannon diversity index
H
Overall Survival probability
p = 0.03
Subtypes
Subtype-1
Subtype-2
Subtype-3
Time (months)
Number at risk
Subtype-1 31 28 17 10
Subtype-2 23 19 15 4
Subtype-3 29 21 14 3
I
Disease-free Survival probability
p = 0.0029
Subtypes
Subtype-1
Subtype-2
Subtype-3
Time (months)
Number at risk
Subtype-1 28 24 16 10 1
Subtype-2 23 19 15 4 0
Subtype-3 28 21 12 3 0
J
Multivariate analysis
Subtypes
Subtype 1 (N=28) reference
Subtype 2 (N=23) 10.87 (0.92 – 128.1) 0.058
Subtype 3 (N=28) 11.26 (1.04 – 122.1) 0.047 *
Tumour size (N=79) 0.81 (0.51 – 1.3) 0.357
Lympho infiltrate
Dense (N=23) reference
Mild# (N=33) 2.60 (0.55 – 12.3) 0.229
Mod (N=19) 2.74 (0.49 – 15.2) 0.251
Menopausal status
Post (N=45) reference
Pre (N=34) 0.43 (0.13 – 1.5) 0.177
Stage
I (N=9) reference
II (N=52) 2.11 (0.18 – 25.0) 0.555
III (N=18) 26.89 (1.60 – 451.1) 0.022 *
Events: 18; Global p–value (Log–Rank): 0.000034587
AIC: 120.97; Concordance Index: 0.85
#Moderate
Hazard ratio
K
p - not significant
INDIA
TCGA
METABRIC
Proportion of subtypes
Subtypes
Subtype-1
Subtype-2
Subtype-3
L
Overall Survival probability
p = 0.023
Subtypes
Subtype-1
Subtype-2
Subtype-3
Time (months)
Number at risk
Subtype-1 123 72 28 0
Subtype-2 68 29 10 0
Subtype-3 108 42 20 1
M
p<0.05
Proportion of samples
n=10
n=13
n=24
n=19
n=8
n=7
Post
Pre
menopausal status
Subtypes
Subtype-1
Subtype-2
Subtype-3
N
p<0.05
Tumour size
Subtypes
Subtype-1
Subtypes-2&3
O
p<0.05
Number of LN involved
Subtypes
Subtype-1
Subtype-2
Subtype-3
P
p<0.05
Proportion of samples
Subtype-1
Subtype-2
Subtype-3
Intrinsic subtypes
HER2-like
Claudin-low
Basal
Q
p<0.05
Proportion of samples
Subtype-1
Subtype-2
Subtype-3
TNBC types
BL1
BL2
IM
LAR
M
MSL
UNS

Figure 3

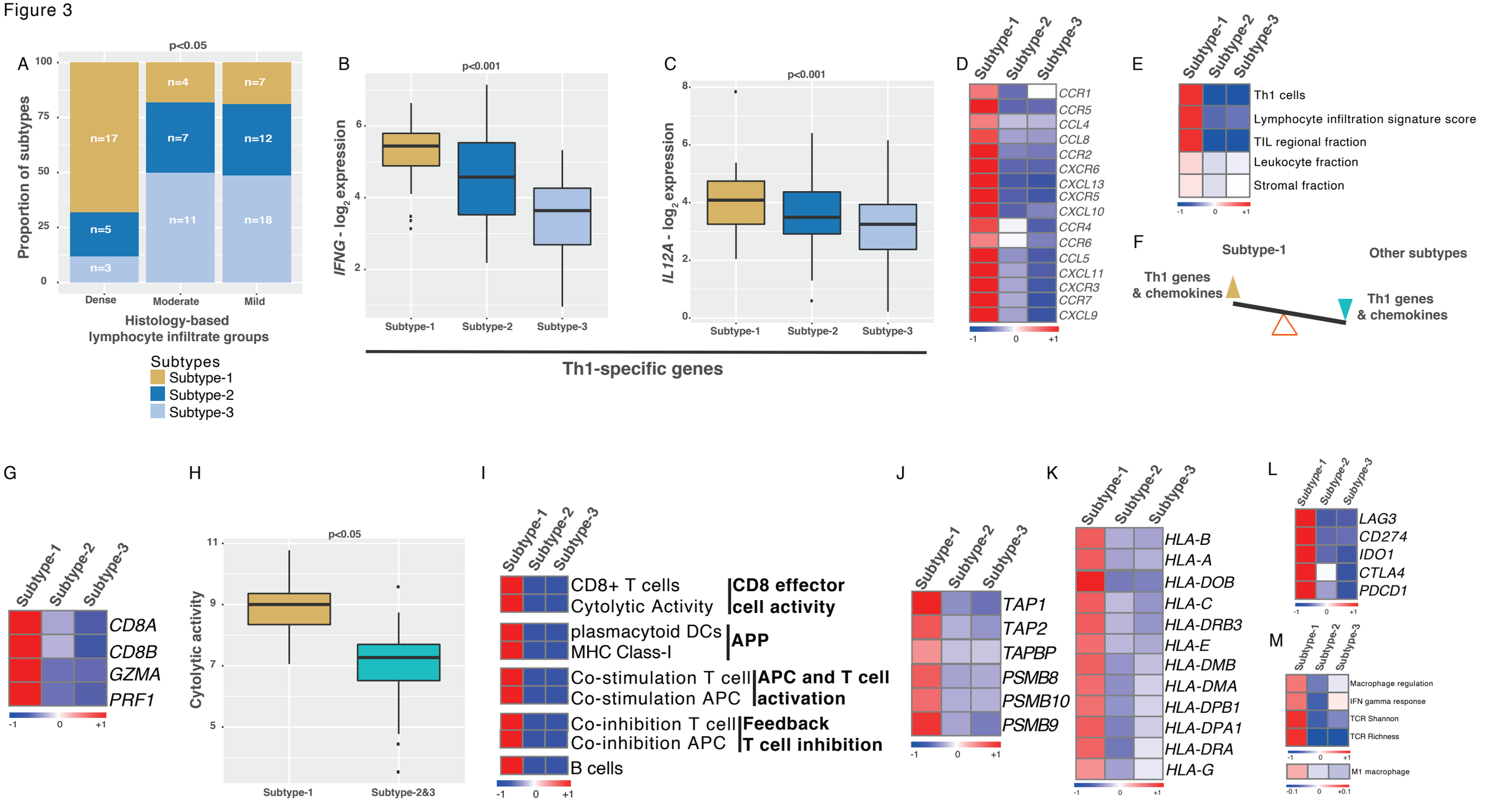
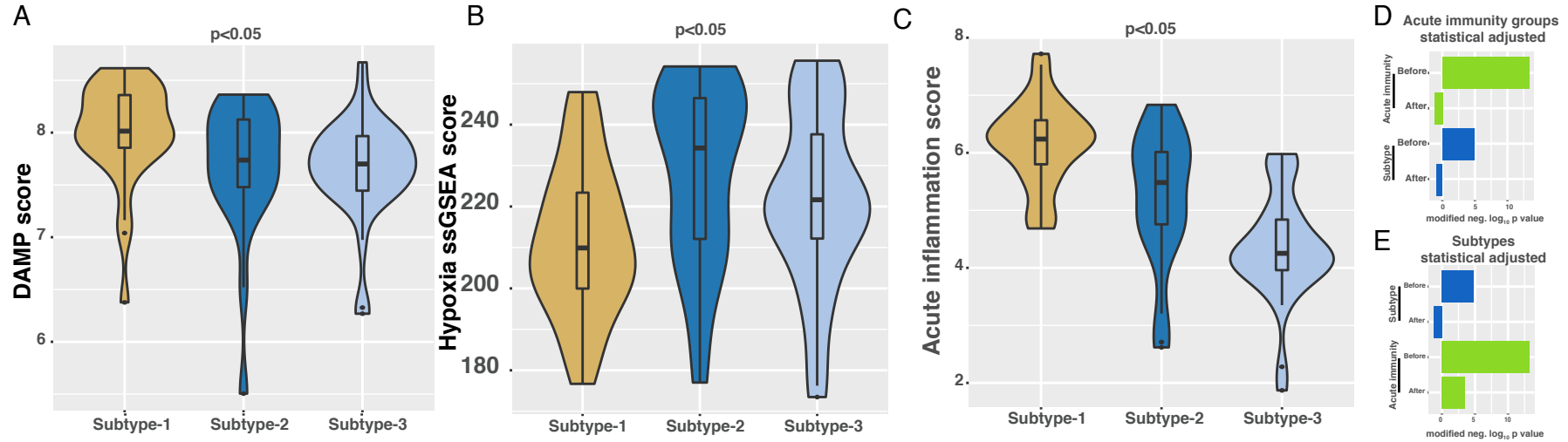

A
p<0.05
Proportion of subtypes
100
75
50
25
0
n=17
n=5
n=3
n=4
n=7
n=11
n=7
n=12
n=18
Dense
Moderate
Mild
Histology-based
lymphocyte infiltrate groups
Subtypes
Subtype-1
Subtype-2
Subtype-3
B
p<0.001
IFNG - log2 expression
Subtype-1
Subtype-2
Subtype-3
C
p<0.001
IL12A - log2 expression
Subtype-1
Subtype-2
Subtype-3
Th1-specific genes
D
Subtype-1
Subtype-2
Subtype-3
CCR1
CCR5
CCL4
CCL8
CCR2
CXCR6
CXCL13
CXCR5
CXCL10
CCR4
CCR6
CCL5
CXCL11
CXCR3
CCR7
CXCL9
-1
0
+1
E
Subtype-1
Subtype-2
Subtype-3
Th1 cells
Lymphocyte infiltration signature score
TIL regional fraction
Leukocyte fraction
Stromal fraction
-1
0
+1
F
Subtype-1
Other subtypes
Th1 genes
& chemokines
Th1 genes
& chemokines
G
Subtype-1
Subtype-2
Subtype-3
CD8A
CD8B
GZMA
PRF1
-1
0
+1
H
p<0.05
Cytolytic activity
11
9
7
5
Subtype-1
Subtype-2&3
I
Subtype-1
Subtype-2
Subtype-3
CD8+ T cells
Cytolytic Activity
CD8 effector
cell activity
plasmacytoid DCs
MHC Class-I
APP
Co-stimulation T cell
Co-stimulation APC
APC and T cell
activation
Co-inhibition T cell
Co-inhibition APC
Feedback
T cell inhibition
B cells
-1
0
+1
J
Subtype-1
Subtype-2
Subtype-3
TAP1
TAP2
TAPBP
PSMB8
PSMB10
PSMB9
-1
0
+1
K
Subtype-1
Subtype-2
Subtype-3
HLA-B
HLA-A
HLA-DOB
HLA-C
HLA-DRB3
HLA-E
HLA-DMB
HLA-DMA
HLA-DPB1
HLA-DPA1
HLA-DRA
HLA-G
-1
0
+1
L
Subtype-1
Subtype-2
Subtype-3
LAG3
CD274
IDO1
CTLA4
PDCD1
-1
0
+1
M
Subtype-1
Subtype-2
Subtype-3
Macrophage regulation
IFN gamma response
TCR Shannon
TCR Richness
-1
0
+1
M1 macrophage
-0.1
0
+0.1

Figure 4



Machine learning-based association analysis

Figure 3

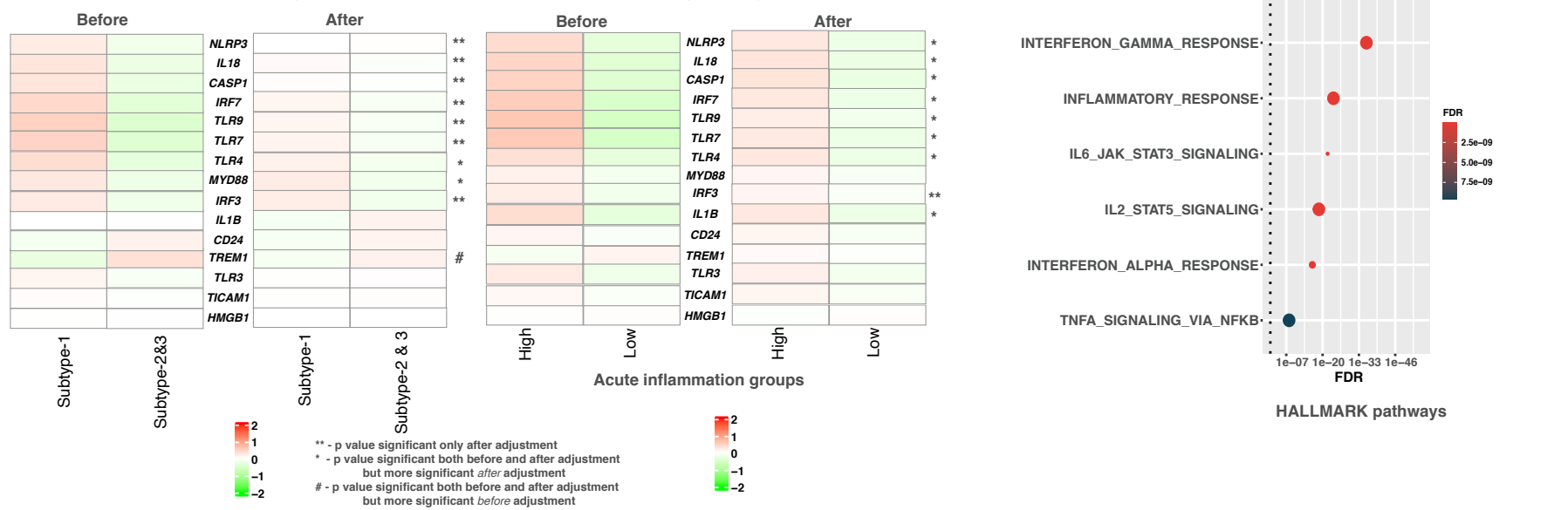


Figure 5

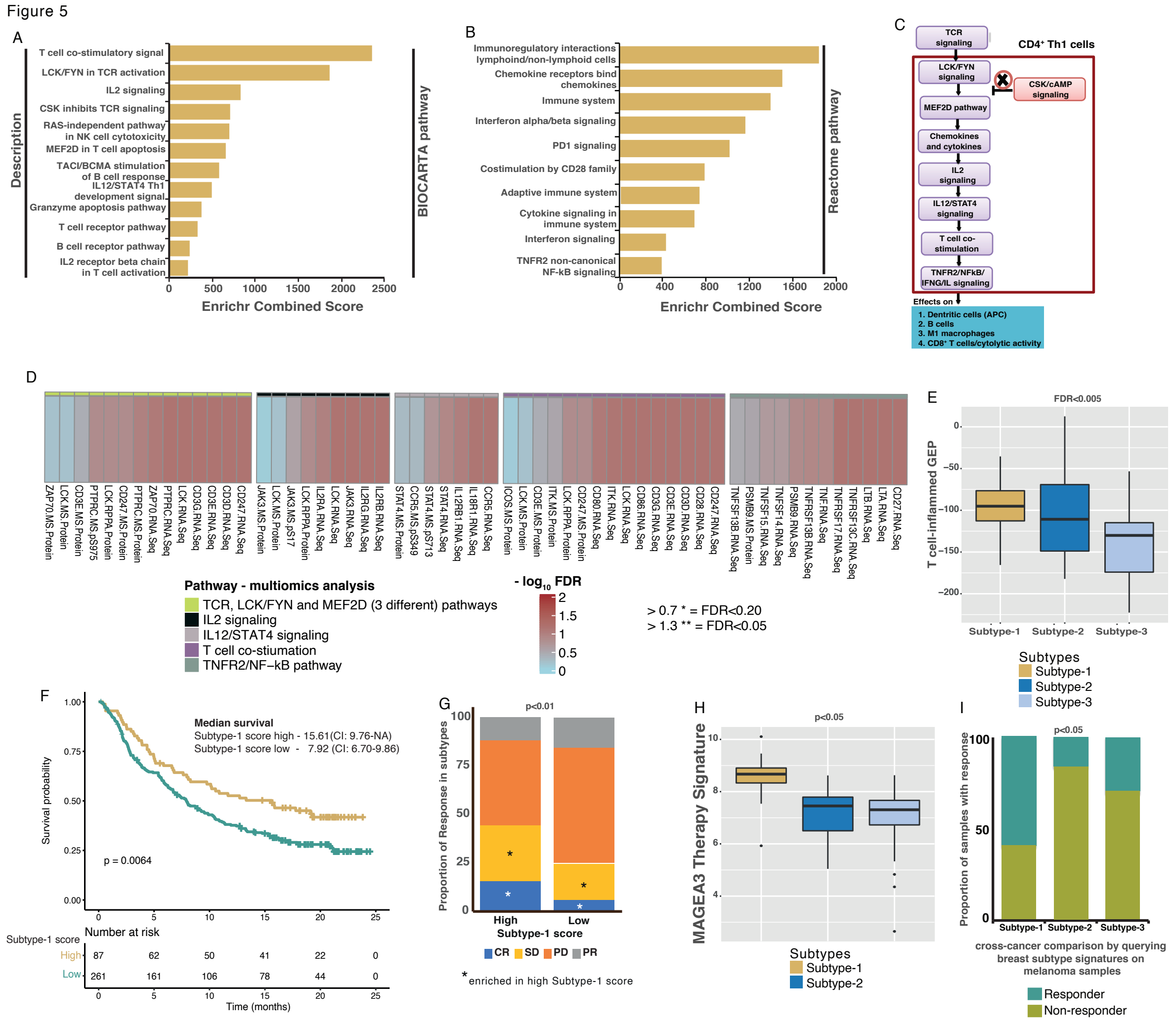
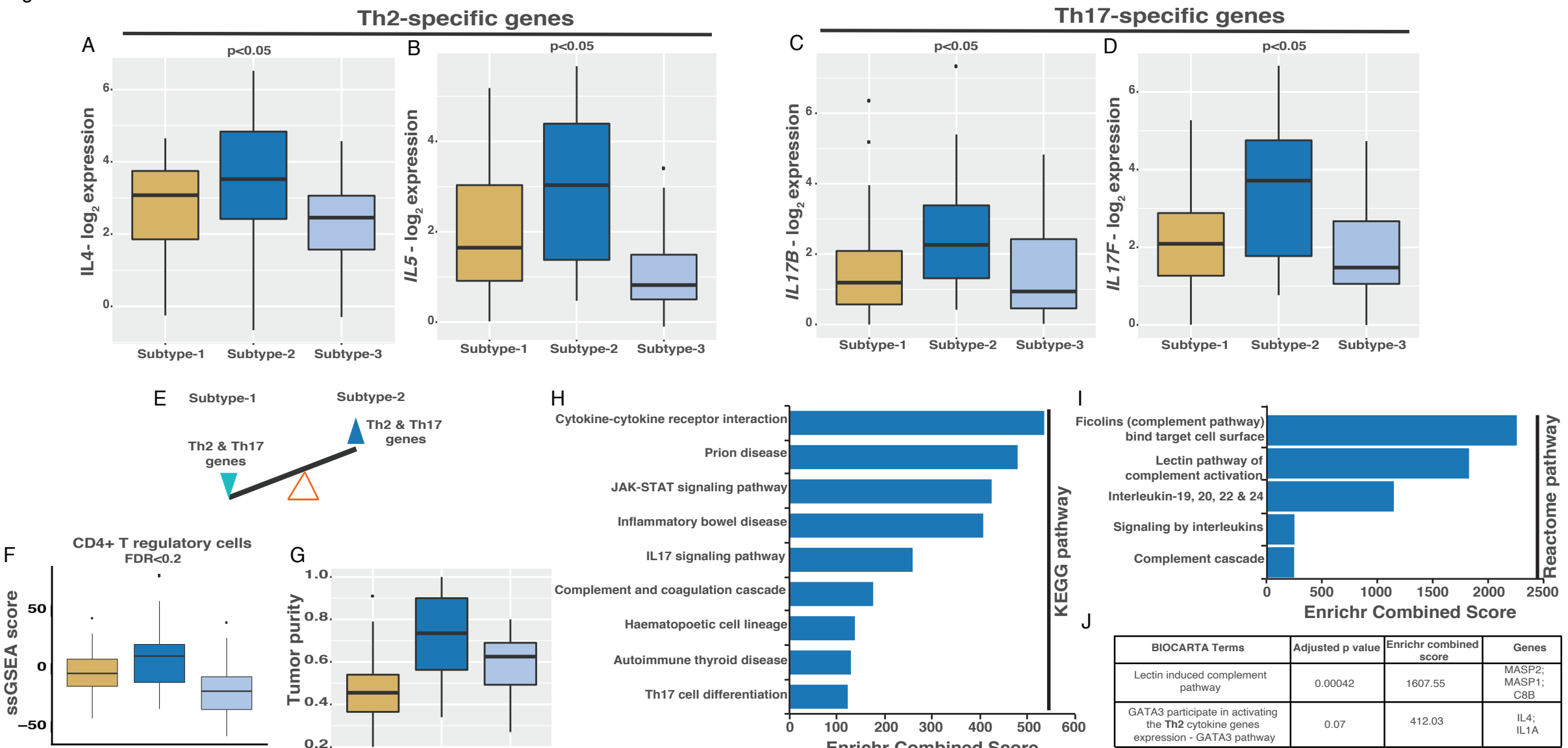
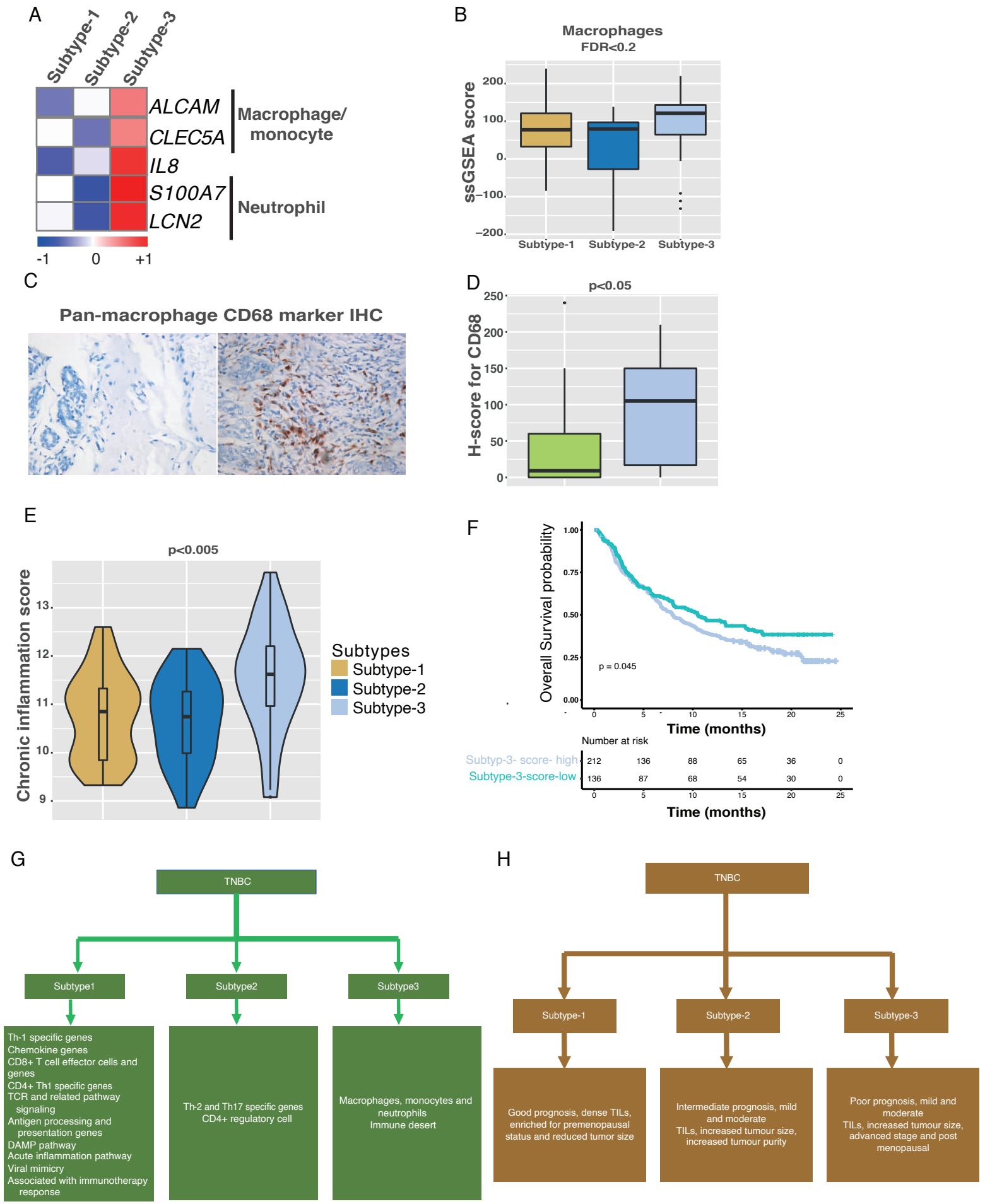

A
Description
T cell co-stimulatory signal
LCK/FYN in TCR activation
IL2 signaling
CSK inhibits TCR signaling
RAS-independent pathway in NK cell cytotoxicity
MEF2D in T cell apoptosis
TACI/BCMA stimulation of B cell response
IL12/STAT4 Th1 development signal
Granzyme apoptosis pathway
T cell receptor pathway
B cell receptor pathway
IL2 receptor beta chain in T cell activation
0 500 1000 1500 2000 2500
Enrichr Combined Score
BIOCARTA pathway
B
Immunoregulatory interactions lymphoind/non-lymphoid cells
Chemokine receptors bind chemokines
Immune system
Interferon alpha/beta signaling
PD1 signaling
Costimulation by CD28 family
Adaptive immune system
Cytokine signaling in immune system
Interferon signaling
TNFR2 non-canonical NF-kB signaling
0 400 800 1200 1600 2000
Enrichr Combined Score
Reactome pathway
C
CD4+ Th1 cells
TCR signaling
LCK/FYN signaling
CSK/cAMP signaling
MEF2D pathway
Chemokines and cytokines
IL2 signaling
IL12/STAT4 signaling
T cell co-stimulation
TNFR2/NFkB/IFNG/IL signaling
Effects on
1. Dentritic cells (APC)
2. B cells
3. M1 macrophages
4. CD8+ T cells/cytolytic activity
D
ZAP70.MS.Protein
LCK.MS.Protein
CD3E.MS.Protein
PTPRC.MS.pS975
LCK.RPPA.Protein
CD247.MS.Protein
PTPRC.MS.Protein
ZAP70.RNA.Seq
PTPRC.RNA.Seq
LCK.RNA.Seq
CD3G.RNA.Seq
CD3E.RNA.Seq
CD3D.RNA.Seq
CD247.RNA.Seq
JAK3.MS.Protein
LCK.MS.Protein
JAK3.MS.pS17
JAK3.RNA.Seq
LCK.RPPA.Protein
LCK.RNA.Seq
IL2RA.RNA.Seq
JAK3.RNA.Seq
IL2RG.RNA.Seq
IL2RB.RNA.Seq
STAT4.MS.Protein
CCR5.MS.pS349
STAT4.MS.pS713
STAT4.RNA.Seq
IL12RB1.RNA.Seq
IL18R1.RNA.Seq
CCR5.RNA.Seq
ICOS.MS.Protein
LCK.MS.Protein
CD3E.MS.Protein
ITK.MS.Protein
LCK.RPPA.Protein
CD247.MS.Protein
CD80.RNA.Seq
ITK.RNA.Seq
LCK.RNA.Seq
CD86.RNA.Seq
CD3G.RNA.Seq
CD3E.RNA.Seq
CD3D.RNA.Seq
CD28.RNA.Seq
CD247.RNA.Seq
TNFSF13B.RNA.Seq
PSMB9.MS.Protein
TNFSF15.RNA.Seq
TNFSF14.RNA.Seq
PSMB9.RNA.Seq
TNFRSF13B.RNA.Seq
TNF.RNA.Seq
TNFRSF17.RNA.Seq
TNFRSF13C.RNA.Seq
LTB.RNA.Seq
LTA.RNA.Seq
CD27.RNA.Seq
Pathway - multiomics analysis
TCR, LCK/FYN and MEF2D (3 different) pathways
IL2 signaling
IL12/STAT4 signaling
T cell co-stiumation
TNFR2/NF–kB pathway
- log10 FDR
2
1.5
1
0.5
0
> 0.7 * = FDR<0.20
> 1.3 ** = FDR<0.05
E
FDR<0.005
T cell-inflammed GEP
0
−50
−100
−150
−200
Subtype-1 Subtype-2 Subtype-3
Subtypes
Subtype-1
Subtype-2
Subtype-3
F
Median survival
Subtype-1 score high - 15.61(CI: 9.76-NA)
Subtype-1 score low - 7.92 (CI: 6.70-9.86)
Survival probability
1.00
0.75
0.50
0.25
0.00
p = 0.0064
0 5 10 15 20 25
Number at risk
Subtype-1 score
High 87 62 50 41 22 0
Low 261 161 106 78 44 0
Time (months)
G
p<0.01
Proportion of Response in subtypes
100
75
50
25
0
High
Low
Subtype-1 score
CR SD PD PR
* enriched in high Subtype-1 score
H
p<0.05
MAGEA3 Therapy Signature
10
8
6
4
Subtypes
Subtype-1
Subtype-2
I
p<0.05
Proportion of samples with response
100
50
0
Subtype-1 Subtype-2 Subtype-3
cross-cancer comparison by querying breast subtype signatures on melanoma samples
Responder
Non-responder

Figure 6



BIOCARTA Terms	Adjusted p value	Enrichr combined score	Genes
Lectin induced complement pathway	0.00042	1607.55	MASP2; MASP1; C8B
GATA3 participate in activating the **Th2** cytokine genes expression - GATA3 pathway	0.07	412.03	IL4; IL1A

Figure 7


A
Subtype-1
Subtype-2
Subtype-3
ALCAM
CLEC5A
IL8
S100A7
LCN2
Macrophage/ monocyte
Neutrophil
-1
0
+1
B
Macrophages
FDR<0.2
ssGSEA score
200
100
0
−100
−200
Subtype-1
Subtype-2
Subtype-3
C
Pan-macrophage CD68 marker IHC
D
p<0.05
H-score for CD68
250
200
150
100
50
0
E
p<0.005
Chronic inflammation score
13
12
11
10
9
Subtypes
Subtype-1
Subtype-2
Subtype-3
F
Overall Survival probability
1.00
0.75
0.50
0.25
0.00
p = 0.045
0
5
10
15
20
25
Time (months)
Number at risk
Subtyp-3- score- high 212 136 88 65 36 0
Subtype-3-score-low 136 87 68 54 30 0
Time (months)
G
TNBC
Subtype1
Subtype2
Subtype3
Th-1 specific genes
Chemokine genes
CD8+ T cell effector cells and genes
CD4+ Th1 specific genes
TCR and related pathway signaling
Antigen processing and presentation genes
DAMP pathway
Acute inflammation pathway
Viral mimicry
Associated with immunotherapy response
Th-2 and Th17 specific genes
CD4+ regulatory cell
Macrophages, monocytes and neutrophils
Immune desert
H
TNBC
Subtype-1
Subtype-2
Subtype-3
Good prognosis, dense TILs, enriched for premenopausal status and reduced tumor size
Intermediate prognosis, mild and moderate
TILs, increased tumour size, increased tumour purity
Poor prognosis, mild and moderate
TILs, increased tumour size, advanced stage and post menopausal