1 Results

Some more guidelines from the School of Geosciences.

This section should summarize the findings of the research referring to all Figure s, tables and statistical results (some of which may be placed in appendices).

- include the primary results, ordered logically - it is often useful to follow the same order as presented in the methods.
- alternatively, you may find that ordering the results from the most important to the least important works better for your project.
- data should only be presented in the main text once, either in tables or Figure s; if presented in Figure s, data can be tabulated in appendices and referred to at the appropriate point in the main text.

Often, it is recommended that you write the results section first, so that you can write the methods that are appropriate to describe the results presented. Then you can write the discussion next, then the introduction which includes the relevant literature for the scientific story that you are telling and finally the conclusions and abstract – this approach is called writing backwards.

1.1 Determination of inflammatory groups representing intratumor inflammatory status

In order to see if inflammatory groups associated to inflammation response can be obtained, first an MDS visualization of the dataset on the Hallmark Inflammatory Response signature (HIR) containing 200 genes, from MSigDB **(Figure** 1**.A)**. The 88 osteosarcoma samples are overall clustered in the middle, while other samples are scattered outwardly. The elbow method **(Figure** 1**.B)** seems to indicate that 2 clusters seems to be the optimal number of clusters after which the wss decreases the least. Visually, 8 clusters is the minimum amount of clusters for which each point visually belongs to its nearest cluster. The gene *ICAM4* was notably not detected in the dataset in the TARGET-OS cohort. Thus, 199 genes from the signature were used.

However, due to the high amount of clusters and combined with a low number of samples and the heterogeneity of the samples, another method based on the intensity of the inflammation has been tried. By doing the mean of the Z-score of the hallmark inflammatory response signature, three groups describing the inflammatory status can be obtained, classified as low, medium and high groups **(Figure** 1**.C)**.

Furthermore, the resulting groups are also partially functionally relevant as they seem to correspond fairly well to the functional groups, defined bythe k-means clustering based on MDS visualization **(Figures** 1**.C, D)**. Each sample is thus attributed to its inflammatory status and this group will be subsequently used for the following results.

1.2 Characterization of osteosarcomas associated to inflammatory status

In order to characterize osteosarcomas, the *Hp Osteosarcoma* gene signature from MSigDB was used to see whether the inflammatory groups can be related to gene expression from this signature. Visually, the heatmap representing the gene signature, annotated with the inflammatory groups, does seem to indicate that the samples express different genes between Low, Medium and High inflammation group **(Figure** 2**A)**. However, the dendrogram clustering the samples indicates that the gene signature does not cluster well with the inflammatory annotations **(Figure** 2**B)**. However, despite high heterogeneity between samples and inflammatory status, there seems to be an overall distinct expression of genes in each inflammatory group.

To further characterize subtypes of osteosarcomas, comparison of the expression of specific osteosarcoma markers relating to osteoblastic, chondroblastic, fibroblastic markers has also been done, through a heatmap

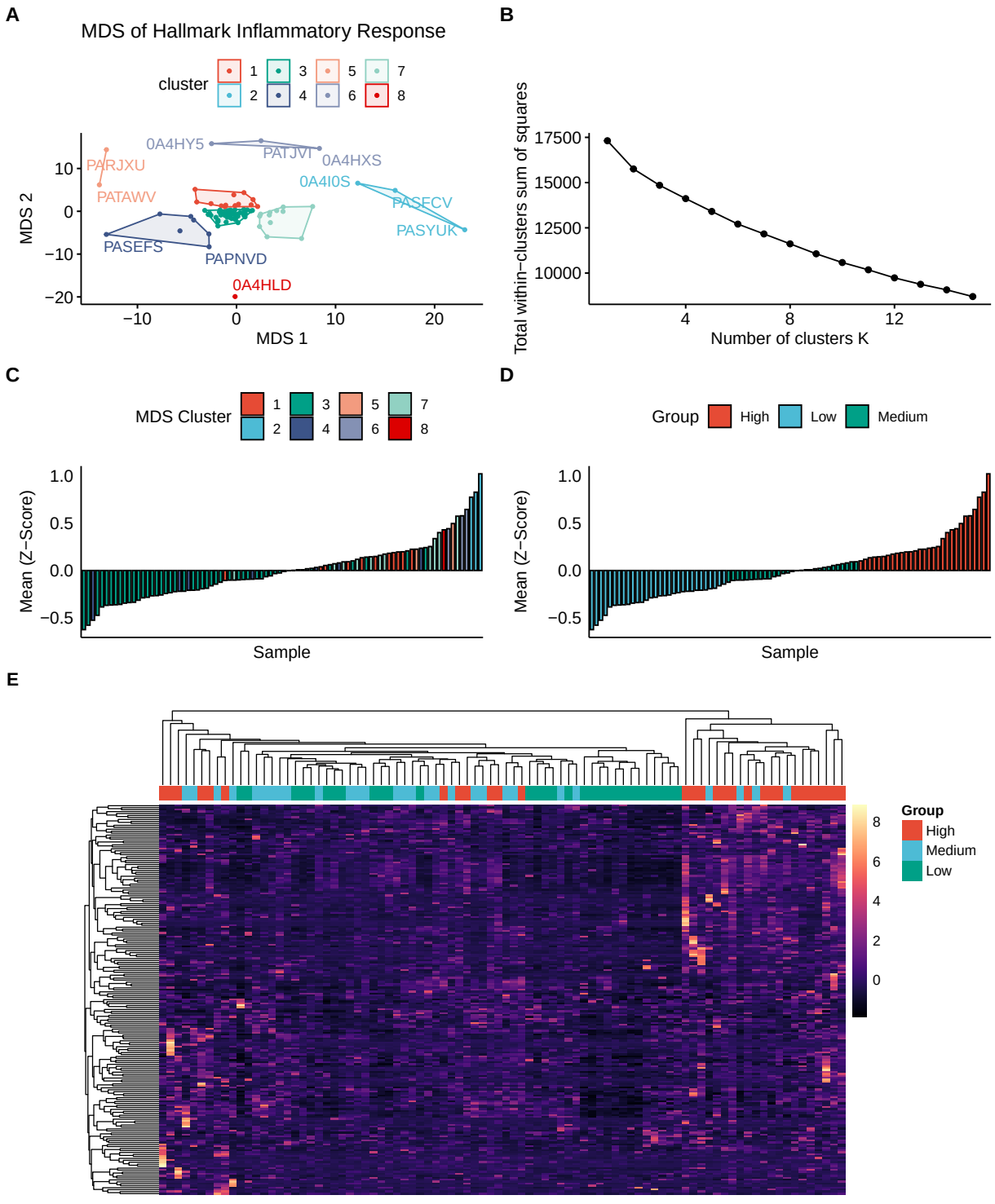

Figure 1: **Construction of inflammatory groups for the 88 samples in TARGET-OS dataset based on the HIR signature from MSigDB.**
(A) MDS visualization with k-means clustering of the HIR signature.
(B) Elbow graph determining the optimal number of k-clusters.
(C) Histogram of the mean of Z-score of the HIR signature, annotated with the 8 k-means clusters.
(D) Histogram of the mean of Z-score of the HIR signature, annotated with the 3 inflammatory status groups.
(E) Heatmap of the HIR signature with samples annotated to their corresponding inflammatory groups. Rows correspond to genes and columns correspond to samples. HIR = Hallmark Inflammatory Response. MDS = Multidimensional Scaling

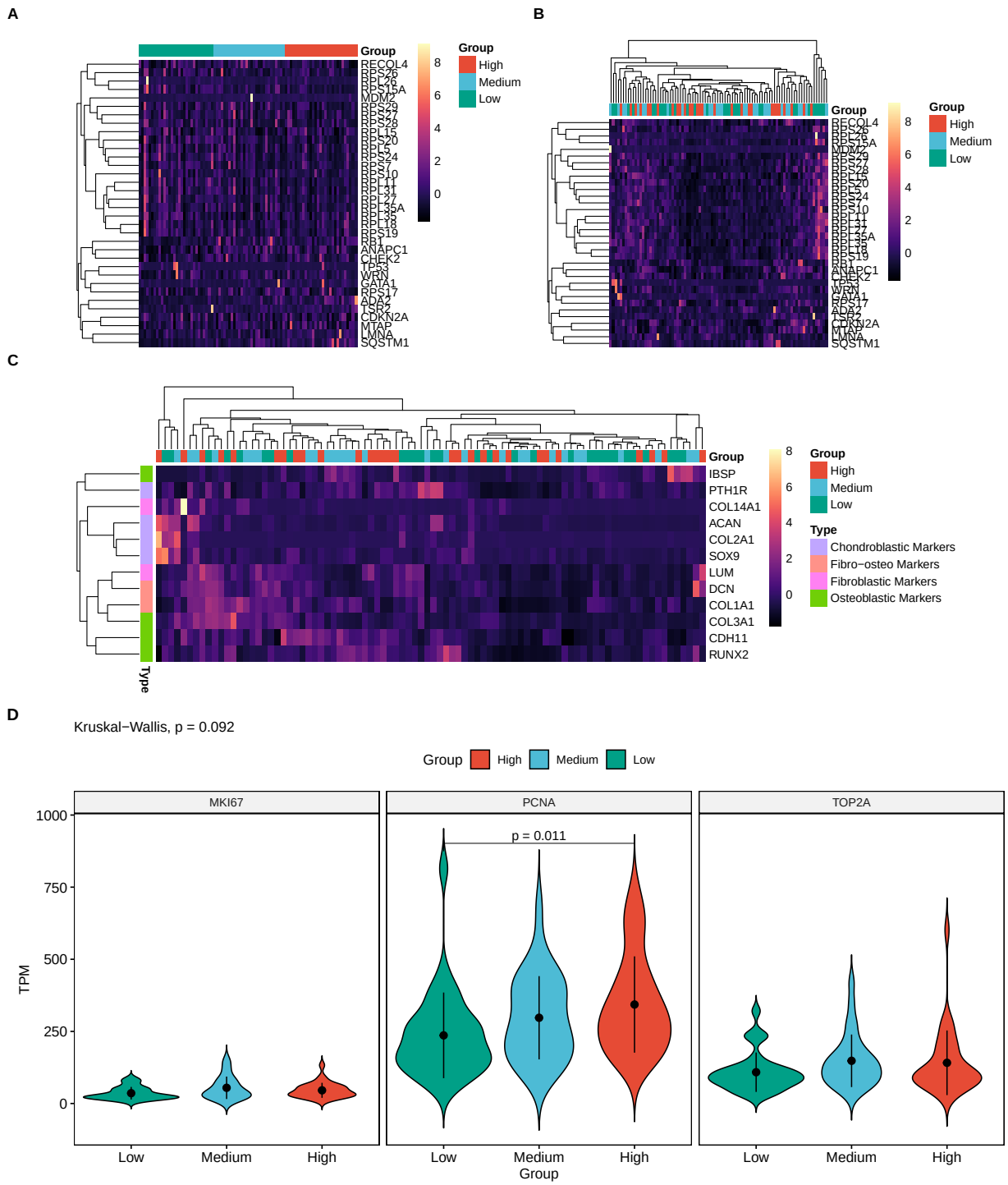

Figure 2: **Displays of various osteosarcoma related genes with samples annotated to their respective inflammatory groups.** Values are Z-scaled for heatmaps.
(A) Heatmap *Hp Osteosarcoma* signature with unclustered samples.
(B) Heatmap of *Hp Osteosarcoma* with clustered samples.
(C) Heatmap of osteosarcoma type markers.
(D) Violin plot of proliferation markers associated with their inflammatory group.

representation. Hierarchical clustering of the samples does not appear to be associated with corresponding inflammatory status. However it does reveal that there are groups of osteoblastic, chondroblastic and fibroblastic osteosarcomas which is expected (**Figure** 2**C**).

To assess the proliferation status of the samples related to the inflammation status, the mean of three markers of proliferation (MKI67, PCNA, TOP2A) have been compared, along with the mean of the three individual markers (**Figure** 2**D**). Statistical Kruskal-Wallis testing is significant ($P = 0.00968$) and post-hoc Dunn analysis reveals that the mean of the proliferation markers between low and high group is significantly different ($P = 0.016$). The data suggests that proliferation is hindered when inflammatory status is high in the osteosarcoma samples.

1.3 Characterization of intra-tumor inflammation associated to inflammatory status

1.3.1 Relationship with ESTIMATE and inflammatory signatures

Using the ESTIMATE algorithm, an immune score has been calculated for each sample which reflects the immune infiltration in a given tumor sample. The violin plot represents the values obtained for each inflammatory group **(Figure** 3**)**. Because the data is normally distributed and has homoscedasticity, ANOVA has been performed ($P = 0.002$) followed by Tukey's test, revealing that there is a statistically significant difference between Low and High group ($P = 0.002$), but not for the other conditions.

```
suppressWarnings(print(vep))
```

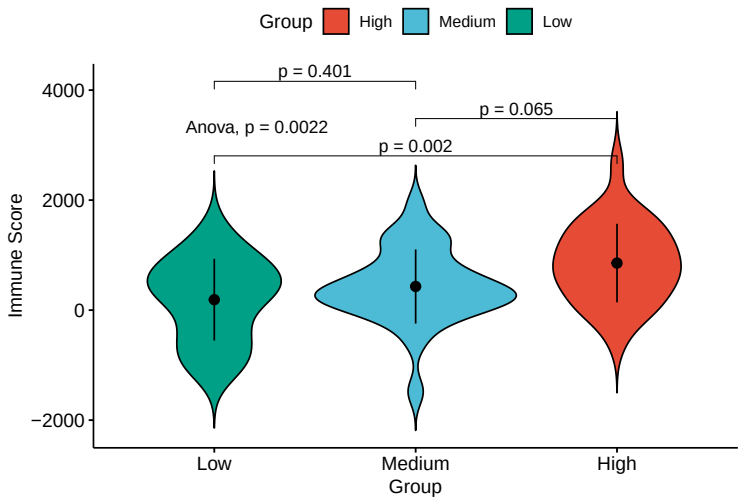

Figure 3: **Violin plot of ESTIMATE score for each inflammatory group.** ANOVA was performed followed by Tukey's post-hoc analysis. *P* ≤ 0.05 is considered statistically significant.

1.3.2 Immune abundance by immune deconvolution algorithm

Immune cell abundance can be determined by various immune deconvolution algorithm on TPM normalized bulk-RNASeq. Here, MCP-counter, CIBERSORTx and xCell have been tried. In MCP-counter, the mean (SD) of Z-score reflecting the abundances of neutrophils is statistically different between all groups, it is increased when the inflammation status gets higher **(Figure** 4**A)**

```
immune_plot <-
  ggarrange(
    mcpn_plot,
    cibn_plot,
    xcelln_plot,
    nrow = 1,
    labels = "AUTO",
    common.legend = TRUE
  )
suppressWarnings(print(immune_plot))
```

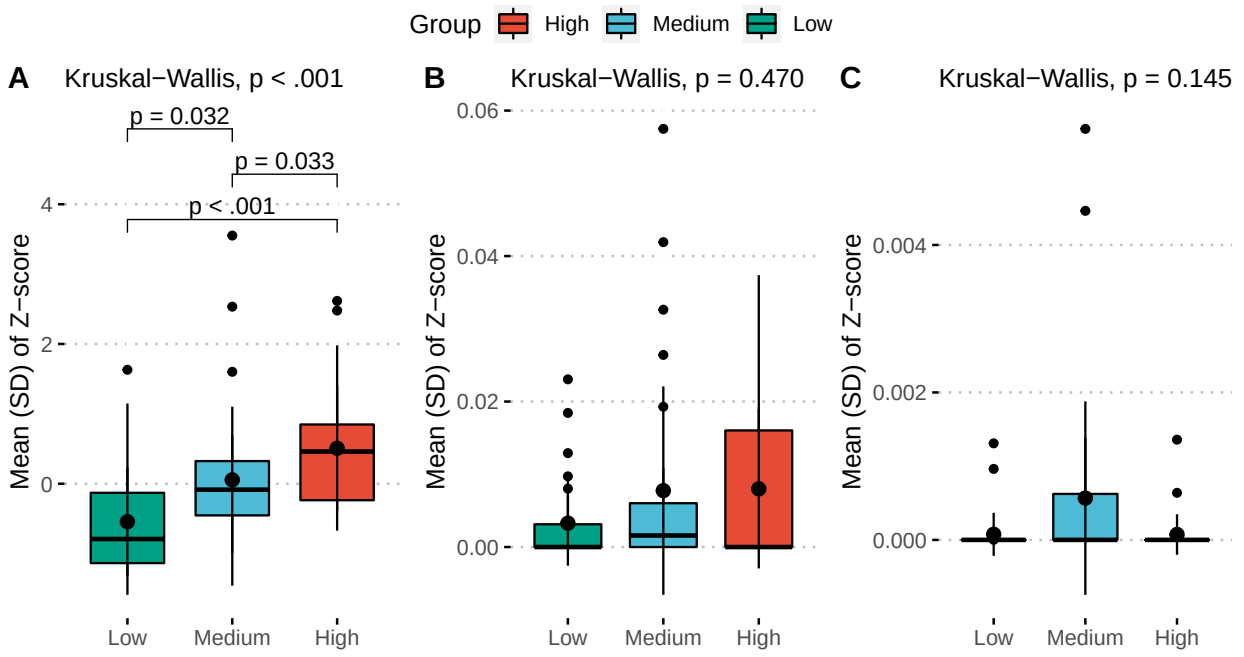

Figure 4: **Immune deconvolution plot of neutrophils with various immune deconvolution algorithm.**
(A) MCP-counter.
(B) CIBERSORTx.
(C) xCell.
Kruskal-Wallis testing is performed on non-parametric data and a post-hoc dunn's test was performed when appropriate. $P \leq 0.05$ is considered statistically significant.

1.3.3 Survival curve

In order to determine if inflammation groups are linked with increased survival odds, a Kaplan-Meier survival analysis was performed between low and high group of inflammation **(Figure** 5**)**. Log-rank analysis shows that the survival curve between low and high group of inflammation are not statistically different ($P = 0.16$). However the P value is low enough that it could be considered a trend. Estimation considers 29 patients in the low group and 28 patients in the high group, and estimates that only 9 patients remain alive in the low group while 20 patients are alive in the high group.

```
suppressWarnings(print(ggsurv))
```

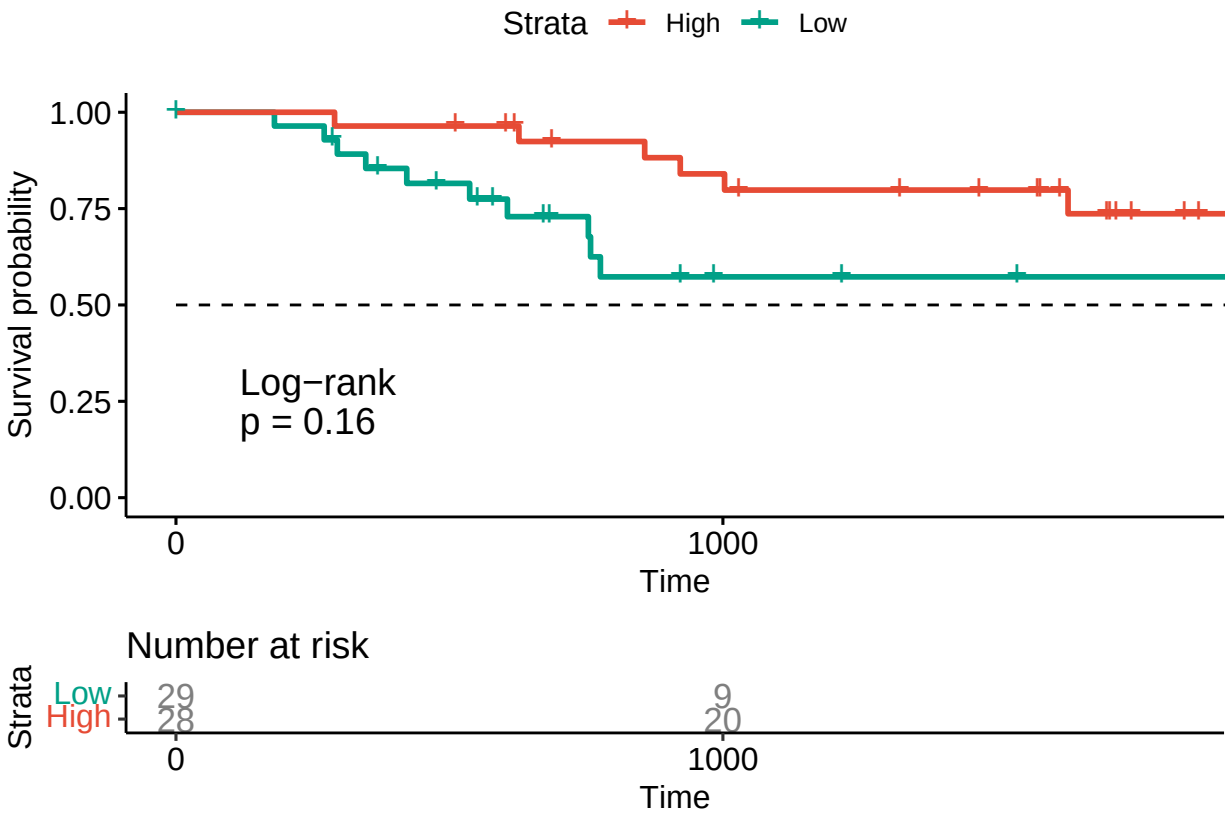

Figure 5: **Kaplan-Meier survival analysis plot of low vs high group indicate a trend associating inflammatory status with survival prognostic.** Horizontal and vertical axes represent survival times and rates, respectively. Blue and red colors represents low and high inflammation group respectively. Plus signs indicate censored values. Depicted P-values were obtained by the log-rank test. The diagram is cut at 1825 days (5 years). $P \leq 0.05$ is considered statistically significant.

1.3.4 Similarity of inflammatory signatures

In order to see if the inflammatory signatures used have overlapping genes or not, a Venn diagram was constructed from the signatures **(Figure @ref(fig:venn-plot*)**. Overall, the three immune signatures depicting immune infiltration (immune score), inflammation response and TNF- pathway have low overlapping genes and can be considered different.

```
suppressWarnings(print(venn_plot))
```

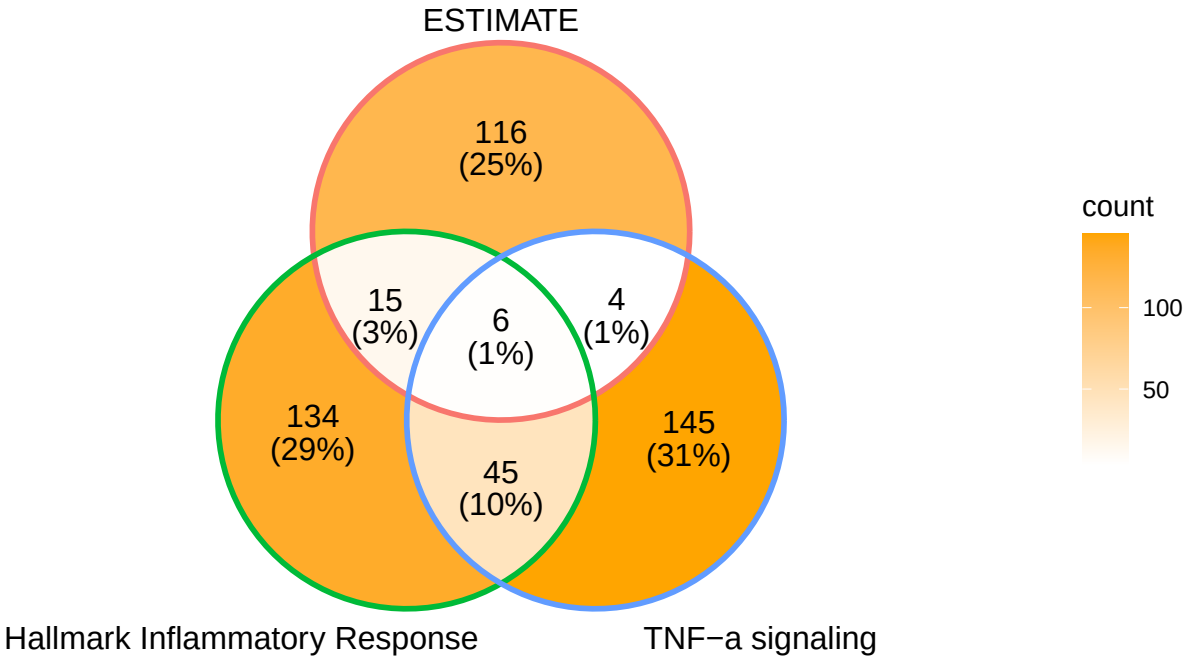

Figure 6: Venn diagram comparing three inflammatory signatures.

1.4 Biological mechanisms underlying the inflammatory groups

1.4.1 Differential Gene Expression Analysis and Enrichment

A differential gene expression analysis was performed on the inflammatory status condition (Low vs High) on the total 19114 genes. DEGs are enriched through multiple databases in order to identify potentially interesting pathways relating to a high level of inflammation **(Figure 7)**. The combined enrichment barplot **(Figure 7A)** returned multiple pathways associated with chemokines, PD-1 signaling, and T cell lymphocyte associated mechanisms. Interestingly, enrichment of downregulated genes in GO Biological Process database returned a lot of pathways linked to immune cell chemotaxis, and particularly neutrophil chemotaxis.

1.4.2 Gene Set Enrichment Analysis (GSEA)

GSEA was performed on 95 selected signatures from MSigDB, based on the theme of osteosarcoma, angiogenesis, neutrophils, migration, hypoxia and inflammation. Only significant pathways are reported in the table 1. Certain pathways are not significant but relevant, such as "Gobp Positive Regulation of Vascular Associated Smooth Muscle Cell Proliferation" ($P = 0.13$), "Gobp Positive Regulation of Inflammatory Response", and all other pathways concerning dendritic, lymphocyte, monocyte, macrophage chemotaxis and migration.

Interestingly, there is no found significant pathway relating to positive regulation of inflammation, but there are significant pathways relating to negative regulation of inflammation, indicating that in the high group, there is negative regulation of inflammation. Similarly, the only signature with a negative enrichment score is "Gobp Positive Regulation of Neutrophil Activation". It is also notable that the signature *Hp Osteosarcoma* in GSEA is not significantly different ($P = 0.38$).

```
gsea_df %>%
  filter(p.adjust <= 0.10) %>%
  dplyr::select(-qvalues) %>%
  mutate(p.adjust = insight::format_p(.$p.adjust)) %>%
  kableExtra::kbl(
    format = "latex",
    caption = glue("Results of GSEA by clusterProfiler, pre-ranked by descending order of log2 of fold c
    digits = 3,
    booktabs = TRUE,
    # longtable = TRUE # Use argument to split table in multiple pages
  )
```

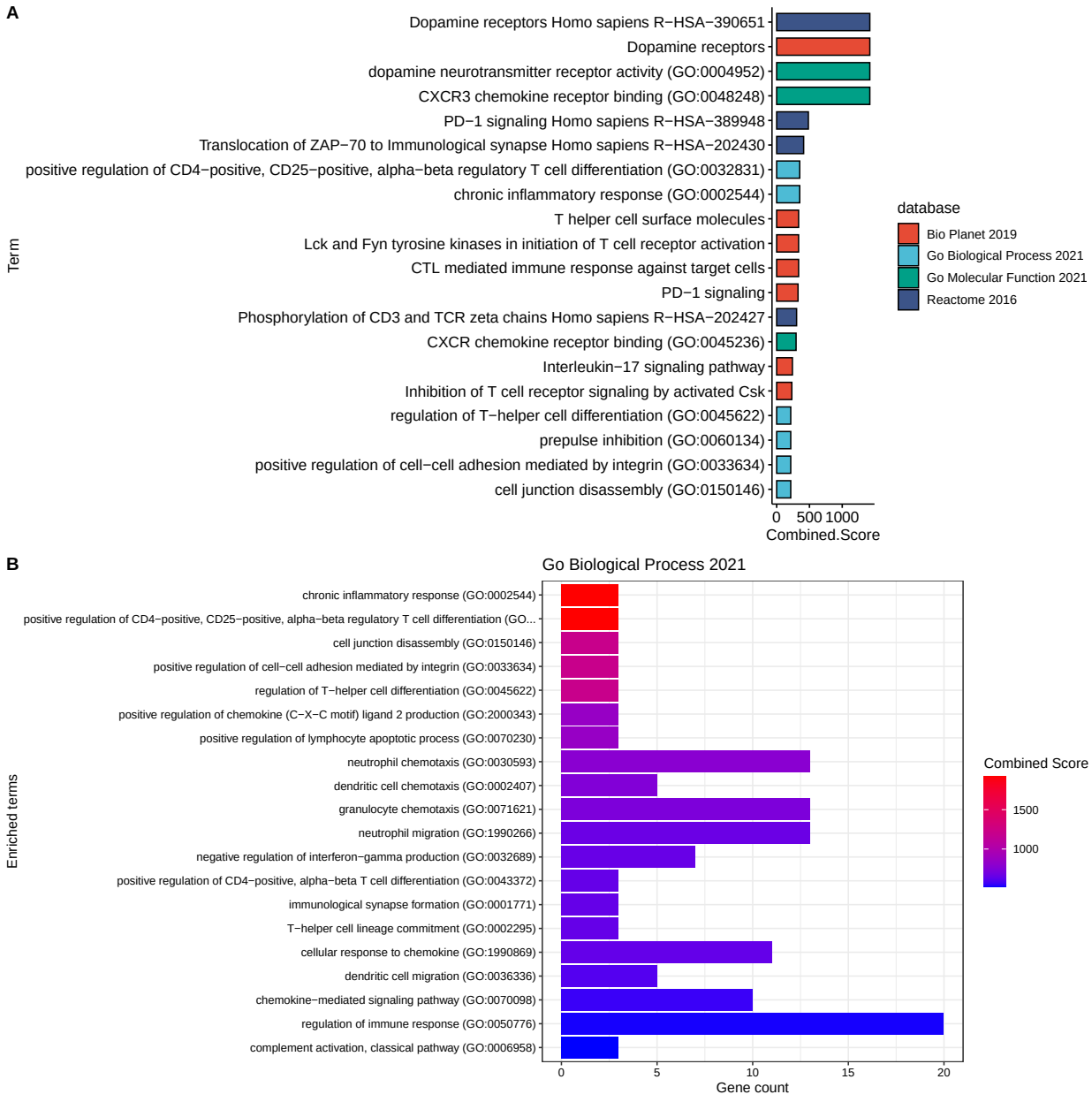

Figure 7: **Top 20 results of enrichment using enrichR for Low vs High condition.**
(A) Combined enrichment for 8 databases.
(B) Enrichment for downregulated genes for GO Biological Process 2021 database.

Table 1: Results of GSEA by clusterProfiler, pre-ranked by descending order of log2 of fold change for genes differentially expressed in low vs high condition. Genes at the top of the list are thus differentially expressed in the high group, and vice versa. Only significant pathways are shown, out of 95 selected pathways. $extitP$ adjust 0.10 is considered significant. NES = Normalized Enrichment Score

Pathway	Set Size	NES	p.adjust	p.sign
Gobp Cell Migration	1431	1.401	p < .001	***
Reactome Neutrophil Degranulation	475	1.488	p < .001	***
Hallmark Hypoxia	199	1.557	p < .001	***
Theilgaard Neutrophil at Skin Wound Dn	226	1.560	p < .001	***
Hay Bone Marrow Neutrophil	439	1.385	p < .001	***
Hallmark Inflammatory Response	199	1.504	p < .001	***
Hp Abnormality of Neutrophils	267	1.461	p < .001	***
Gobp Negative Regulation of Inflammatory Response	144	1.512	p = 0.001	**
Hp Abnormal Neutrophil Count	189	1.460	p = 0.001	**
Gobp Endothelial Cell Migration	216	1.425	p = 0.002	**
Kegg Leukocyte Transendothelial Migration	113	1.500	p = 0.004	**
Gobp Negative Regulation of Neuroinflammatory Response	14	1.663	p = 0.007	**
Wp Neovascularisation Processes	37	1.571	p = 0.023	*
Gobp Sprouting Angiogenesis	127	1.416	p = 0.023	*
Hallmark Tnfa Signaling via Nfkb	200	1.344	p = 0.023	*
Theilgaard Neutrophil at Skin Wound Up	75	1.485	p = 0.026	*
Travaglini Lung Neutrophil Cell	332	1.230	p = 0.038	*
Gobp Negative Regulation of Neutrophil Activation	5	1.497	p = 0.038	*
Sig Chemotaxis	45	1.482	p = 0.047	*
Reactome Cellular Response to Hypoxia	75	1.437	p = 0.055	*
Hay Bone Marrow Immature Neutrophil	189	1.276	p = 0.067	*
Gobp Positive Regulation of Neutrophil Activation	6	-1.687	p = 0.093	*
Wp Angiogenesis	24	1.455	p = 0.093	*