Aim 1 Differential Discovery Analysis

Setup

```r
# libraries
library(diffcyt)
library(tidyverse)
library(tidymodels)
library(lme4)

# source utils
source('~/GitHub/classes/BIOMEDIN_212/r_scripts/pap_utils.R', echo = FALSE)

# paths
data_path <-
  file.path("~", "GitHub", "classes", "BIOMEDIN_212", "data-raw", "cell_table.csv")

metadata_path <-
  file.path(
    "~", "GitHub", "classes",
    "BIOMEDIN_212", "data-raw", "fov_labels.csv"
  )

surfactant_path <-
  file.path(
    "~", "GitHub", "classes",
    "BIOMEDIN_212", "data-raw", "surfactant_masks"
  )

# globals
healthy_fovs_in_pap_patients <-
  c(9, 15, 20, 29, 40)

functional_markers <-
   c(
     "cd11c", "cd123", "cd14",
     "cd16", "cd163", "cd20",
     "cd206", "cd209", "cd3",
     "cd31", "cd4", "cd45",
     "cd45ro", "cd57", "cd68",
     "cd8", "calprotectin",
     "epox", "foxp3",
     "grz_b", "h3k27me3", "h3k9ac",
     "hh3", "hla_dr", "ho_1",
     "ido", "if_ng", "ki67",
     "lag3", "mmp9",
     "na_kat_pase", "pd1", "pan_ck",
```

```
    "sma", "si", "tim3",
    "tryptase", "vim",
    "i_nos", "p_s6"
  )
```

Read in data

```r
# metadata
metadata <-
  metadata_path %>%
  read_csv() %>%
  rename(fov_id = point) %>%
  janitor::clean_names()

# mibi data
mibi_data <-
  data_path %>%
  read_csv() %>%
  rename(
    fov_id = point,
    cell_id = label,
    cluster_id = pixelfreq_hclust_cap,
    cluster_name = name,
    centroid_x = `centroid-0`,
    centroid_y = `centroid-1`
  ) %>%
  janitor::clean_names()

# surfactant data
surf_data <-
  tibble(
    filenames =
      surfactant_path %>%
      list.files(),
    paths =
      surfactant_path %>%
      list.files(full.names = TRUE),
    data = map(.x = paths, .f = pap_read_tif)
  )

surf_data <-
  surf_data %>%
  unnest(cols = data) %>%
  transmute(
    fov_id = str_extract(filenames, pattern = "[:digit:]+"),
    x,
    y,
    values
  )
```

Pre-process data

```r
# join the mibi single-cell data with the FOV metadata
mibi_data <-
  mibi_data %>%
  left_join(metadata)

# count how many cells there are in each FOV type
mibi_data %>%
  count(category)
```

```
## # A tibble: 4 x 2
##   category        n
##   <chr>       <int>
## 1 nonSJIA-PAP 10584
## 2 Normal       4987
## 3 Pneumonia    6161
## 4 SJIA-PAP    57324
```

Because of our limited sample size, we more or less have to combine the "Normal" and "Pneumonia" category FOVs into a single category ("Control"). However, we should acknowledge that these two controls are not created equal - in fact, if we perform a simple t-test between the Pneumonia and Normal patient FOVs' proportion of each our immune cell clusters, we can see that there are significant differences (at the level of p = 0.05) even after Benjamini-Hochberg adjustment.

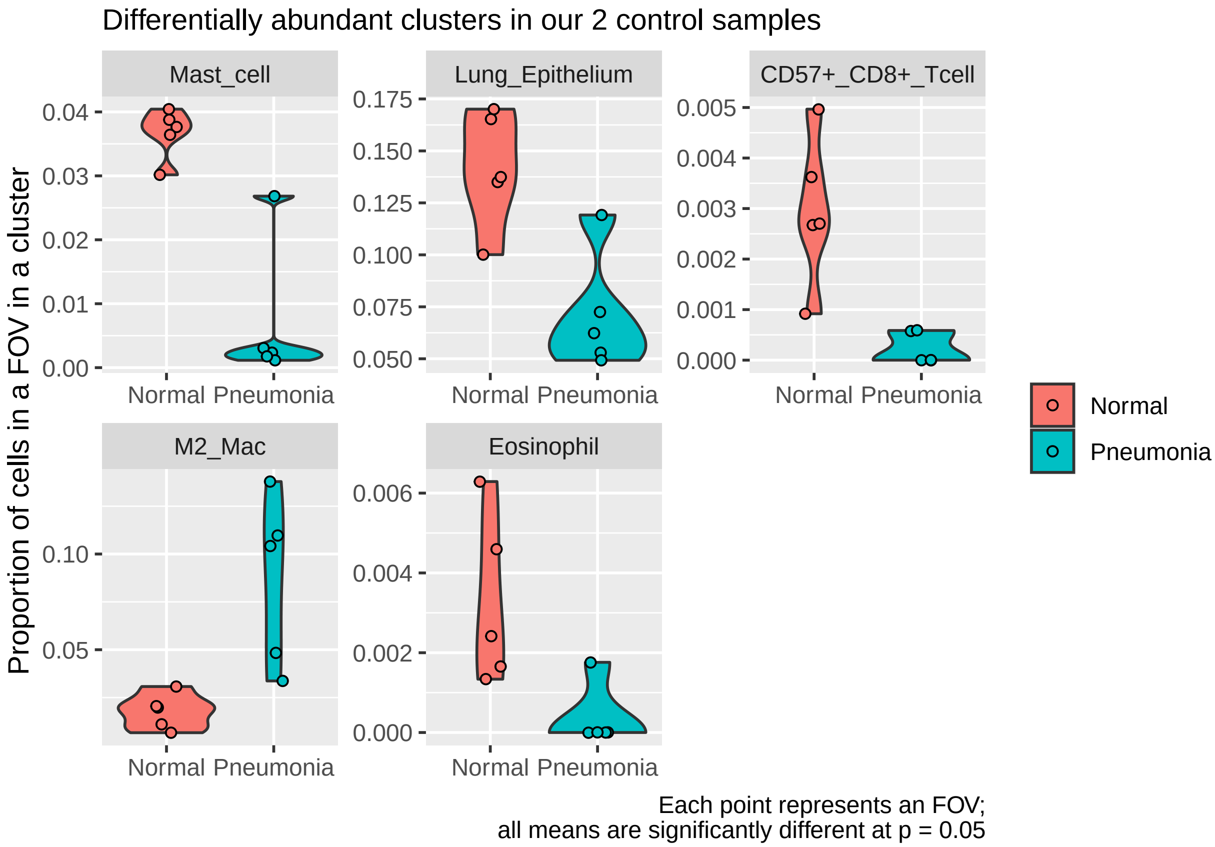
```r
cancer_pneumonia_counts <-
  mibi_data %>%
  filter(category %in% c("Pneumonia", "Normal")) %>%
  mutate(cluster_name = as.factor(cluster_name)) %>%
  count(patient_id, fov_id, category, cluster_name, .drop = FALSE) %>%
  group_by(fov_id) %>%
  mutate(prop = n / sum(n))

t_tests <-
  cancer_pneumonia_counts %>%
  group_by(cluster_name) %>%
  nest() %>%
  mutate(
    p_value =
      map_dbl(
        .x = data,
        .f = ~
          t.test(
            x =
              .x %>%
              dplyr::filter(patient_id == 13) %>%
              pull(prop),
            y =
              .x %>%
              dplyr::filter(patient_id == 14) %>%
              pull(prop)
          ) %>%
          tidy() %>%
          pull(p.value)
      ) %>%
```

```
      p.adjust(method = "BH")
  )

t_tests %>%
  select(-data) %>%
  arrange(p_value) %>%
  mutate(significant = if_else(p_value < 0.05, "*", "")) %>%
  knitr::kable()
```

cluster_name	p_value	significant
Mast_cell	0.0024518	*
Lung_Epithelium	0.0042509	*
CD57+_CD8+_Tcell	0.0129929	*
M2_Mac	0.0230855	*
Eosinophil	0.0341076	*
Endothelial	0.0561127	
CD8+_Tcell	0.0632200	
CD209+_Mac	0.0735467	
iNOS+_Pneumocyte	0.1045573	
CD16+_ImmuneOther	0.1383749	
Fibroblast	0.1780802	
CD11c+_mDC	0.2475057	
Neutrophil	0.2509420	
Bcell	0.2793292	
iNOS+_Mac	0.5925798	
CD57+_ImmuneOther	0.6663467	
CD4+_Tcell	0.7789749	
Mesenchymal	0.9513561	
Treg	0.9617596	
CD14+_Mono	0.9904512	

```
cluster_order <-
  t_tests %>%
  arrange(p_value) %>%
  pull(cluster_name) %>%
  as.character()

sig_clusters <-
  t_tests %>%
  filter(p_value < 0.05) %>%
  pull(cluster_name) %>%
  as.character()

cancer_pneumonia_counts %>%
  mutate(cluster_name = factor(cluster_name, levels = cluster_order)) %>%
  filter(cluster_name %in% sig_clusters) %>%
  ggplot(aes(y = prop, x = category, fill = category)) +
  geom_violin() +
  geom_jitter(shape = 21, width = 0.1) +
  facet_wrap(facets = vars(cluster_name), scales = "free") +
  labs(
    subtitle = "Differentially abundant clusters in our 2 control samples",
```

```r
    x = NULL,
    y = "Proportion of cells in a FOV in a cluster",
    fill = NULL,
    caption = "Each point represents an FOV;\nall means are significantly different at p = 0.05"
  )
```



Keeping this in mind, we proceed with annotating the `outcome` variable such that the pneumonia sample and cancer sample are treated equally as "controls" (we would be relatively underpowered otherwise in later comparisons, although this lumping is sub-optimal).

```r
mibi_data <-
  mibi_data %>%
  mutate(
    outcome =
      if_else(category %in% c("Normal", "Pneumonia"), "Control", category)
  )
```

Basic data summary

Number of unique FOVs in each condition

```
mibi_data %>%
  distinct(outcome, fov_id) %>%
  count(outcome, name = "num_fovs") %>%
  arrange(-num_fovs)
```

```
## # A tibble: 3 x 2
##   outcome     num_fovs
##   <chr>          <int>
## 1 SJIA-PAP          48
## 2 Control           10
## 3 nonSJIA-PAP        9
```

Number of unique patients in each condition

```
mibi_data %>%
  distinct(outcome, patient_id) %>%
  count(outcome, name = "num_patients") %>%
  arrange(-num_patients)
```

```
## # A tibble: 3 x 2
##   outcome     num_patients
##   <chr>              <int>
## 1 SJIA-PAP              12
## 2 Control                2
## 3 nonSJIA-PAP            2
```

Number of unique cells in each condition

```
mibi_data %>%
  count(outcome, name = "num_cells") %>%
  arrange(-num_cells)
```

```
## # A tibble: 3 x 2
##   outcome     num_cells
##   <chr>           <int>
## 1 SJIA-PAP        57324
## 2 Control         11148
## 3 nonSJIA-PAP     10584
```

Number of cells for each patient

```
mibi_data %>%
  count(patient_id, name = "num_cells") %>%
  arrange(-num_cells)
```

```
## # A tibble: 16 x 2
##    patient_id num_cells
##         <dbl>     <int>
##  1         11      8710
##  2         16      7705
```

```
##   3          13      6161
##   4          10      5811
##   5           9      5294
##   6           8      5061
##   7          14      4987
##   8           4      4972
##   9          12      4935
##  10           2      4590
##  11           5      4140
##  12           7      4115
##  13           3      4025
##  14           6      3433
##  15          15      2879
##  16           1      2238
```

Number of cells in each FOV

```r
mibi_data %>%
  count(fov_id, name = "num_cells") %>%
  arrange(-num_cells)
```

```
## # A tibble: 67 x 2
##    fov_id num_cells
##     <dbl>     <int>
##  1     14      2634
##  2     44      2341
##  3     12      2019
##  4     32      1924
##  5     43      1891
##  6      1      1885
##  7     25      1759
##  8     26      1754
##  9     49      1752
## 10     38      1726
## # ... with 57 more rows
```

Differential Abundance Analysis - between patients

In Aim 1, we proposed a differential abundance analysis of different immune cell subtypes (represented by the column `cluster_name` in `mibi_data`) across different types of MIBI images. The first of these analyses is to compare the abundance of each immune cell subtype between independent patients, each of which has either SJIA-PAP, PAP caused by something other than SJIA (nonSJIA-PAP), pneumonia, or lung cancer (which we code as "Normal" in `mibi_data`). We combine the last two conditions into the "control" category because neither of them have PAP, so they make as much sense as any sample we have access to to form our basis of comparison.

To perform our differential abundance analysis, we use the statistical framework proposed in the {{diffcyt}} framework.

Specifically, we use generalized linear mixed models (GLMMs) to test for differences in cluster abundance and cluster marker expression. The benefit of using mixed-models in this context is that, unlike more traditional differential abundance/expression testing tools commonly applied to cytometry data like CITRUS, GLMMs can account for complex experimental designs such as paired or block designs with known covariates representing batch effects or individual-to-individual variation. In the case of the present study, using random effects to model the variance in cluster abundance and marker expression that arises from random variation between individual patients (from whom we draw multiple FOVs), we can more reliably detect differences attributable solely to the effect of the `outcome` variable.

To do this, we can use the `{diffcyt}` R package to test for differential abundance of clusters across different levels of `outcome` using binomial regression. For each cluster, we can fit a binomial regression model in which we model the log-odds (and thus indirectly the proportion of cells in a given cluster) of each cluster in a given patient i and a given FOV j p_{ij} according to the following equation:

$$logit(p_{ij}) = log(\frac{p_{ij}}{1-p_{ij}}) = \beta_0 + \alpha_i + \beta_1 X_j$$

In the equation above, we use the following definitions:

- p_{ij}: The proportion of cells in a given cluster in patient i and FOV j
- α_i: A random intercept for each patient i in which $\alpha_i^{(p)} \sim N(0, \sigma_p)$, where σ_p is estimated during model fitting.
- X_j: an indicator variable representing whether or not an FOV j was taken from an SJIA-PAP patient (1 if yes, 0 otherwise). Depending on which comparisons we're making, what X_j stands for can change (but it always represents which `outcome` FOV j has been annotated with.
- All β's are linear model parameters optimized during model fitting.

Using the above setup, we can apply null-hypothesis significance testing to β_1 (under the null hypothesis that $\beta_1 = 0$): if β_1 is significantly different from 0 in the model, we can state that the proportion of cells in our cluster differs significantly between the levels of `outcome` we're investigating while controlling for individual-to-individual variation.

PAP vs. non-PAP

Using this framework, we can first compare the PAP samples (either SJIA-PAP or nonSJIA-PAP) to the control samples in our cohort. Note that we set a filter so that clusters that have fewer than 3 cells in 5 samples are removed from the analysis, as clusters with this few cells can't be used to estimate reliable proportions for a cell subtype's relative abundance in the sample it was collected from.

```
pap_daa <-
  mibi_data %>%
  select(fov_id, cluster_name, outcome, patient_id, any_of(functional_markers)) %>%
  mutate(outcome = if_else(outcome %in% c("SJIA-PAP", "nonSJIA-PAP"), "PAP", outcome)) %>%
  pap_perform_daa(
```

```
    data_tibble = .,
    sample_col = fov_id,
    cluster_col = cluster_name,
    fixed_effect_cols = outcome,
    random_effect_cols = c(patient_id),
    include_observation_level_random_effects = FALSE
  )

pap_daa$da_results %>%
  topTable(all = TRUE) %>%
  as_tibble() %>%
  arrange(p_adj) %>%
  mutate(significance = if_else(p_adj < 0.05, "*", "")) %>%
  knitr::kable()
```

cluster_id	p_val	p_adj	significance
CD209+_Mac	0.0284889	0.2848894	
Neutrophil	0.0149365	0.2848894	
Bcell	0.3262681	0.7449216	
CD11c+_mDC	0.1519862	0.7449216	
CD14+_Mono	0.4472275	0.7449216	
CD57+_CD8+_Tcell	0.4812898	0.7449216	
CD8+_Tcell	0.2688716	0.7449216	
Endothelial	0.2369884	0.7449216	
Fibroblast	0.2432184	0.7449216	
iNOS+_Mac	0.1652710	0.7449216	
iNOS+_Pneumocyte	0.4402886	0.7449216	
Mesenchymal	0.4841990	0.7449216	
Treg	0.3633710	0.7449216	
CD16+_ImmuneOther	0.5616607	0.8023724	
CD4+_Tcell	0.6354191	0.8074845	
CD57+_ImmuneOther	0.6459876	0.8074845	
Eosinophil	0.8001735	0.9413806	
Lung_Epithelium	0.9541837	0.9541837	
M2_Mac	0.9149582	0.9541837	
Mast_cell	0.8741013	0.9541837	
Giant_cell	NA	NA	NA

From these results, we can see that, when taking indivual random-effects into account, there are no statistically significant differentially abundance clusters between PAP and non-PAP samples (at least at the level of power we have available to us in this study).

SJIA-PAP vs. Controls

The second comparison we can run is between SJIA-PAP samples and control samples.

```
daa_sjia_pap_vs_controls <-
  mibi_data %>%
  select(fov_id, cluster_name, outcome, patient_id, any_of(functional_markers)) %>%
  #filter(!(fov_id %in% healthy_fovs_in_pap_patients)) %>%
  filter(outcome != "nonSJIA-PAP") %>%
  pap_perform_daa(
    data_tibble = .,
```

```
    sample_col = fov_id,
    cluster_col = cluster_name,
    fixed_effect_cols = outcome,
    random_effect_cols = c(patient_id),
    include_observation_level_random_effects = FALSE
  )

daa_sjia_pap_vs_controls$da_results %>%
  topTable(all = TRUE) %>%
  as_tibble() %>%
  arrange(p_adj) %>%
  mutate(significance = if_else(p_adj < 0.05, "*", "")) %>%
  knitr::kable()
```

cluster_id	p_val	p_adj	significance
Neutrophil	0.0000533	0.0010658	*
CD11c+_mDC	0.1296667	0.6439611	
CD14+_Mono	0.3199656	0.6439611	
CD209+_Mac	0.1100076	0.6439611	
CD8+_Tcell	0.2086889	0.6439611	
Endothelial	0.2859825	0.6439611	
Fibroblast	0.2608195	0.6439611	
iNOS+_Mac	0.1769249	0.6439611	
Mesenchymal	0.3219806	0.6439611	
Treg	0.2466686	0.6439611	
CD57+_CD8+_Tcell	0.3558237	0.6469522	
Bcell	0.4690123	0.7785946	
CD4+_Tcell	0.5060865	0.7785946	
CD16+_ImmuneOther	0.7383804	0.9488822	
CD57+_ImmuneOther	0.8849907	0.9488822	
Eosinophil	0.9014381	0.9488822	
Lung_Epithelium	0.6759856	0.9488822	
M2_Mac	0.8072448	0.9488822	
Mast_cell	0.7877623	0.9488822	
iNOS+_Pneumocyte	0.9523543	0.9523543	

In these results, we can see that neutrophils are differentially abundant in SJIA-PAP and control samples.

SJIA-PAP vs. nonSJIA-PAP

The third comparison we can run is between SJIA-PAP and nonSJIA-PAP samples.

```
daa_sjia_pap_vs_nonsjia_pap <-
  mibi_data %>%
  select(fov_id, cluster_name, outcome, patient_id, any_of(functional_markers)) %>%
  filter(outcome != "Control") %>%
  pap_perform_daa(
    data_tibble = .,
    sample_col = fov_id,
    cluster_col = cluster_name,
    fixed_effect_cols = outcome,
    random_effect_cols = c(patient_id),
    include_observation_level_random_effects = FALSE
```

```r
  )

daa_sjia_pap_vs_nonsjia_pap$da_results %>%
  topTable(all = TRUE) %>%
  as_tibble() %>%
  arrange(p_adj) %>%
  mutate(significance = if_else(p_adj < 0.05, "*", ""))
```

```
## # A tibble: 21 x 4
##    cluster_id         p_val p_adj significance
##    <fct>              <dbl> <dbl> <chr>
##  1 CD209+_Mac        0.126  0.595 ""
##  2 CD4+_Tcell        0.0965 0.595 ""
##  3 CD57+_ImmuneOther 0.0777 0.595 ""
##  4 M2_Mac            0.149  0.595 ""
##  5 Mesenchymal       0.0569 0.595 ""
##  6 Fibroblast        0.184  0.614 ""
##  7 CD16+_ImmuneOther 0.247  0.618 ""
##  8 Treg              0.217  0.618 ""
##  9 Neutrophil        0.358  0.795 ""
## 10 Bcell             0.494  0.824 ""
## # ... with 11 more rows
```

And once again we can see that there are no differentially abundant clusters between these sample types.

nonSJIA-PAP vs. Controls

The final between-patients comparison we can run is between nonSJIA-PAP samples and control samples.

```r
daa_control_vs_nonsjia_pap <-
  mibi_data %>%
  select(fov_id, cluster_name, outcome, patient_id, any_of(functional_markers)) %>%
  #filter(!(fov_id %in% healthy_fovs_in_pap_patients)) %>%
  filter(outcome != "SJIA-PAP") %>%
  pap_perform_daa(
    data_tibble = .,
    sample_col = fov_id,
    cluster_col = cluster_name,
    fixed_effect_cols = outcome,
    random_effect_cols = c(patient_id),
    include_observation_level_random_effects = FALSE
  )

daa_control_vs_nonsjia_pap$da_results %>%
  topTable(all = TRUE) %>%
  as_tibble() %>%
  arrange(p_adj) %>%
  mutate(significance = if_else(p_adj < 0.05, "*", ""))
```

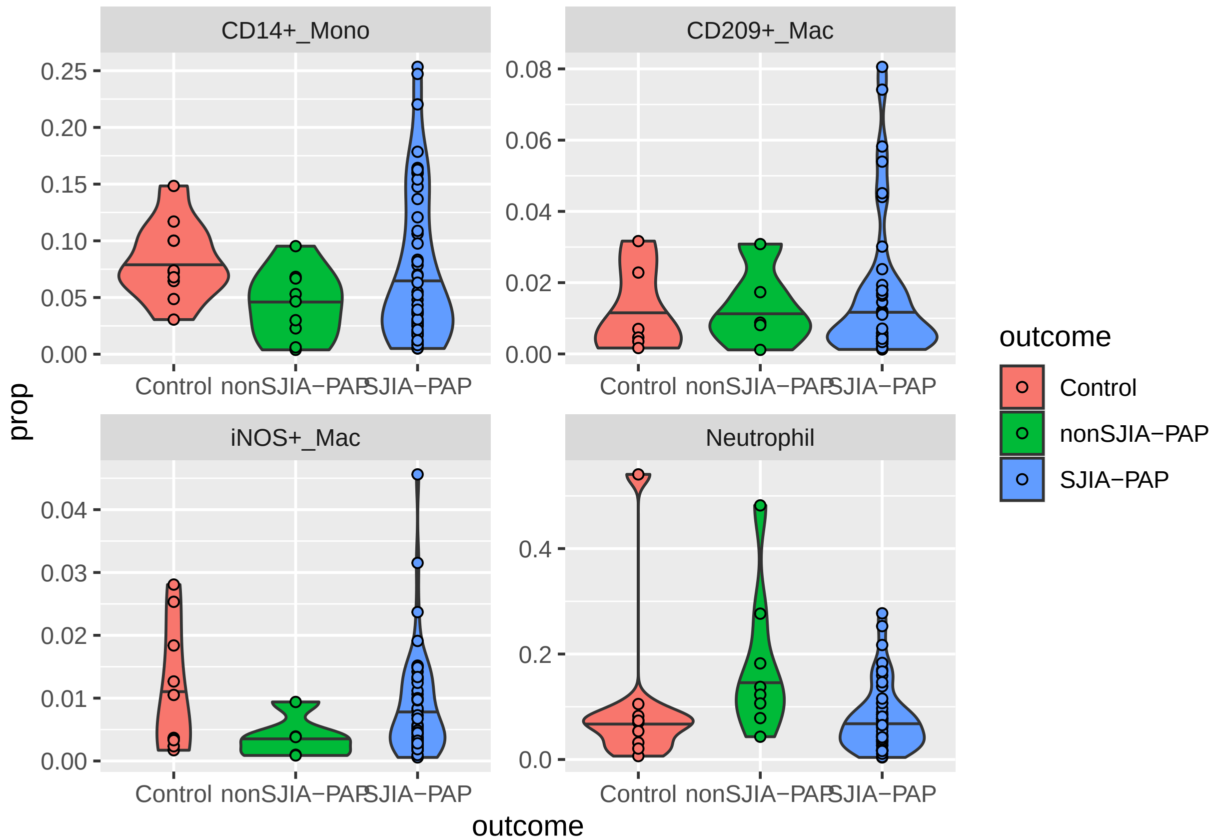
```
## # A tibble: 21 x 4
##    cluster_id            p_val   p_adj significance
##    <fct>                 <dbl>   <dbl> <chr>
##  1 iNOS+_Pneumocyte  0.000208 0.00416 "*"
##  2 CD14+_Mono        0.00768  0.0487  "*"
##  3 Endothelial       0.00974  0.0487  "*"
```

```
##   4 iNOS+_Mac         0.00658  0.0487  "*"
##   5 CD57+_ImmuneOther 0.0125   0.0502  ""
##   6 CD16+_ImmuneOther 0.0346   0.115   ""
##   7 Bcell             0.119    0.283   ""
##   8 Fibroblast        0.127    0.283   ""
##   9 Treg              0.122    0.283   ""
##  10 CD11c+_mDC        0.312    0.567   ""
## # ... with 11 more rows
```

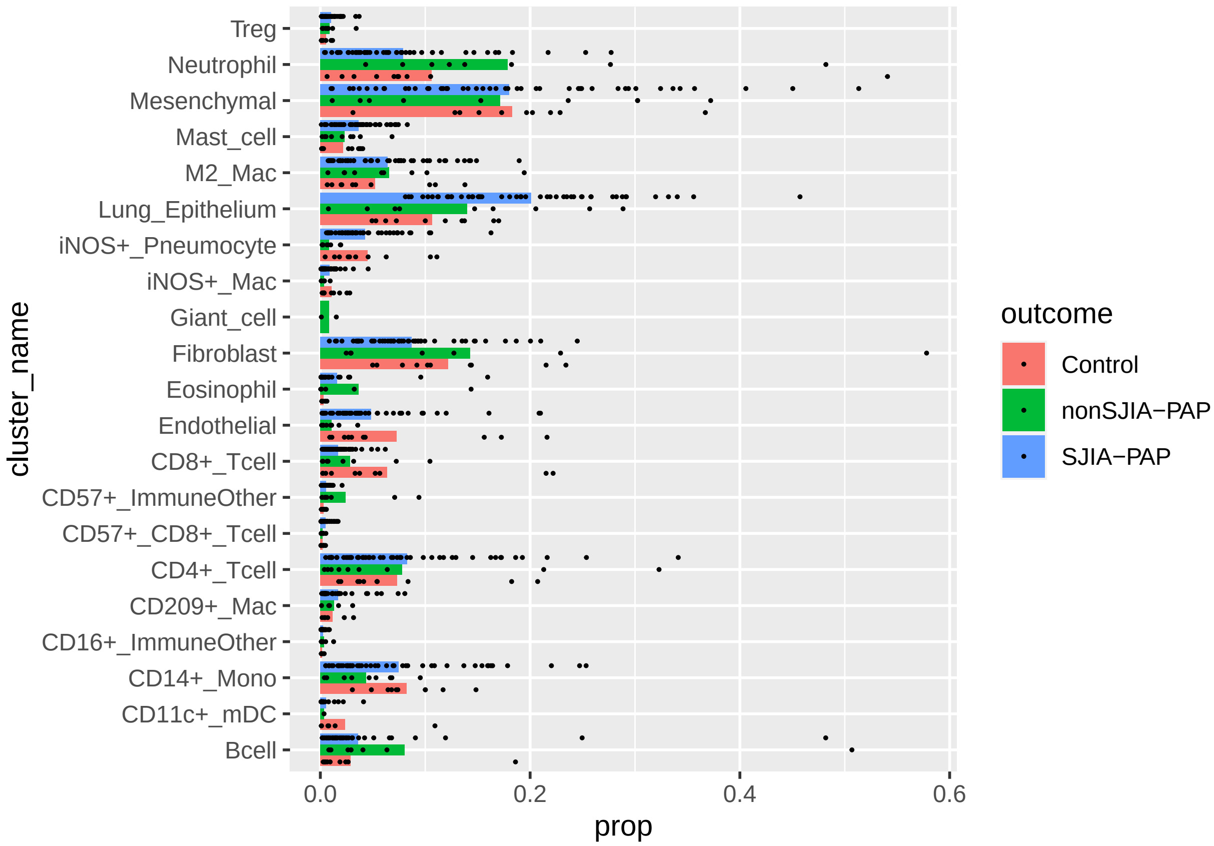
And in this case we can see that there are several cell subtypes that are differentially abundant (pneumocytes, monocytes, endothelial cells, and iNOS+ Macrophages).

Visualization

```
interesting_clusters <-
  c("Neutrophil", "CD209+_Mac", "CD14+_Mono", "iNOS+_Mac")

mibi_data %>%
  count(fov_id, cluster_name, patient_id, outcome) %>%
  group_by(fov_id) %>%
  mutate(
    total_cells = sum(n),
    prop = n / total_cells
  ) %>%
  filter(cluster_name %in% interesting_clusters) %>%
  ggplot(aes(x = outcome, y = prop, fill = outcome)) +
  geom_violin(draw_quantiles = 0.5) +
  geom_point(shape = 21, position = position_dodge(width = 0.3)) +
  facet_wrap(facets = vars(cluster_name), scales = "free")
```




```
mibi_data %>%
  count(fov_id, cluster_name, patient_id, outcome) %>%
  group_by(fov_id) %>%
  mutate(
    total_cells = sum(n),
    prop = n / total_cells
  ) %>%
  group_by(cluster_name, outcome) %>%
  summarize(prop = mean(prop)) %>%
  ggplot(aes(y = cluster_name, x = prop, fill = outcome)) +
  geom_col(position = "dodge") +
  geom_point(
    position = position_dodge(width = 1),
    size = 0.2,
    data =
      mibi_data %>%
      count(fov_id, cluster_name, patient_id, outcome) %>%
      group_by(fov_id) %>%
      mutate(
        total_cells = sum(n),
        prop = n / total_cells
      ) %>%
      ungroup()
  )
```



Treg
Neutrophil
Mesenchymal
Mast_cell
M2_Mac
Lung_Epithelium
iNOS+_Pneumocyte
iNOS+_Mac
Giant_cell
Fibroblast
Eosinophil
Endothelial
CD8+_Tcell
CD57+_ImmuneOther
CD57+_CD8+_Tcell
CD4+_Tcell
CD209+_Mac
CD16+_ImmuneOther
CD14+_Mono
CD11c+_mDC
Bcell
cluster_name
0.0
0.2
0.4
0.6
prop
outcome
Control
nonSJIA−PAP
SJIA−PAP

Differential Abundance Analysis - within patients

In addition to the between-patients comparisons, we can also run another set of comparisons that leverages a within-subjects design to increase statistical power. As it turns out, for several of our SJIA-PAP samples, one of the FOVs collected was annotated as a "healthy" section of tissue relative to the others (which had more of the hallmark histopathological features of SJIA-PAP). We can compare the abundance of each of our immune cell subpopulations within the same patients by comparing the "healthy" FOV to the other FOVs taken from the same patient.

```
# find patients who had at least one "healthy" FOV
interesting_patients <-
  mibi_data %>%
  filter(fov_id %in% healthy_fovs_in_pap_patients) %>%
  distinct(patient_id) %>%
  pull(patient_id)

interesting_patients
```

```
## [1] 16  8  9 11  1
```

Processing

```
paired_patients <-
  mibi_data %>%
  filter(patient_id %in% interesting_patients) %>%
  # annotate FOVs that are "healthy-looking" according to our pathologist
  mutate(
    fov_condition =
          if_else(fov_id %in% healthy_fovs_in_pap_patients, "healthy", "pap")
  )
```

Statistical analysis

```
paired_daa_results <-
  paired_patients %>%
  filter(outcome != "nonSJIA-PAP") %>%
  pap_perform_daa(
    data_tibble = .,
    sample_col = fov_id,
    cluster_col = cluster_name,
    fixed_effect_cols = fov_condition,
    random_effect_cols = patient_id,
    include_observation_level_random_effects = FALSE
  )

paired_daa_results %>%
  pluck("da_results") %>%
  topTable(all = TRUE) %>%
  as_tibble() %>%
  mutate(significant = if_else(p_adj < 0.05, "*", "")) %>%
  arrange(p_adj) %>%
  knitr::kable()
```

cluster_id	p_val	p_adj	significant
Bcell	0.0000000	0.0000000	*
Eosinophil	0.0000000	0.0000000	*
Lung_Epithelium	0.0000000	0.0000000	*
Mesenchymal	0.0000001	0.0000005	*
CD14+_Mono	0.0000008	0.0000025	*
Fibroblast	0.0000007	0.0000025	*
M2_Mac	0.0022011	0.0056600	*
iNOS+_Mac	0.0037262	0.0083839	*
Neutrophil	0.0102007	0.0204013	*
CD4+_Tcell	0.1098600	0.1797709	
Treg	0.1097409	0.1797709	
Endothelial	0.1863928	0.2795892	
Mast_cell	0.2171053	0.3006073	
CD209+_Mac	0.2976274	0.3826638	
CD8+_Tcell	0.3671814	0.4130791	
iNOS+_Pneumocyte	0.3620723	0.4130791	
CD57+_ImmuneOther	0.4157340	0.4401889	
CD57+_CD8+_Tcell	0.7218197	0.7218197	
CD11c+_mDC	NA	NA	NA
CD16+_ImmuneOther	NA	NA	NA

From these results, we can see that there are several immune cell subtypes that, when using a paired design, we find are enriched in parts of the SJIA-PAP lung that actually show histopathological signs of disease compared to paired parts of the SJIA-PAP lung that do not show histopathological signs of disease.

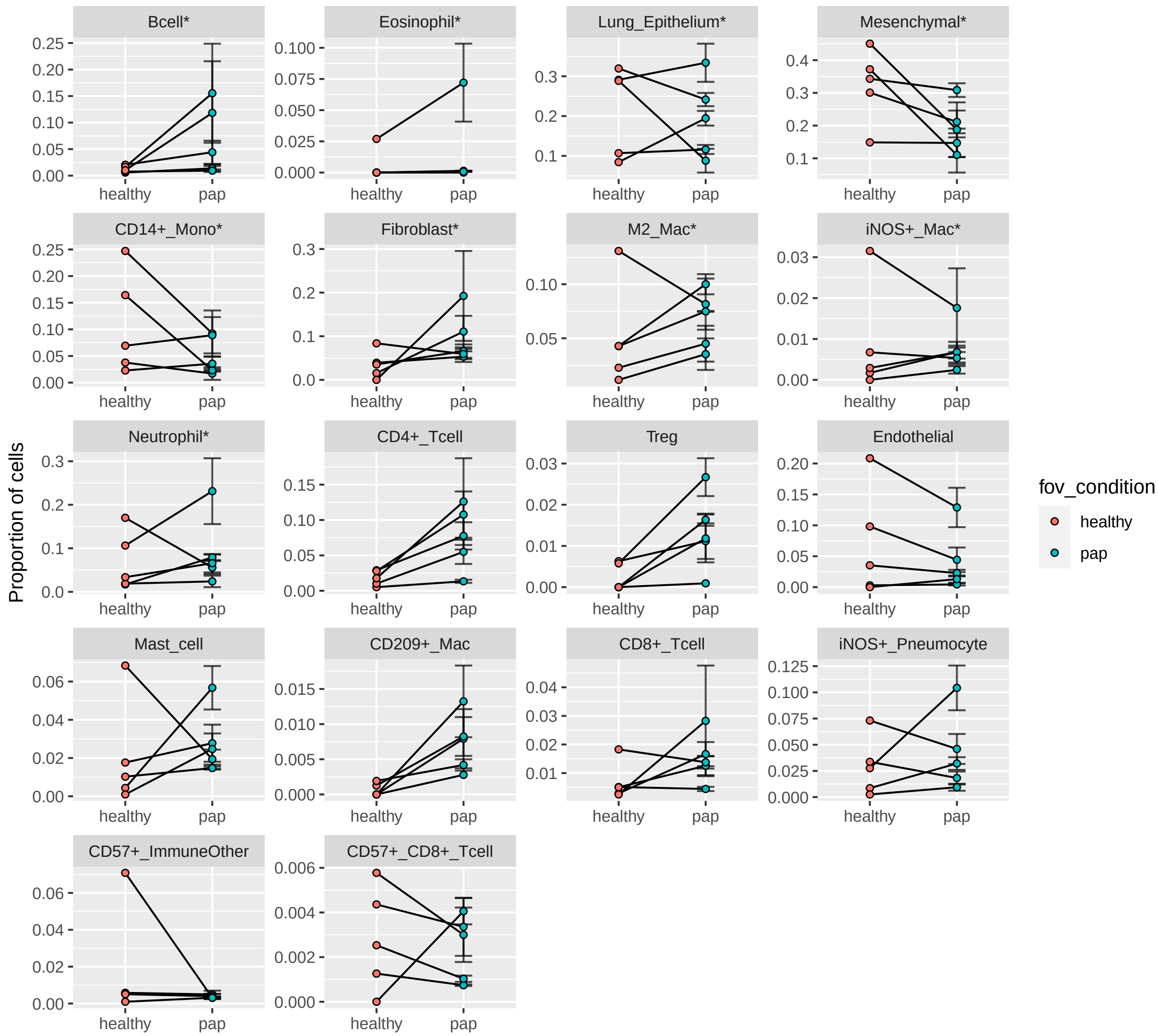
We can visualize these differences below.

Visualization

```
paired_p_values <-
  paired_daa_results %>%
  pluck("da_results") %>%
  topTable(all = TRUE) %>%
  as_tibble() %>%
  mutate(
    significant = if_else(p_adj < 0.05, "*", ""),
    new_cluster_name = if_else(significant == "*", str_c(cluster_id, "*"), as.character(cluster_id))
  ) %>%
  arrange(p_adj) %>%
  rename(cluster_name = cluster_id)

sig_clusters <-
  paired_p_values %>%
  filter(significant == "*") %>%
  pull(cluster_name)

# calculate the number of fovs used for each patient in each "condition"
num_fov_tibble <-
  paired_patients %>%
  distinct(fov_id, patient_id, fov_condition) %>%
  count(patient_id, fov_condition, name = "num_fovs")
```

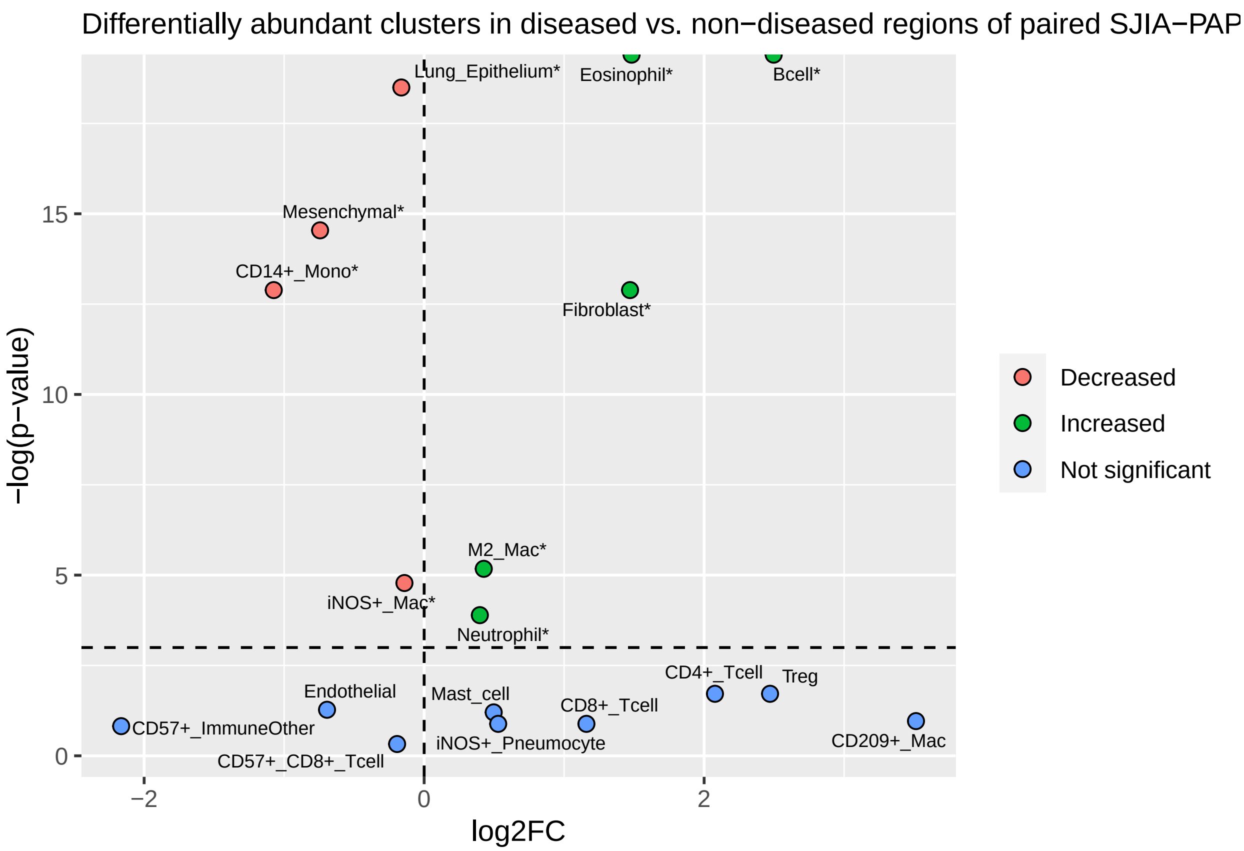
```
paired_plot_data <-
  paired_patients %>%
  mutate(
    cluster_name =
      factor(cluster_name, levels = pull(paired_p_values, cluster_name)) ,
  ) %>%
  count(cluster_name, patient_id, fov_id, fov_condition, .drop = FALSE) %>%
  group_by(fov_id) %>%
  mutate(
    total_fov_cells = sum(n),
    prop = n / total_fov_cells
  ) %>%
  ungroup() %>%
  group_by(fov_condition, patient_id, cluster_name) %>%
  summarize(
    sd = sd(prop, na.rm = TRUE),
    prop = mean(prop, na.rm = TRUE),
  ) %>%
  drop_na(cluster_name) %>%
  ungroup() %>%
  #mutate(across(c(patient_id, fov_condition), .f = as.factor)) %>%
  complete(patient_id, fov_condition, cluster_name, fill = list(prop = 0)) %>%
  left_join(num_fov_tibble) %>%
  mutate(
    sem = sd / sqrt(num_fovs)
  )
```

```
paired_plot_data %>%
  left_join(paired_p_values) %>%
  mutate(new_cluster_name = fct_reorder(new_cluster_name, p_adj)) %>%
  drop_na(new_cluster_name) %>%
  ggplot(aes(y = prop, x = fov_condition, fill = fov_condition)) +
  geom_line(aes(group = patient_id), color = "black") +
  geom_errorbar(
    aes(x = fov_condition, y = prop, ymin = prop - sem, ymax = prop + sem),
    width = 0.2,
    alpha = 0.7
  ) +
  geom_point(shape = 21) +
  #scale_y_continuous(oob = scales::oob_squish_infinite) +
  facet_wrap(facets = vars(new_cluster_name), scales = "free", ncol = 4) +
  labs(
    x = NULL,
    y = "Proportion of cells"
  )
```




```
paired_plot_data %>%
  left_join(paired_p_values) %>%
  mutate(new_cluster_name = fct_reorder(new_cluster_name, p_adj)) %>%
  drop_na(new_cluster_name) %>%
  select(patient_id, fov_condition, new_cluster_name, prop, p_adj) %>%
  pivot_wider(
    names_from = fov_condition,
    values_from = prop
  ) %>%
  group_by(new_cluster_name) %>%
  summarize(across(c(healthy, pap), mean, na.rm = TRUE)) %>%
  mutate(
    fc = pap / healthy,
    log2_fc = log(fc, base = 2)
  ) %>%
  left_join(paired_p_values) %>%
  mutate(
    neg_log_p_val = -log(p_adj),
```

```r
  cluster_type =
    case_when(
      p_adj > 0.05 ~ "Not significant",
      fc > 1       ~ "Increased",
      fc < 1       ~ "Decreased"
    )
) %>%
ggplot(aes(x = log2_fc, y = neg_log_p_val, fill = cluster_type)) +
geom_hline(yintercept = -log(0.05), color = "black", linetype = "dashed") +
geom_vline(xintercept = 0, color = "black", linetype = "dashed") +
geom_point(shape = 21, size = 2.5) +
ggrepel::geom_text_repel(aes(label = new_cluster_name), size = 2.5, color = "black") +
labs(
  subtitle = "Differentially abundant clusters in diseased vs. non-diseased regions of paired SJIA-PAI
  x = "log2FC",
  y = "-log(p-value)",
  fill = NULL
)
```



Differential Expression Analysis - between patients

[Blurb about how we're doing the same as the between-patients design above, but this time using a LMM to predict median marker expression for each cluster]

PAP vs. non-PAP

```
pap_dea <-
  mibi_data %>%
  select(fov_id, cluster_name, outcome, patient_id, any_of(functional_markers)) %>%
  mutate(outcome = if_else(outcome %in% c("SJIA-PAP", "nonSJIA-PAP"), "PAP", outcome)) %>%
  pap_perform_dea(
    data_tibble = .,
    sample_col = fov_id,
    cluster_col = cluster_name,
    fixed_effect_cols = outcome,
    random_effect_col = c(patient_id),
    min_cells = 5,
    min_samples = 5
  )

# only show the top 25 most significant results
pap_dea$de_results %>%
  topTable(top_n = 25) %>%
  as_tibble() %>%
  arrange(p_adj) %>%
  mutate(significance = if_else(p_adj < 0.05, "*", "")) %>%
  knitr::kable()
```

cluster_id	marker_id	p_val	p_adj	significance
CD4+_Tcell	ho_1	0.0000000	0.0000003	*
CD8+_Tcell	ido	0.0000000	0.0000120	*
Treg	calprotectin	0.0000001	0.0000227	*
CD8+_Tcell	sma	0.0000037	0.0007343	*
Neutrophil	cd31	0.0000072	0.0011498	*
CD8+_Tcell	cd45ro	0.0000337	0.0044886	*
Neutrophil	hh3	0.0001206	0.0137804	*
CD8+_Tcell	cd14	0.0001441	0.0144104	*
CD8+_Tcell	cd31	0.0004699	0.0376558	*
Mast_cell	calprotectin	0.0004707	0.0376558	*
M2_Mac	cd31	0.0006038	0.0439119	*
CD14+_Mono	cd31	0.0007405	0.0447756	*
Neutrophil	hla_dr	0.0007836	0.0447756	*
CD8+_Tcell	vim	0.0007542	0.0447756	*
Neutrophil	cd45ro	0.0014519	0.0725940	
CD11c+_mDC	hla_dr	0.0013935	0.0725940	
CD4+_Tcell	cd45ro	0.0017349	0.0816402	
iNOS+_Mac	vim	0.0024281	0.1079161	
Treg	cd163	0.0032848	0.1274656	
Eosinophil	ido	0.0033460	0.1274656	
CD8+_Tcell	p_s6	0.0032649	0.1274656	
CD4+_Tcell	calprotectin	0.0039012	0.1300399	
CD8+_Tcell	hh3	0.0038458	0.1300399	
CD8+_Tcell	pan_ck	0.0035863	0.1300399	

cluster_id	marker_id	p_val	p_adj	significance
Bcell	cd8	0.0041655	0.1321142	

SJIA-PAP vs. Controls

```
dea_sjia_pap_vs_controls <-
  mibi_data %>%
  select(fov_id, cluster_name, outcome, patient_id, any_of(functional_markers)) %>%
  #filter(!(fov_id %in% healthy_fovs_in_pap_patients)) %>%
  filter(outcome != "nonSJIA-PAP") %>%
  pap_perform_dea(
    data_tibble = .,
    sample_col = fov_id,
    cluster_col = cluster_name,
    fixed_effect_cols = outcome,
    random_effect_col = c(patient_id),
    min_cells = 5,
    min_samples = 5
  )

dea_sjia_pap_vs_controls$de_results %>%
  topTable(top_n = 25) %>%
  as_tibble() %>%
  arrange(p_adj) %>%
  mutate(significance = if_else(p_adj < 0.05, "*", "")) %>%
  knitr::kable()
```

cluster_id	marker_id	p_val	p_adj	significance
CD4+_Tcell	ho_1	0.0000000	0.0000040	*
CD8+_Tcell	sma	0.0000000	0.0000040	*
CD4+_Tcell	calprotectin	0.0000000	0.0000053	*
CD8+_Tcell	ido	0.0000007	0.0001413	*
CD8+_Tcell	cd45ro	0.0000088	0.0014110	*
Neutrophil	cd31	0.0000346	0.0046129	*
CD8+_Tcell	cd14	0.0000745	0.0085159	*
CD8+_Tcell	cd31	0.0001685	0.0149745	*
Treg	calprotectin	0.0001672	0.0149745	*
Neutrophil	hla_dr	0.0003534	0.0235578	*
CD8+_Tcell	pan_ck	0.0003145	0.0235578	*
CD8+_Tcell	vim	0.0003497	0.0235578	*
Neutrophil	hh3	0.0006470	0.0398161	*
CD8+_Tcell	cd4	0.0013638	0.0727353	
CD4+_Tcell	cd45ro	0.0012871	0.0727353	
CD8+_Tcell	hh3	0.0016627	0.0831329	
CD8+_Tcell	calprotectin	0.0026299	0.1237621	
Treg	cd163	0.0043787	0.1821522	
Neutrophil	cd45ro	0.0047842	0.1821522	
Mast_cell	calprotectin	0.0050092	0.1821522	
CD11c+_mDC	hla_dr	0.0047077	0.1821522	
iNOS+_Mac	vim	0.0046123	0.1821522	
CD209+_Mac	hla_dr	0.0052589	0.1829181	
CD11c+_mDC	h3k27me3	0.0060519	0.2017299	

cluster_id	marker_id	p_val	p_adj	significance
M2_Mac	cd31	0.0069729	0.2147075	

SJIA-PAP vs. nonSJIA-PAP

```
dea_sjia_pap_vs_nonsjia_pap <-
  mibi_data %>%
  select(fov_id, cluster_name, outcome, patient_id, any_of(functional_markers)) %>%
  #filter(!(fov_id %in% healthy_fovs_in_pap_patients)) %>%
  filter(outcome != "Control") %>%
  pap_perform_dea(
    data_tibble = .,
    sample_col = fov_id,
    cluster_col = cluster_name,
    fixed_effect_cols = outcome,
    random_effect_col = c(patient_id),
    min_cells = 20,
    min_samples = 10
  )

dea_sjia_pap_vs_nonsjia_pap$de_results %>%
  topTable(top_n = 25) %>%
  as_tibble() %>%
  arrange(p_adj) %>%
  mutate(significance = if_else(p_adj < 0.05, "*", "")) %>%
  knitr::kable()
```

cluster_id	marker_id	p_val	p_adj	significance
Mesenchymal	lag3	0.0000161	0.0090116	*
iNOS+_Pneumocyte	lag3	0.0001722	0.0482035	*
Mesenchymal	vim	0.0003095	0.0577704	
CD8+_Tcell	cd123	0.0010664	0.0996289	
CD8+_Tcell	lag3	0.0008544	0.0996289	
iNOS+_Pneumocyte	vim	0.0010675	0.0996289	
Endothelial	cd206	0.0020487	0.1409582	
CD4+_Tcell	calprotectin	0.0018725	0.1409582	
Endothelial	ido	0.0025171	0.1409582	
Mast_cell	vim	0.0024047	0.1409582	
CD4+_Tcell	cd123	0.0046176	0.1879792	
M2_Mac	cd123	0.0039847	0.1879792	
Mast_cell	lag3	0.0046995	0.1879792	
Neutrophil	lag3	0.0040967	0.1879792	
CD8+_Tcell	hla_dr	0.0059429	0.2218684	
Mast_cell	cd123	0.0068880	0.2410811	
Fibroblast	lag3	0.0074621	0.2458120	
CD14+_Mono	cd123	0.0147420	0.2948396	
Mesenchymal	cd123	0.0118887	0.2948396	
CD8+_Tcell	cd45ro	0.0141040	0.2948396	
Endothelial	cd45ro	0.0136826	0.2948396	
iNOS+_Pneumocyte	hh3	0.0120917	0.2948396	
Mast_cell	hh3	0.0146146	0.2948396	
Mesenchymal	hh3	0.0123219	0.2948396	

cluster_id	marker_id	p_val	p_adj	significance
M2_Mac	lag3	0.0110886	0.2948396	

nonSJIA-PAP vs. Controls

```
dea_control_vs_nonsjia_pap <-
  mibi_data %>%
  select(fov_id, cluster_name, outcome, patient_id, any_of(functional_markers)) %>%
  #filter(!(fov_id %in% healthy_fovs_in_pap_patients)) %>%
  filter(outcome != "SJIA-PAP") %>%
  pap_perform_dea(
    data_tibble = .,
    sample_col = fov_id,
    cluster_col = cluster_name,
    fixed_effect_cols = outcome,
    random_effect_col = c(patient_id),
    min_cells = 20,
    min_samples = 10
  )

dea_control_vs_nonsjia_pap$de_results %>%
  topTable(top_n = 25) %>%
  as_tibble() %>%
  arrange(p_adj) %>%
  mutate(significance = if_else(p_adj < 0.05, "*", "")) %>%
  knitr::kable()
```

cluster_id	marker_id	p_val	p_adj	significance
Neutrophil	cd14	0.0000717	0.0177454	*
CD8+_Tcell	cd45	0.0002662	0.0177454	*
M2_Mac	cd45	0.0001778	0.0177454	*
M2_Mac	cd68	0.0001100	0.0177454	*
CD4+_Tcell	hh3	0.0002257	0.0177454	*
CD4+_Tcell	vim	0.0001781	0.0177454	*
Neutrophil	cd163	0.0006408	0.0366184	*
M2_Mac	cd163	0.0010146	0.0474647	*
Mast_cell	tryptase	0.0010680	0.0474647	*
CD4+_Tcell	cd45	0.0011993	0.0479737	*
CD8+_Tcell	cd8	0.0016696	0.0513734	
M2_Mac	calprotectin	0.0016307	0.0513734	
M2_Mac	na_kat_pase	0.0014304	0.0513734	
Mast_cell	hh3	0.0021202	0.0605763	
CD8+_Tcell	cd45ro	0.0030115	0.0803060	
M2_Mac	cd11c	0.0037483	0.0881944	
CD4+_Tcell	lag3	0.0036630	0.0881944	
Neutrophil	cd45ro	0.0040223	0.0893840	
Mast_cell	ido	0.0054926	0.1156347	
M2_Mac	ho_1	0.0069143	0.1279904	
Mesenchymal	na_kat_pase	0.0068203	0.1279904	
CD4+_Tcell	pd1	0.0070395	0.1279904	
CD4+_Tcell	cd123	0.0075248	0.1292888	
M2_Mac	vim	0.0080806	0.1292888	

cluster_id	marker_id	p_val	p_adj	significance
Neutrophil	vim	0.0077610	0.1292888	

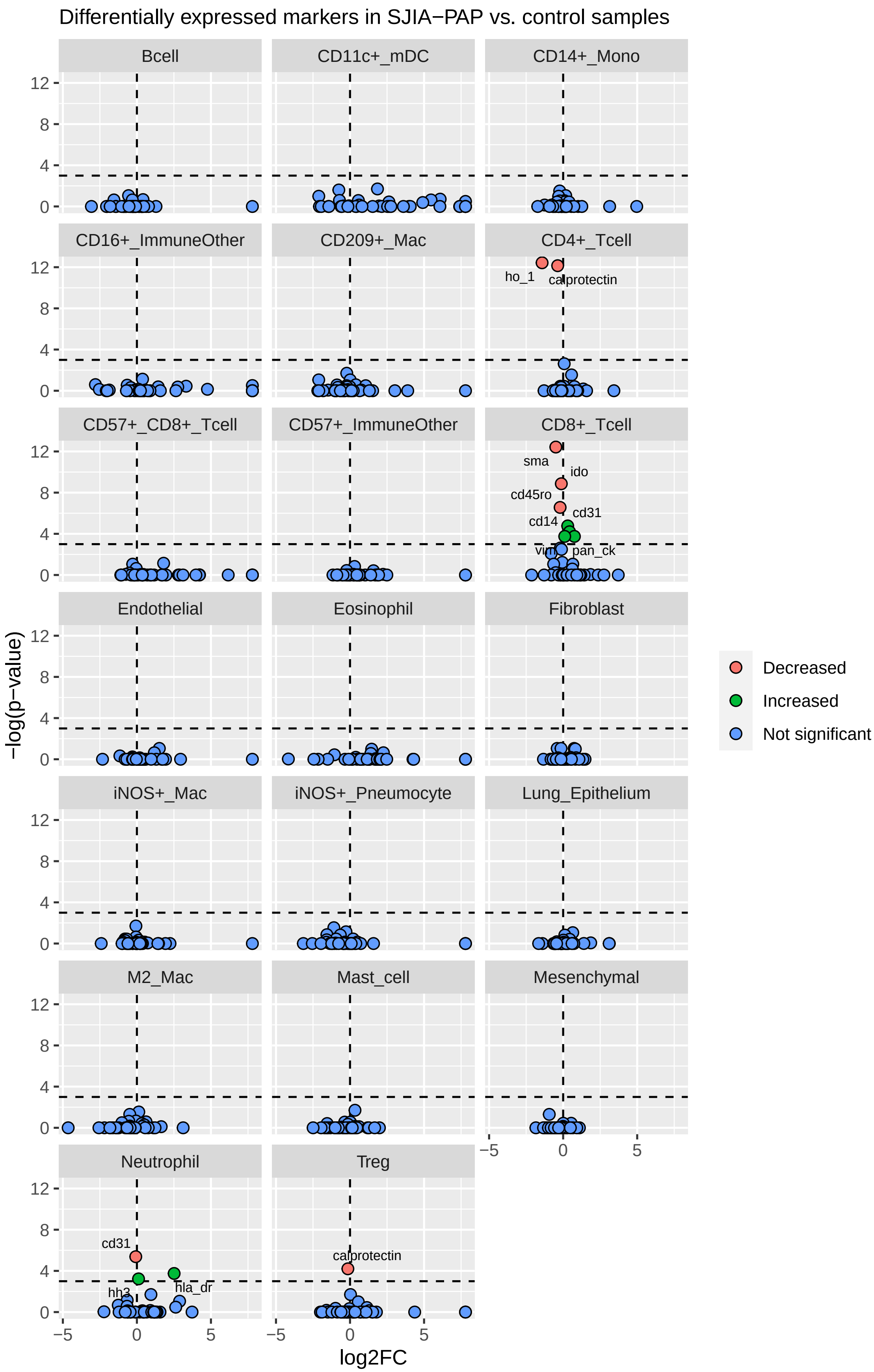
Visualization

```
dea_sjia_pap_vs_control_results <-
  dea_sjia_pap_vs_controls$de_results %>%
  topTable(all = TRUE) %>%
  as_tibble() %>%
  arrange(p_adj) %>%
  mutate(significance = if_else(p_adj < 0.05, "*", ""))  %>%
  rename(cluster_name = cluster_id)

# number of significantly different markers in each cluster
dea_sjia_pap_vs_control_results %>%
  filter(significance == "*") %>%
  count(cluster_name)
```

```
## # A tibble: 4 x 2
##   cluster_name     n
##   <fct>        <int>
## 1 CD4+_Tcell       2
## 2 CD8+_Tcell       7
## 3 Neutrophil       3
## 4 Treg             1
```

```
feature_volcano_tibble <-
  mibi_data %>%
  filter(outcome != "nonSJIA-PAP") %>%
  group_by(cluster_name, outcome, patient_id) %>%
  summarize(across(any_of(functional_markers), .f = mean, na.rm = TRUE)) %>%
  ungroup() %>%
  pivot_longer(
    cols = any_of(functional_markers),
    names_to = "marker_id",
    values_to = "median"
  ) %>%
  group_by(outcome, marker_id, cluster_name) %>%
  summarize(median = mean(median, na.rm = TRUE)) %>%
  ungroup() %>%
  pivot_wider(
    names_from = outcome,
    values_from = median
  ) %>%
  mutate(
    fc = `SJIA-PAP` / Control,
    log2_fc = log(fc, base = 2)
  ) %>%
  #filter(!is.nan(fc)) %>%
  left_join(
    dea_sjia_pap_vs_control_results %>%
      drop_na()
  ) %>%
```

```r
  arrange(p_adj) %>%
  mutate(
    neg_log_p_val = -log(p_adj),
    feature_type =
      case_when(
        p_adj > 0.05 ~ "Not significant",
        fc < 1       ~ "Decreased",
        fc > 1       ~ "Increased"
      ),
    feature = str_c(marker_id, cluster_name, sep = "@"),
  ) %>%
  drop_na(feature_type, significance)

feature_volcano_tibble %>%
  ggplot(aes(x = log2_fc, y = neg_log_p_val, fill = feature_type)) +
  geom_hline(yintercept = -log(0.05), color = "black", linetype = "dashed") +
  geom_vline(xintercept = 0, color = "black", linetype = "dashed") +
  geom_point(shape = 21, size = 2.5) +
  ggrepel::geom_text_repel(
    aes(label = marker_id),
    data = filter(feature_volcano_tibble, feature_type != "Not significant"),
    size = 2.5,
    color = "black"
  ) +
  scale_x_continuous(oob = scales::oob_squish_infinite) +
  scale_y_continuous(oob = scales::oob_squish_infinite) +
  facet_wrap(facets = vars(cluster_name), ncol = 3) +
  labs(
    subtitle = "Differentially expressed markers in SJIA-PAP vs. control samples",
    x = "log2FC",
    y = "-log(p-value)",
    fill = NULL
  )
```



Differentially expressed markers in SJIA−PAP vs. control samples
Bcell
CD11c+_mDC
CD14+_Mono
CD16+_ImmuneOther
CD209+_Mac
CD4+_Tcell
ho_1
calprotectin
CD57+_CD8+_Tcell
CD57+_ImmuneOther
CD8+_Tcell
sma
ido
cd45ro
cd31
cd14
vim
pan_ck
Endothelial
Eosinophil
Fibroblast
iNOS+_Mac
iNOS+_Pneumocyte
Lung_Epithelium
M2_Mac
Mast_cell
Mesenchymal
Neutrophil
cd31
hh3
hla_dr
Treg
calprotectin
−log(p−value)
log2FC
Decreased
Increased
Not significant

Differential Expression Analysis - within patients

```
paired_dea_results <-
  paired_patients %>%
  filter(outcome != "nonSJIA-PAP") %>%
  select(
    fov_id,
    cluster_name,
    fov_condition,
    patient_id,
    any_of(functional_markers)
  ) %>%
  pap_perform_dea(
    data_tibble = .,
    sample_col = fov_id,
    cluster_col = cluster_name,
    fixed_effect_cols = fov_condition,
    random_effect_col = c(patient_id),
    min_cells = 5,
    min_samples = 5
  )

paired_dea_results %>%
  pluck("de_results") %>%
  topTable(top_n = 25) %>%
  as_tibble() %>%
  mutate(significant = if_else(p_adj < 0.10, "*", "")) %>%
  arrange(p_adj) %>%
  knitr::kable()
```

cluster_id	marker_id	p_val	p_adj	significant
Eosinophil	cd31	0.0000922	0.0313411	*
CD209+_Mac	ho_1	0.0000870	0.0313411	*
Eosinophil	hh3	0.0002986	0.0676921	*
Eosinophil	vim	0.0008043	0.1367321	
Eosinophil	pan_ck	0.0010167	0.1382757	
CD14+_Mono	hh3	0.0024189	0.2741378	
Mesenchymal	cd45ro	0.0031148	0.3025807	
iNOS+_Pneumocyte	cd45ro	0.0044107	0.3749120	
M2_Mac	cd45ro	0.0052723	0.3983509	
Bcell	cd31	0.0089437	0.4933762	
Eosinophil	cd45ro	0.0089218	0.4933762	
Mast_cell	cd45ro	0.0074826	0.4933762	
CD8+_Tcell	hh3	0.0094322	0.4933762	
CD4+_Tcell	cd45ro	0.0132830	0.6021623	
Neutrophil	cd45ro	0.0132431	0.6021623	
CD8+_Tcell	cd45ro	0.0173989	0.6262957	
Treg	hh3	0.0163440	0.6262957	
Neutrophil	lag3	0.0153198	0.6262957	
Endothelial	pan_ck	0.0184205	0.6262957	
iNOS+_Mac	pan_ck	0.0179964	0.6262957	
CD14+_Mono	sma	0.0211224	0.6839636	
Neutrophil	cd123	0.0315969	0.7036612	

cluster_id	marker_id	p_val	p_adj	significant
CD209+_Mac	cd14	0.0254273	0.7036612	
CD14+_Mono	cd31	0.0295660	0.7036612	
CD4+_Tcell	cd31	0.0320787	0.7036612	

Spatial Analysis

[surfactant analysis]