Jazz differential discovery analysis

Setup

```
# load utility functions
source(here::here("scripts", "setup", "aml_utils.R")) #may need to change on scg machines

# Libraries
libraries <-
  c(
    "flowCore",
    "diffcyt",
    "SummarizedExperiment",
    "tidyverse",
    "readxl",
    "ggridges",
    "rlang",
    "ggthemes",
    "DataExplorer",
    "ComplexHeatmap",
    "foreach",
    "doParallel",
    "ggrepel",
    "ggiraph",
    "ggiraphExtra",
    "FlowSOM",
    "tidytof",
    "tidymodels",
    "ggforce",
    "lme4",
    "Rtsne",
    "umap",
    "broomExtra",
    "patchwork"
  )

call_libraries(libraries)


# Parameters
set_global_variables(locale = "galaxia")
is_sampled <- TRUE

# Paths
jazz_path <- file.path("~", "Box", "Tim", "Lab", "Data", "jazz_data")
healthy_path <- here::here("data-raw", "healthy_myeloid")
```

```r
source('~/GitHub/aml-cells/scripts/feature_extraction/feature_extraction_utils.R')


# Misc. setup
marker_setup()
patient_setup()

CLASSIFIER_MARKERS <-
  CLASSIFIER_MARKERS %>%
  str_to_lower() %>%
  str_remove(pattern = "-")

correction_factor <- 0.6
```

Reading in data

First, we read in the data - for this experiment, we have CyTOF data collected from several primary patient AML samples that have been treated for 0, 24, or 72 hours with Jazz Pharmaceutical's new FDA-approved drug Vyxeos (daunorubicin and cytarabine).

We also read in some healthy reference data from a different set of experiments in the Davis Lab (with a slightly different myeloid panel). There are a variety of analyses that these healthy data can help us with, like using the developmental classifier published here.

To help with reading in the data, I'll write a simple function that can rename the columns in both data sets into something more human-readable (because the names right off of the machine are sometimes a bit messy).

```
tof_names <- function(my_names) {
  my_names %>%
    str_remove(pattern = "-") %>%
    str_to_lower() %>%
    str_c("_", .) %>%
    str_extract(pattern = "_[:alnum:]*$") %>%
    str_remove(pattern = "_")
}
```

Jazz data

First we read in the data collected from cancer samples treated with Vyxeos.

```
jazz_data <-
  jazz_path %>%
  tof_read_fcs() %>%
  # This is the line that I've added based on the panel analysis below!
  mutate(`162Dy_CD38` = correction_factor * `162Dy_CD38`) %>%
  tof_preprocess()

# store information about the panel used for the jazz data
jazz_panel <-
  tibble(
    raw_names = colnames(jazz_data),
    metal =
      str_extract(raw_names, "[:alnum:]+_?") %>%
      str_extract("[:digit:]+"),
    jazz_marker =
      str_extract(raw_names, "_[:alnum:]+") %>%
      str_remove("_")
  ) %>%
  select(-raw_names) %>%
  drop_na(metal)

# read in jazz data
jazz_data <-
  jazz_data %>%
  rename_with(.fn = tof_names) %>%
  select(-contains("bc")) %>%
  mutate(
    patient =
      str_extract(string = name, pattern = "_[:digit:]+") %>%
      str_remove("_") %>%
```

```
      replace_na("healthy"),
    timepoint =
      str_extract(string = name, pattern = "[:digit:]+hr") %>%
      replace_na("healthy")
  )
```

Healthy data

Next, we read in the data from healthy myeloid samples.

```
healthy_data <-
  healthy_path %>%
  tof_read_fcs() %>%
  tof_preprocess()

# store information about the panel used for the jazz data
healthy_panel <-
  tibble(
    raw_names = colnames(healthy_data),
    metal =
      str_extract(raw_names, "[:alnum:]+_?") %>%
      str_extract("[:digit:]+"),
    healthy_marker =
      str_extract(raw_names, "_[:alnum:]+") %>%
      str_remove("_")
  ) %>%
  select(-raw_names) %>%
  drop_na(metal)

# finish preprocessing healthy data
healthy_data <-
  healthy_data %>%
  select(-contains("Pd"), -contains("157gd")) %>%
  rename_with(.fn = tof_names) %>%
  mutate(
    name =
      name %>%
      str_extract("[:alpha:]+.fcs$") %>%
      str_remove(".fcs")
  ) %>%
  rename(cell_type = name)
```

What channels to the healthy data and the jazz data have in common?

When analyzing data collected from more than one CyTOF run, it's important to compare the panels used to collect both data sets. Specifically, you should check the following:

1. If the same markers are present in both/all panels
2. If the same metals were paired with the same markers in both/all panels.

We look into the first issue here:

```
healthy_data %>%
  colnames() %>%
  setdiff(colnames(jazz_data))
```

```
##  [1] "127i"      "ccr2"      "cd64"      "cebpa"     "cd10"      "gata1"
##  [7] "cd41"      "perk"      "cd109"     "191ir"     "193ir"     "cisplatin"
## [13] "cell_type"
```

```
jazz_data %>%
  colnames() %>%
  setdiff(colnames(healthy_data))
```

```
##  [1] "idu"        "cll1"       "prb"        "cyclinb1"   "cd32"
##  [6] "pu1"        "pdl1"       "ph2ax"      "phh3"       "dna1"
## [11] "dna2"       "viability"  "omiqfilter" "name"       "patient"
## [16] "timepoint"
```

And we can see that there are a few markers that are present in the healthy data panel that aren't present in the jazz panel and vice-versa.

Do both data sets contain the columns we need to perform the classification?

In this analysis specifically, we're interested using the developmental classifier, which uses the markers that I've saved in the global variable `CLASSIFIER_MARKERS`. We can check that both the healthy and Jazz data panels have all of these markers:

```
all(CLASSIFIER_MARKERS %in% colnames(healthy_data))
```

```
## [1] TRUE
```

```
all(CLASSIFIER_MARKERS %in% colnames(jazz_data))
```

```
## [1] FALSE
```

```
CLASSIFIER_MARKERS[!(CLASSIFIER_MARKERS %in% colnames(jazz_data))]
```

```
## [1] "cd41"
```

It looks like most of the markers are present in both panels, except the Jazz data doesn't have `CD41`. We can simply omit this marker from the classification and proceed as normal.

```
CLASSIFIER_MARKERS <- CLASSIFIER_MARKERS[CLASSIFIER_MARKERS != "cd41"]
```

We should also check that the classifier markers in both panels are paired with the same metal. If they aren't, we will have to correct for the differential sensitivity of the different metals in the CyTOF's detector..

```
panel_tibble <-
  healthy_panel %>%
  full_join(jazz_panel) %>%
  mutate(across(everything(), str_to_lower)) %>%
  filter(
    (healthy_marker %in% CLASSIFIER_MARKERS) |
      (jazz_marker %in% CLASSIFIER_MARKERS)
  )

panel_tibble %>%
  knitr::kable()
```

metal	healthy_marker	jazz_marker
89	cd45	cd45
113	cd61	cd61
139	cd45ra	cd45ra
146	cd90	cd90

metal	healthy_marker	jazz_marker
147	cd38	cyclinb1
148	cd34	cd34
151	cd11c	cd11c
152	cd13	cd13
160	cd14	cd14
162	gata	cd38
170	cd135	cd135
174	hla	hladr
209	cd11b	cd11b

We can see that `CD38` is on different metals in the healthy data compared to the jazz data. This means that we can try two approaches: omitting it (and seeing what our accuracy on the healthy data is) or trying a correction factor (see this paper).

What I've done in this case is gone back to my code above and added a correction factor to the pre-processing of the Jazz data (so nothing else is needed here - see the comment in the preprocessing pipeline above).

Clustering

After reading in and pre-processing the data, we want to cluster the Jazz cells to perform differential discovery later in our analysis pipeline. Here, we will explore two clustering methods:

- Developmental classification (using mahalanobis distance)
- FlowSOM clustering

We can then interrogate both of the clustering results manually to decide which we will use throughout the remainder of our analysis.

Developmental Classification

We start by using the developmental classifier described in Good et al.

Build classifier

Because `CD38` was measured on different channels in the Jazz and healthy reference data sets, an easy way to not worry about this problem is simply to perform the classification without using `CD38` at all. So, we can fit the classifier with and without `CD38` to see if its accuracy is comparable (and if there isn't a big change in accuracy when `CD38` is removed, we can remove it without worrying).

First, we fit the developmental classifier without `CD38`.

```
classifier_markers <-
  CLASSIFIER_MARKERS[CLASSIFIER_MARKERS != "cd38"]

classifier_markers
```

```
##  [1] "cd45"   "cd34"   "cd61"   "cd14"   "cd135"  "cd45ra" "cd90"   "hladr"
##  [9] "cd13"   "cd11b"  "cd11c"
```

```
classifier_fit_no_cd38 <-
  tidytof:::tof_classifier_build(
  tof_tibble = healthy_data,
  population_vector = healthy_data$cell_type,
  classifier_markers = classifier_markers
)
```

Then we fit it with `CD38`.

```
classifier_fit <-
  tidytof:::tof_classifier_build(
    tof_tibble = healthy_data,
    population_vector = healthy_data$cell_type,
    classifier_markers = CLASSIFIER_MARKERS
  )
```

Apply classifier

After fitting these two classifiers, we can then apply them to the healthy data and compare their accuracies (relative to the gold standard of manual gating).

First, we apply the classifier fit without `CD38`.

```
classifier_healthy_results_no_cd38 <-
  tidytof:::tof_classifier_apply(
    tof_tibble = healthy_data,
    classifier_fit = classifier_fit_no_cd38,
```

```
    num_cores = 6,
    parallel_var = cell_type
  )
```

Then, we apply the classifier fit without CD38.

```
classifier_healthy_results <-
  tidytof:::tof_classifier_apply(
    tof_tibble = healthy_data,
    classifier_fit = classifier_fit,
    num_cores = 6,
    parallel_var = cell_type
  )
```

Then, we can compare accuracies on the healthy data for the classifiers that include and exclude CD38.

```
healthy_data <-
  healthy_data %>%
  mutate(
    cluster_no_cd38 = classifier_healthy_results_no_cd38$mahalanobis_cluster,
    cluster = classifier_healthy_results$mahalanobis_cluster
  ) %>%
  mutate(
    cell_type = factor(cell_type, levels = CLASSIFIER_POPULATIONS),
    cluster_no_cd38 = factor(cluster_no_cd38, levels = CLASSIFIER_POPULATIONS),
    cluster = factor(cluster, levels = CLASSIFIER_POPULATIONS)
  )

accuracy_no_cd38 <-
  healthy_data %>%
  group_by(cell_type) %>%
  accuracy(truth = cell_type, estimate = cluster_no_cd38)

accuracy_with_cd38 <-
  healthy_data %>%
  group_by(cell_type) %>%
  accuracy(truth = cell_type, estimate = cluster)
```

```
accuracy_no_cd38 %>%
  knitr::kable()
```

cell_type	.metric	.estimator	.estimate
HSC	accuracy	multiclass	0.9393842
MPP	accuracy	multiclass	0.5215730
CMP	accuracy	multiclass	0.8239872
GMP	accuracy	multiclass	0.9705393
MEP	accuracy	multiclass	0.8005520
Monocyte	accuracy	multiclass	0.8577643
DC	accuracy	multiclass	0.9453490
Macrophage	accuracy	multiclass	0.8882588
Thrombocyte	accuracy	multiclass	0.9834695

```
accuracy_with_cd38 %>%
  knitr::kable()
```

cell_type	.metric	.estimator	.estimate
HSC	accuracy	multiclass	0.9571175
MPP	accuracy	multiclass	0.8666981
CMP	accuracy	multiclass	0.8943305
GMP	accuracy	multiclass	0.9774658
MEP	accuracy	multiclass	0.9160125
Monocyte	accuracy	multiclass	0.8577318
DC	accuracy	multiclass	0.9566391
Macrophage	accuracy	multiclass	0.9178655
Thrombocyte	accuracy	multiclass	0.9835667

We can see that including CD38 is actually pretty important for the accuracy of the MPP cell type in particular. So we should move forward with the version that includes `CD38`.

```
jazz_classifier_results <-
  tidytof:::tof_classifier_apply(
    tof_tibble = jazz_data,
    classifier_fit = classifier_fit,
    num_cores = 5,
    parallel_var = patient
  )

jazz_data <-
  jazz_data %>%
  mutate(
    cluster =
      jazz_classifier_results$mahalanobis_cluster %>%
      factor(levels = CLASSIFIER_POPULATIONS)
  )
```

After applying this classifier to the full Jazz dataset, we can see that we have the following breakdown of cells in each cluster:

```
jazz_data %>%
  count(cluster) %>%
  knitr::kable()
```

cluster	n
HSC	755
MPP	17343
CMP	28659
GMP	818255
MEP	178127
Monocyte	21051
DC	10661
Macrophage	395106
Thrombocyte	1

FlowSOM clustering

An alternative to using the developmental clustering at all is simply to use unsupervised (flowSOM) clustering directly on the Jazz dataset and use those cluster labels to identify which cells are significantly different (in cell abundance or cell state) across treatment timepoints.

```
# remove healthy cells that were collected with the jazz data
jazz_healthy <-
  jazz_data %>%
  filter(timepoint == "healthy")

jazz_data <-
  jazz_data %>%
  filter(timepoint != "healthy")

# perform the flowsom clustering on the jazz samples
set.seed(2021)
flowsom_results <-
  jazz_data %>%
  flowSOM_cluster(cluster_markers = CLASSIFIER_MARKERS)

jazz_data <-
  jazz_data %>%
  mutate(
    flowsom_clusters = as.factor(flowsom_results$my_clusters),
    flowsom_metaclusters = as.factor(flowsom_results$my_metaclusters)
  )
```

And after the flowSOM cluster, we can see the following breakdown between the metaclusters:

```
jazz_data %>%
  count(flowsom_metaclusters) %>%
  knitr::kable()
```

flowsom_metaclusters	n
1	232995
2	425210
3	326170
4	37855
5	125923
6	77211
7	70417
8	98946
9	5942

One nice thing about the flowSOM metaclustering is that, while the number of clusters is similar to the developmental clustering, the cells are spread more evenly across them (which makes comparing between clusters, patients, and samples a bit easier).

Exploratory Data Analysis

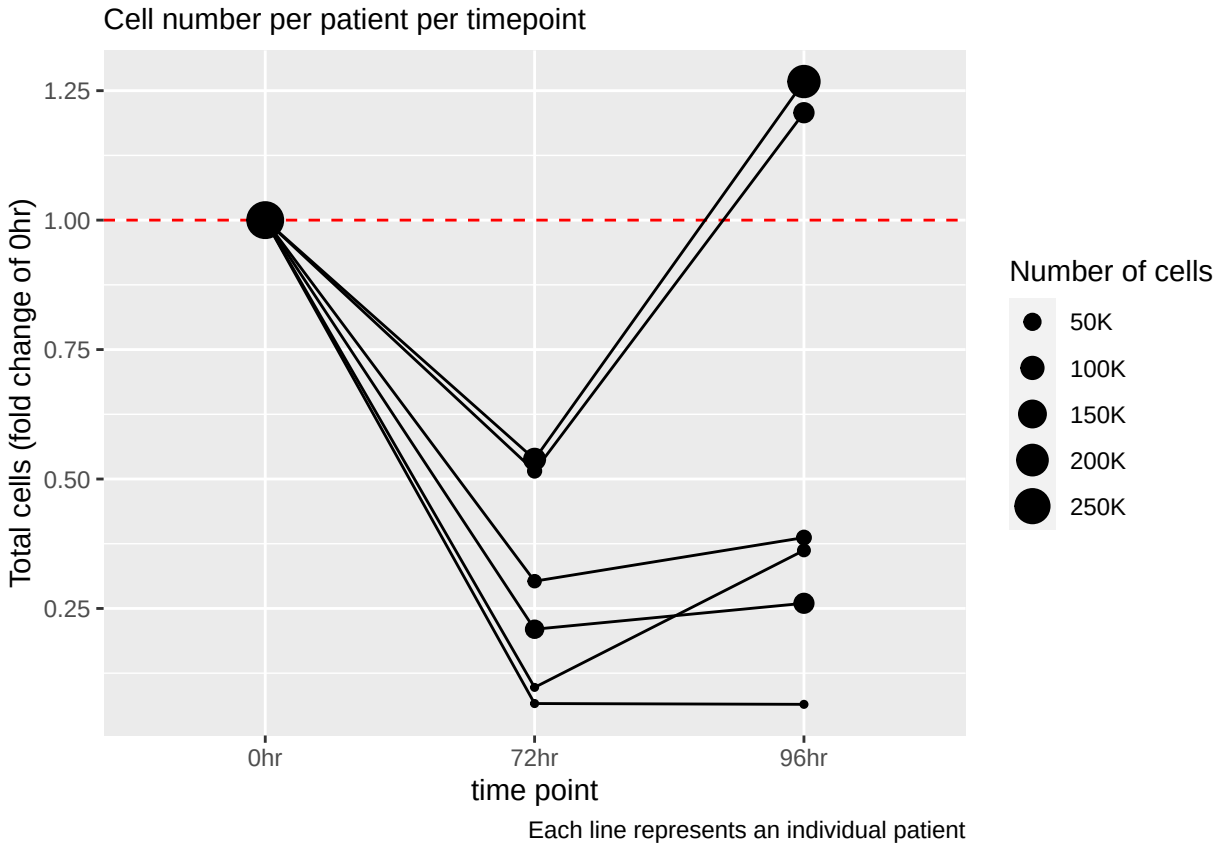
Now that we have some clustering results, we can do some exploratory data analysis and visualization about what the clusters look like. Specifically, we can answer the following questions:

1. Did cells die over the course of Vyxeos treatment?
2. Which cell types look like they died the most over the course of Vyxeos treatment?
3. What markers seem to be associated with cell types that expanded (as opposed to contracted) over the course of Vyxeos treatment?

We explore each of these questions one at a time below.

1. Did cells die over the course of Vyxeos treatment?

```
all_props <-
  jazz_data %>%
  filter(timepoint != "healthy", cluster != "Thrombocyte") %>%
  count(patient, timepoint)

all_first_timepoint <-
  all_props %>%
  filter(timepoint == "0hr") %>%
  select(
    baseline_count = n,
    patient
  )

all_props %>%
  left_join(all_first_timepoint) %>%
  mutate(
    raw_num = n,
    n = n / baseline_count
  ) %>%
  ggplot(aes(x = timepoint, y = n)) +
  geom_hline(yintercept = 1, color = "red", linetype = "dashed") +
  geom_line(aes(group = patient)) +
  geom_point(aes(size = raw_num)) +
  scale_size_area(
    breaks = seq(0, 300000, 50000),
    labels = scales::label_number(scale = 1/1000, suffix = "K")
  ) +
  labs(
    subtitle = "Cell number per patient per timepoint",
    x = "time point",
    y = "Total cells (fold change of 0hr)",
    size = "Number of cells",
    caption = "Each line represents an individual patient"
  )
```



From this plot, we can see that in most patients, there is a 50% reduction or more in acquired cell number in the first 72 hours after treatment begins. This isn't necessarily reflective of the exact proportion of cells that died over the course of treatment (because the vials were treated in parallel, not in series) but it should give a broad sense of if the treatment worked (there should be fewer cells in vials that were exposed to the treatment than cells that weren't).

It doesn't appear that there are further decreases from the 72hr to 96hr timepoints, and in 2 patients there was actually a much larger number of cells present at 96hr relative to either of the previous time points.

I'm not that sure what to make of the increases in cells overall in some patients at the 96h timepoint, and I think there might be something going on experimentally that I don't have a lot of insight about. For the rest of this analysis, I will compare the 0hr timepoint and the 72 hour timepoint, since that seems to be when most of the cell death happens. From there, I can then compare the 72hr and 96hr timepoints to see which cells persist (or expand), since that seems to be what's happening in the final day of treatment.

(Note: Jolanda mentioned that this rebound might be due to a need to redose the drug at some point during the treatment period - and that the cells rebounding at the later timepoint could simply be because the drug is no longer present at an active concentration.)

2. Which cell types die over the course of treatment?

From here, we can start to ask ourselves not only if cells died over the course of treatment, but which cell types died (or expanded) preferentially.

First, a useful function to automate plotting.

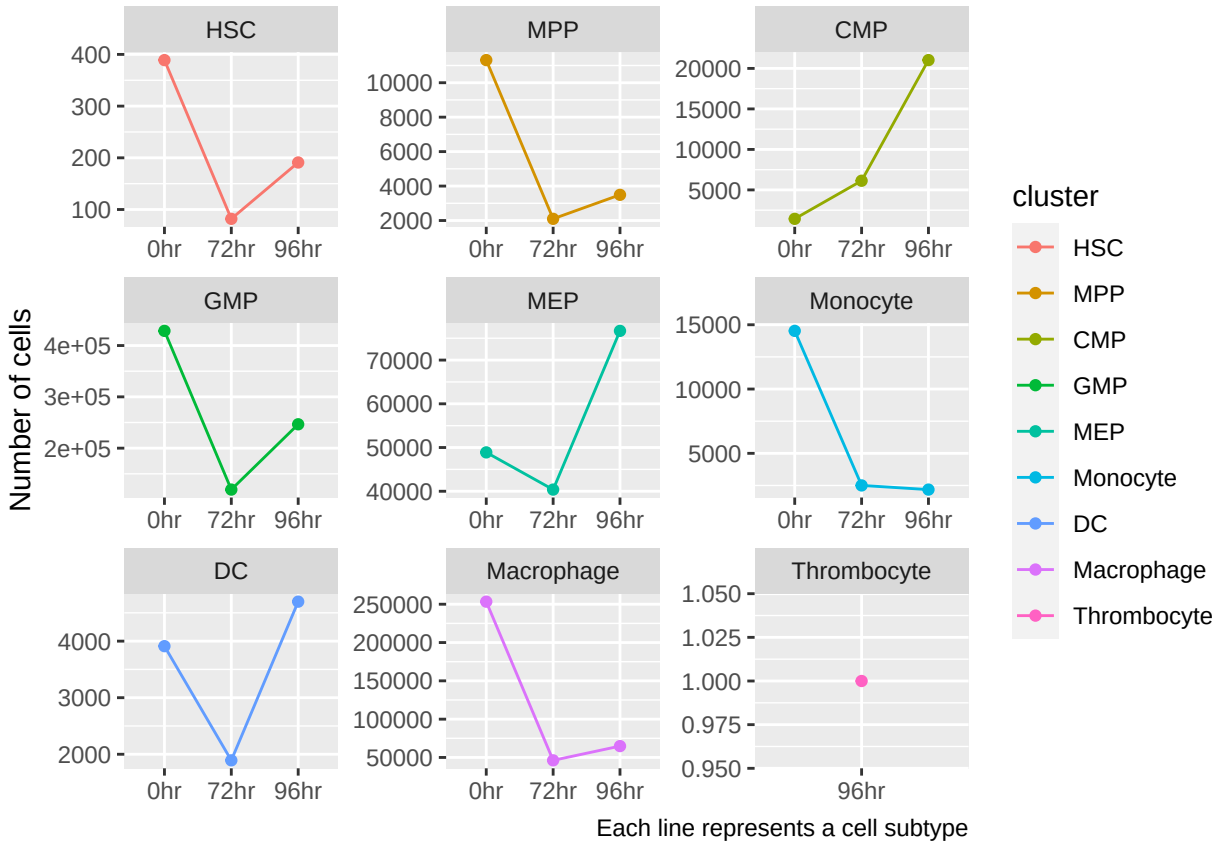
```
# a function that plots the raw counts of cells in each cluster (collected across all patients)
# under a specific clustering approach (cluster_col)
```

```
# Input: cluster_col - an unquoted name of the column in jazz_data containing
#                      the cluster IDs you want to visualize
#
# Output: A line plot

jazz_raw_cluster_plot <- function(cluster_col) {

  # save names of clusters that decrease over the treatment timeline
  clusters_that_decrease <-
    jazz_data %>%
    count(timepoint, {{cluster_col}}) %>%
    pivot_wider(
      names_from = timepoint,
      values_from = n
    ) %>%
    filter(`0hr` > `96hr`) %>%
    pull({{cluster_col}})

  clusters_that_decrease

  # raw counts
  raw_count_plot <-
    jazz_data %>%
    filter(timepoint != "healthy") %>%
    count(timepoint, {{cluster_col}}) %>%
    mutate(
      decrease = if_else({{cluster_col}} %in% clusters_that_decrease, "Decrease", "Increase")
    ) %>%
    ggplot(aes(x = timepoint, y = n, color = {{cluster_col}}, fill = {{cluster_col}})) +
    geom_line(aes(group = {{cluster_col}})) +
    geom_point() +
    labs(
      x = NULL,
      y = "Number of cells",
      caption = "Each line represents a cell subtype"
    )

  return(raw_count_plot)
}
```

Developmental classifier

Using this function, we can look at how developmental subpopulations changed over the course of treatment

```
raw_count_plot <- jazz_raw_cluster_plot(cluster_col = cluster)

raw_count_plot +
  facet_wrap(facets = vars(cluster), scales = "free")
```



Using just these plots, it's hard to draw any definitive conclusions about which cell subtypes are most affected by treatment. That being said, this eye-in-the-sky visualization (pooled across all patients) can show us broad trends. Note that the axes for each plot are different because certain subpopulations are much larger than others.

In general, there are a lot of cells in our dataset that end up getting classified into the GMP-like subtype, which makes sense based on what we know about AML blasts (and what their broad phenotype often looks like). We can also see that most of the larger cell types seem to respond to the treatment, but not necessarily equally. The most pronounced decreases in cell presence are in Monocyte-like cells and Macrophage-like cells, with HSC-like cells and MPP-like cells a close second. The most pronounced increases in cell abundance seem to be in CMP-like and MEP-like cells, with potentially DC-like and GMP-like cells also expanding at later time points. We can also see that there aren't very many Thrombocyte-like cells in the dataset in general.

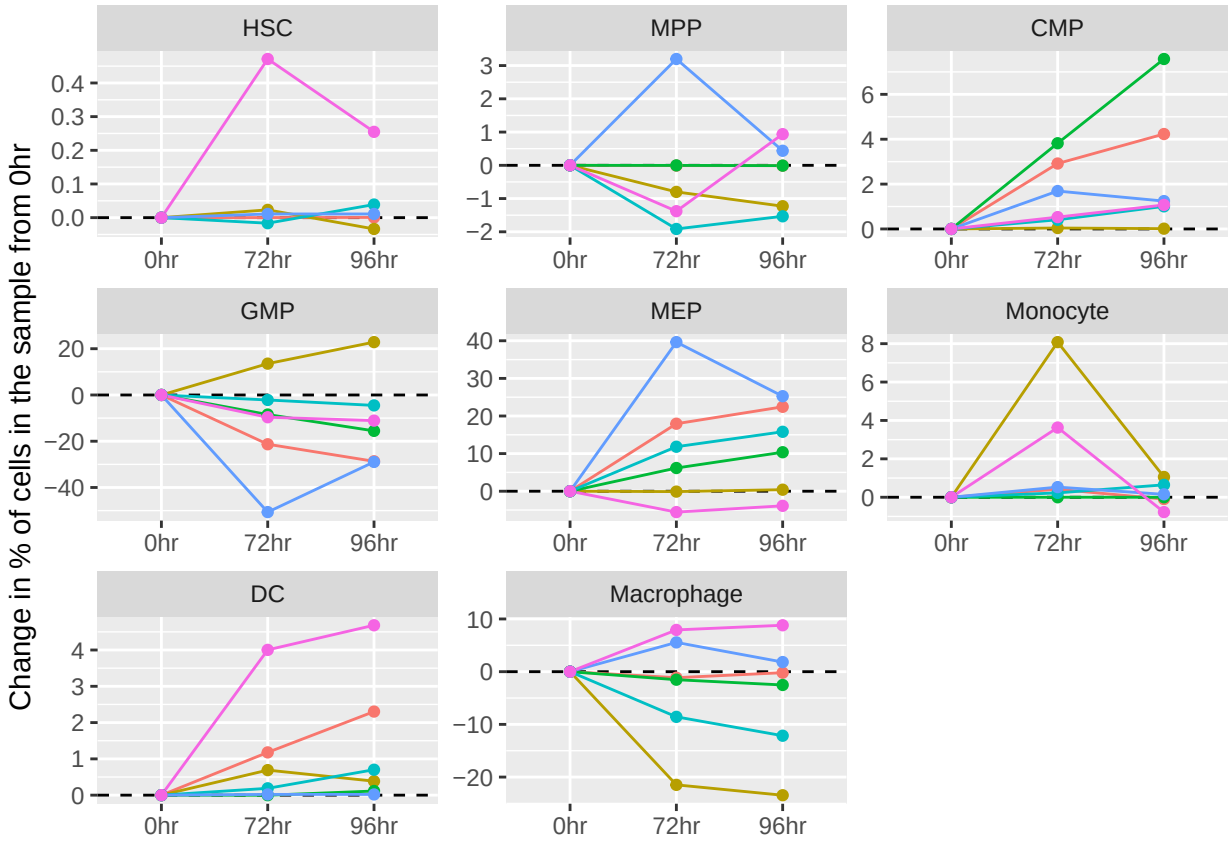
We can also look at this same pattern on a patient-specific basis.

```
# a function that plots the raw counts of cells in each cluster (collected across all patients) # under

# Input: cluster_col - an unquoted name of the column in jazz_data containing
#                      the cluster IDs you want to visualize
#
# Output: A line plot

jazz_patient_cluster_plot <- function(cluster_col, ncol, nrow) {
  props <-
    jazz_data %>%
    filter(timepoint != "healthy", {{cluster_col}} != "Thrombocyte") %>%
    count(patient, timepoint, {{cluster_col}}) %>%
    group_by(patient, timepoint) %>%
```

```
  mutate(total = sum(n)) %>%
  mutate(across(where(is.numeric), ~ 100 * .x / total)) %>%
  select(-total) %>%
  ungroup()

first_timepoint <-
  props %>%
  filter(timepoint == "0hr") %>%
  rename(baseline_count = n) %>%
  ungroup() %>%
  select(-timepoint)

mean_first_timepoints <-
  first_timepoint %>%
  group_by({{cluster_col}}) %>%
  summarize(mean_baseline = mean(baseline_count))

my_plot <-
  props %>%
  left_join(first_timepoint) %>%
  mutate(
    cluster = factor({{cluster_col}}, levels = CLASSIFIER_POPULATIONS),
    #baseline_count = replace_na(baseline_count, replace = 5),
    #n = n / baseline_count,
    n = n - baseline_count
  ) %>%
  ggplot(aes(x = timepoint, y = n, color = patient, fill = patient)) +
  geom_hline(
    yintercept = 0,
    linetype = "dashed"
  ) +
  geom_line(aes(group = str_c(patient, {{cluster_col}}))) +
  geom_point() +
  labs(
    x = NULL,
    y = "Change in % of cells in the sample from 0hr"
  ) +
  theme(legend.position = "none")

num_pages <-
  jazz_data %>%
  pull({{cluster_col}}) %>%
  unique() %>%
  length()

num_pages <- ceiling(num_pages / (ncol * nrow))

props_plot <-
  map(
    .x = 1:num_pages,
    .f = ~ my_plot +
      facet_wrap_paginate(
        facets = vars({{cluster_col}}),
```

```
          scales = "free",
          ncol = ncol,
          nrow = nrow,
          page = .x
        )
    )

  return(props_plot)

}
```

```
jazz_patient_cluster_plot(cluster, ncol = 3, nrow = 3) %>%
  walk(print)
```

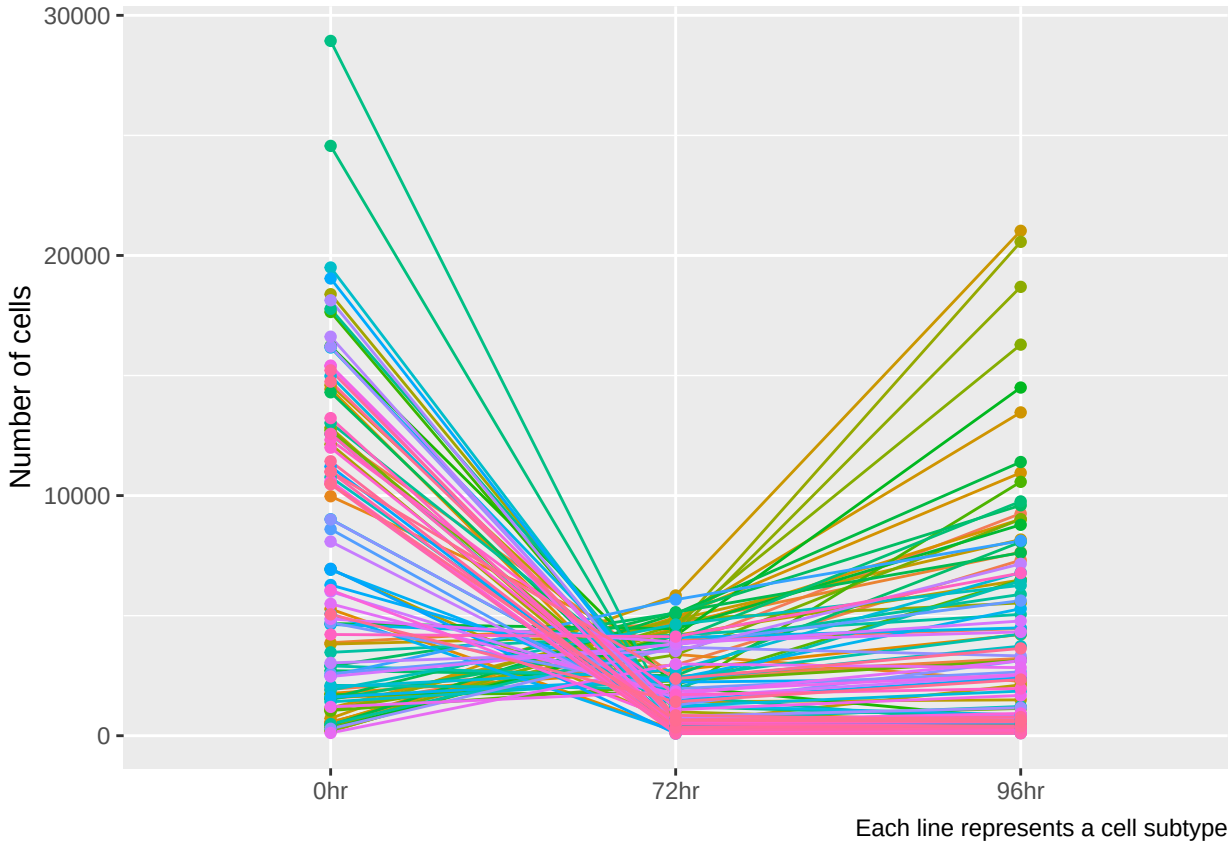


In general, we can see the same trends as observed above, with a high degree of variability between patients (especially in HSC-like cells, a very small cluster, and macrophage-like cells, a very heterogeneous cluster).

FlowSOM clustering

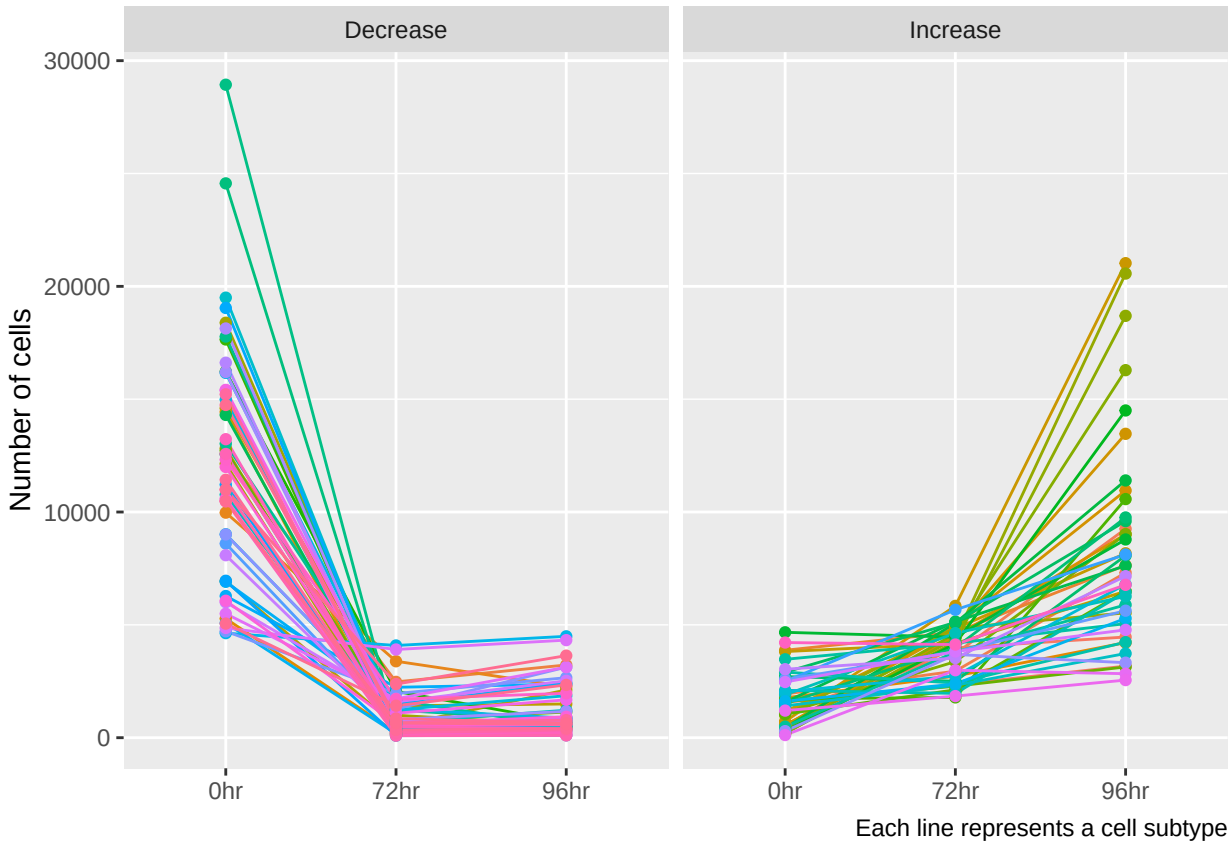
We can make the same plots as above, but using FlowSOM to identify clusters instead of developmental classification/clustering.

```
flowsom_raw_cluster_plot <-
  jazz_raw_cluster_plot(flowsom_clusters) +
  theme(legend.position = "none")

flowsom_raw_cluster_plot
```



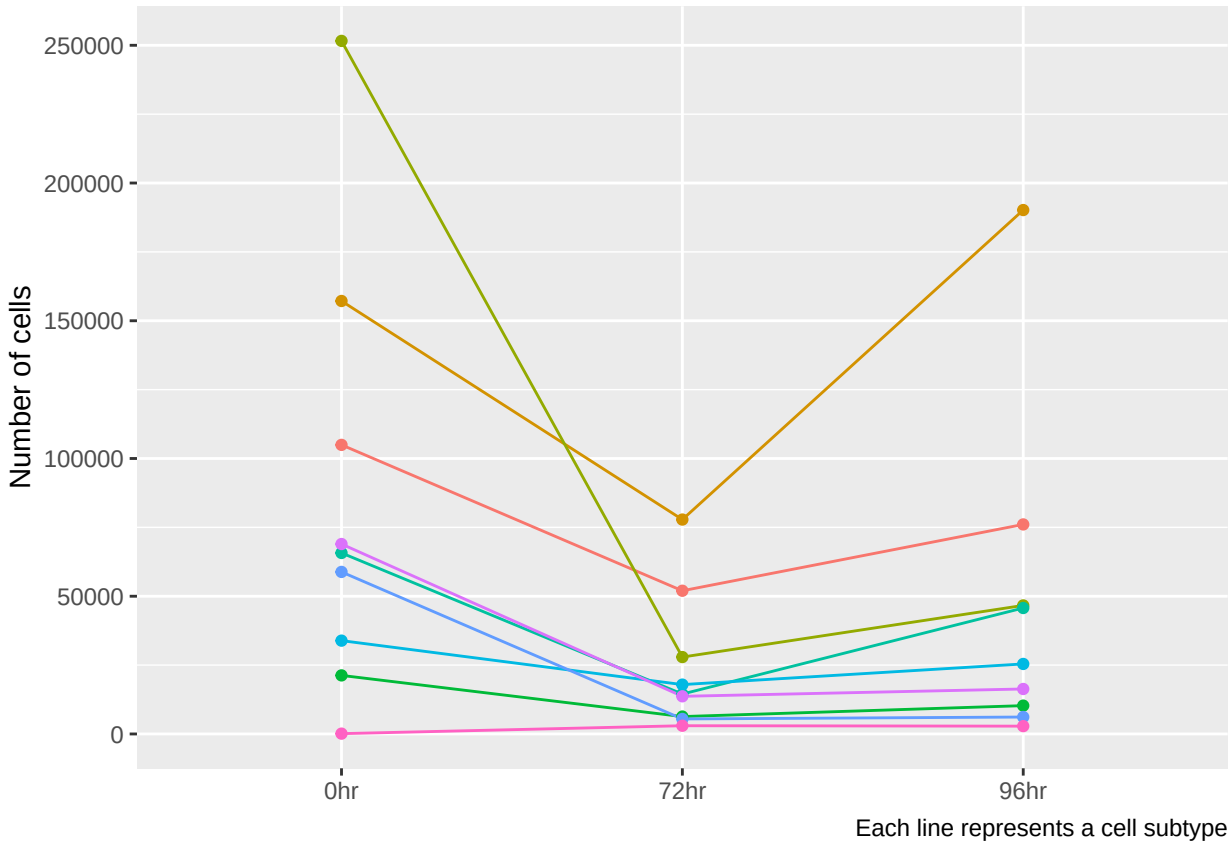
Using flowSOM clustering, we can see that there are clearly some populations that are more present before treatment that then mostly die over the course of treatment, whereas some populations are relatively small before treatment but expand over the course of treatment. We can get a slightly better look like this:

```
flowsom_raw_cluster_plot +
  facet_grid(cols = vars(decrease)) +
  labs(y = "Number of cells")
```

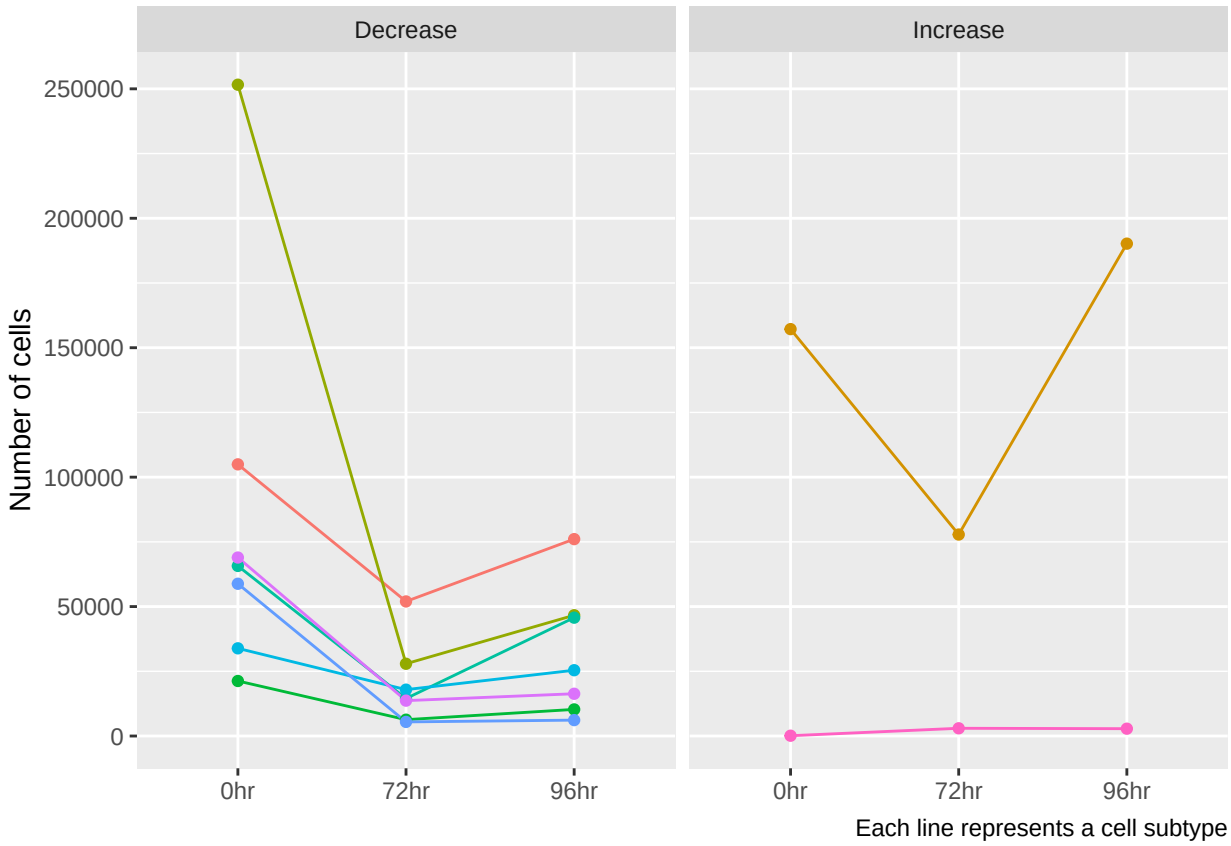


We can also look at the metaclusters that flowsom gives us to see if there's a simplified version of this story...

```
flowsom_metacluster_raw_cluster_plot <-
  jazz_raw_cluster_plot(flowsom_metaclusters) +
  theme(legend.position = "none")

flowsom_metacluster_raw_cluster_plot
```



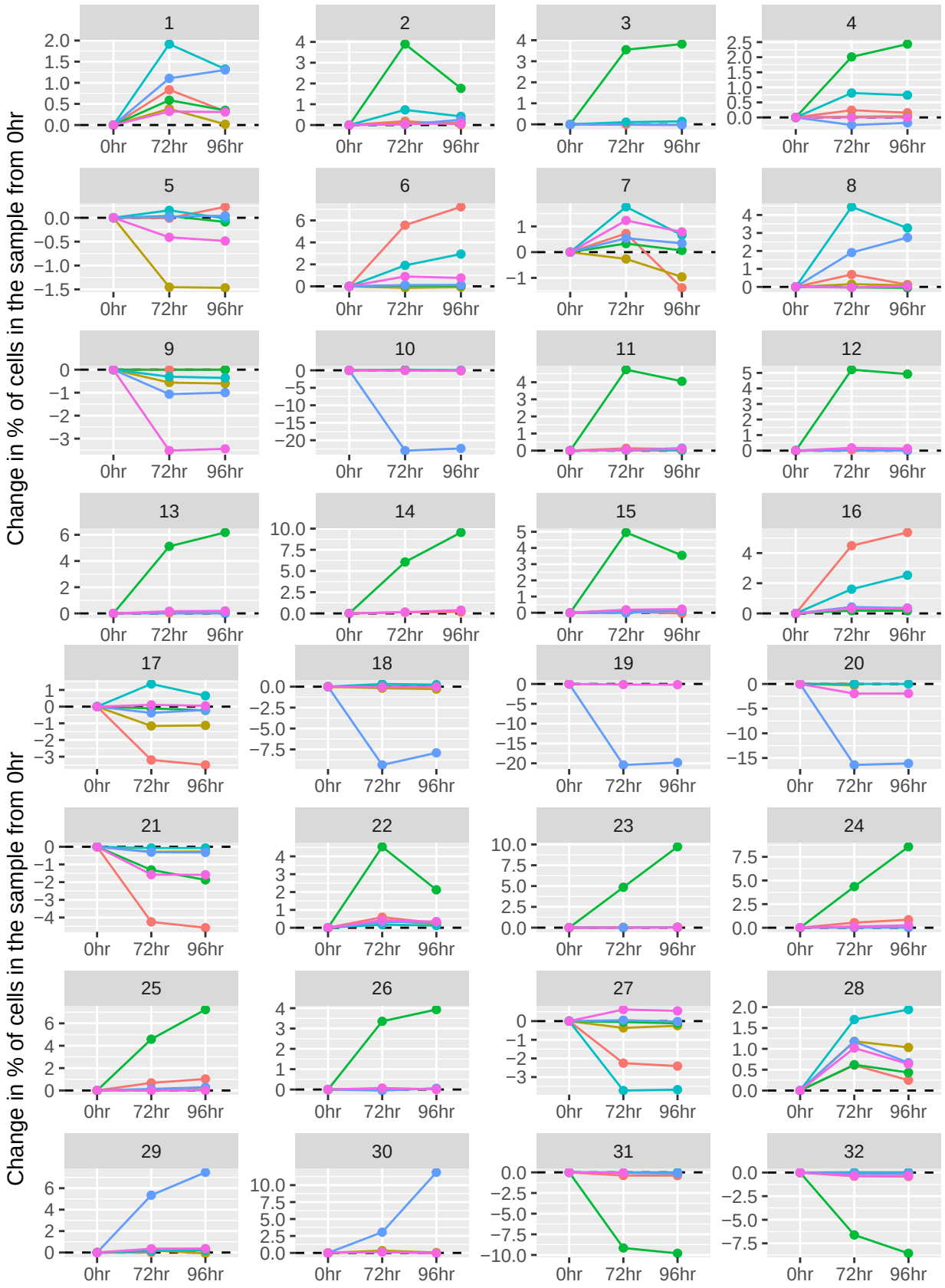

```
flowsom_metacluster_raw_cluster_plot +
  facet_grid(cols = vars(decrease)) +
  labs(y = "Number of cells")
```

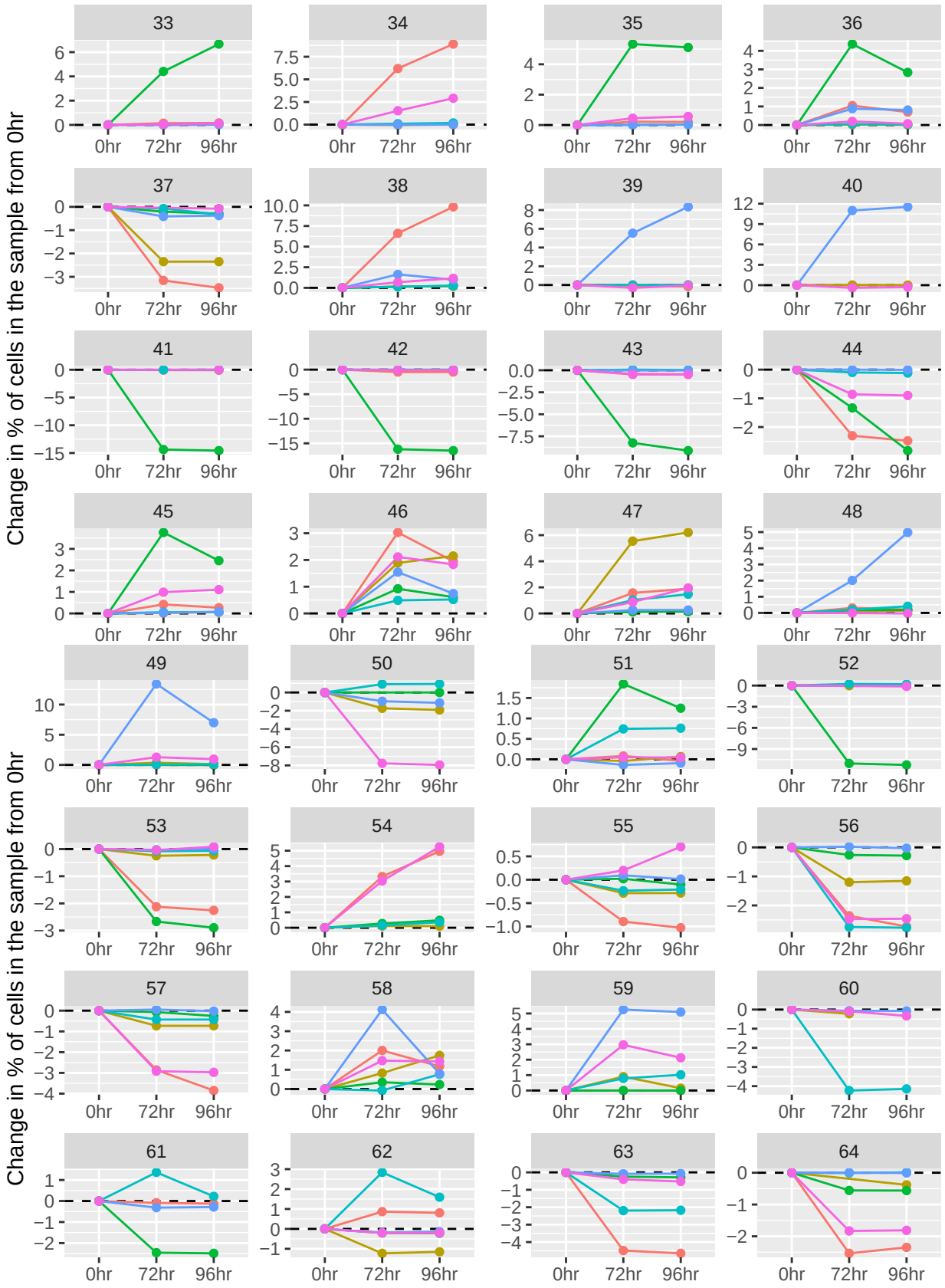


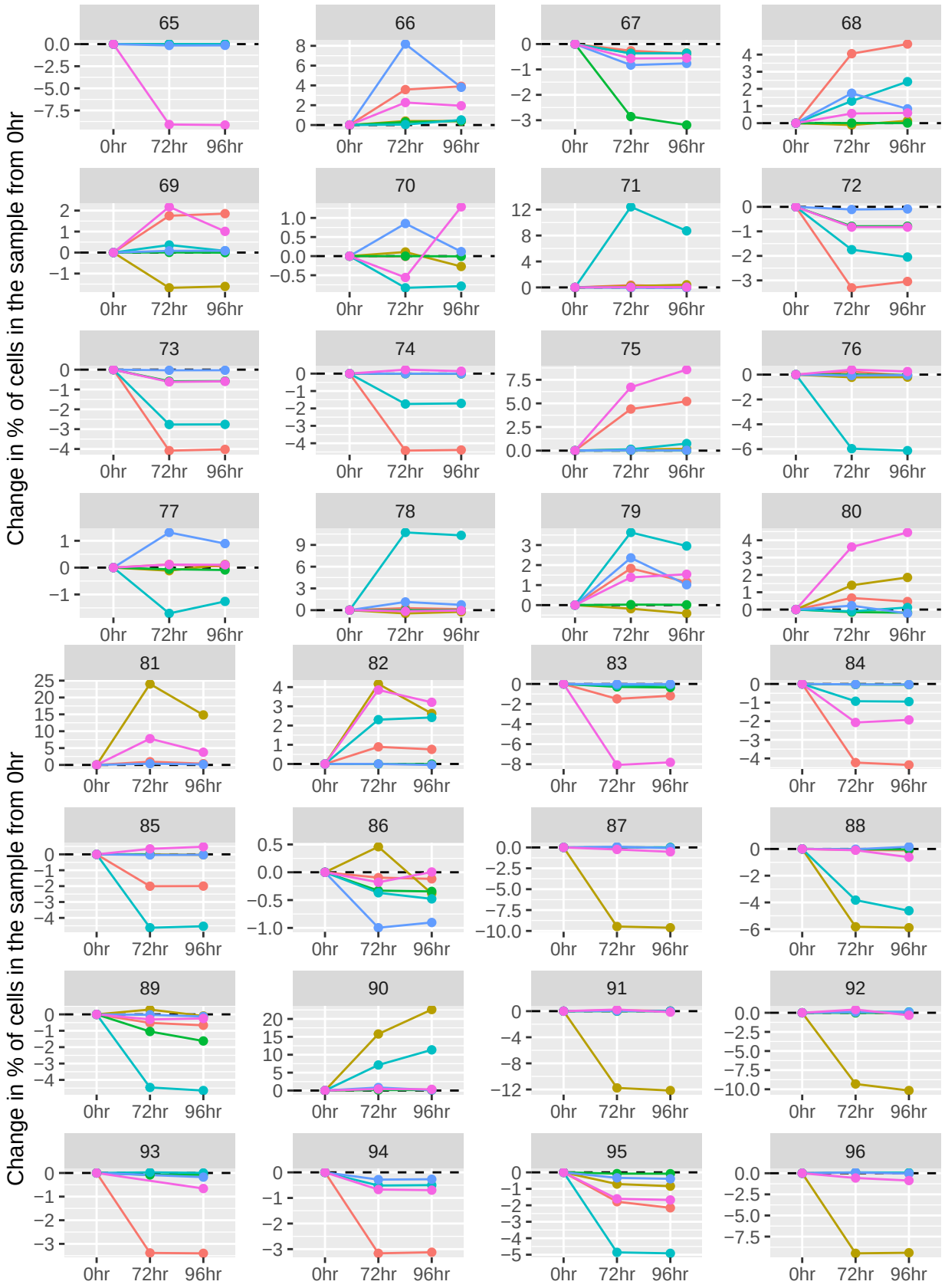
And it doesn't really seem like there's anything too interesting going on here that wasn't apparent from the individual clusters defined above (in particular, it looks like the metaclustering may give us a **worse** separation between which cells respond and don't respond to the treatment).

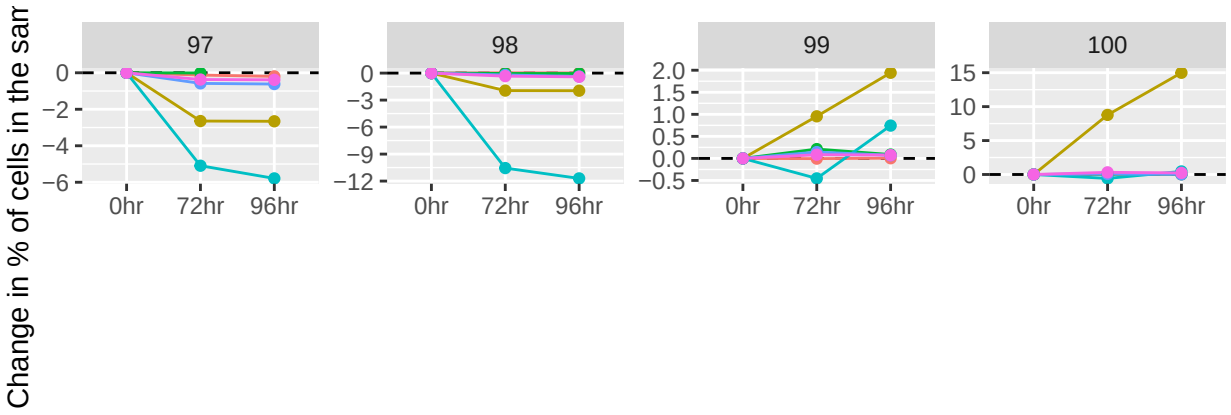
For a closer look, we can look at the patient-wise trends in each of these clusters as well...

```
jazz_patient_cluster_plot(flowsom_clusters, nrow = 4, ncol = 4) %>%
  walk(print)
```


Change in % of cells in the sample from 0hr
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
0hr
72hr
96hr


Change in % of cells in the sample from 0hr
Change in % of cells in the sample from 0hr
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
0hr
72hr
96hr

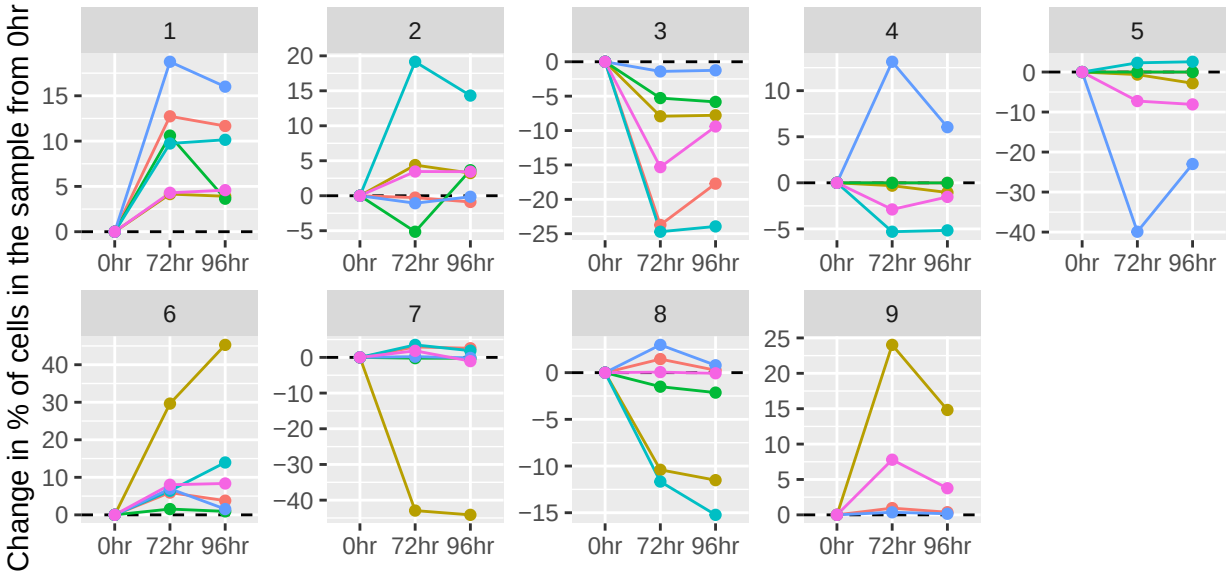

Change in % of cells in the sample from 0hr
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
0hr
72hr
96hr



From these plots, we can see the the raw flowSOM clustering is probably overclustering our data: we can see this in that many clusters (such as clusters 2, 18-20, 90-92) mostly change in only 1 or 2 patients, but not present in others. While having such a large number of clusters could allow us to identify very specific cell subtypes, we'll have problems with generalizability of those subtypes to other samples if the clusters we identify are too sample-specific.

One way to address this issue is to metacluster the flowSOM clusters, which will yield a smaller number of clusters that are more equally shared between samples.

```
jazz_patient_cluster_plot(flowsom_metaclusters, nrow = 3, ncol = 5) %>%
  walk(print)
```



In either case, we can see that there are certainly clusters that tend to respond to the treatment more than others.

3. What markers are associated with cell types that expanded?

As a quick way of visualizing marker expression between clusters that expanded or contracted during Vyxeos treatment, we can summarize the data as follows:

1. Identify the cells in each type of cluster (we did this during our clustering step above).
2. If a cluster expanded at the 96hr timepoint relative to the 0hr timepoint, label all cells in that clusters as "Vyxeos-resistant" (because they did not die during treatment). Otherwise, label them as "Vyxeos-sensitive."

3. Average the expression of all Vyxeos-resistant and Vyxeos-sensitive cells at each of the timepoints included in the study.

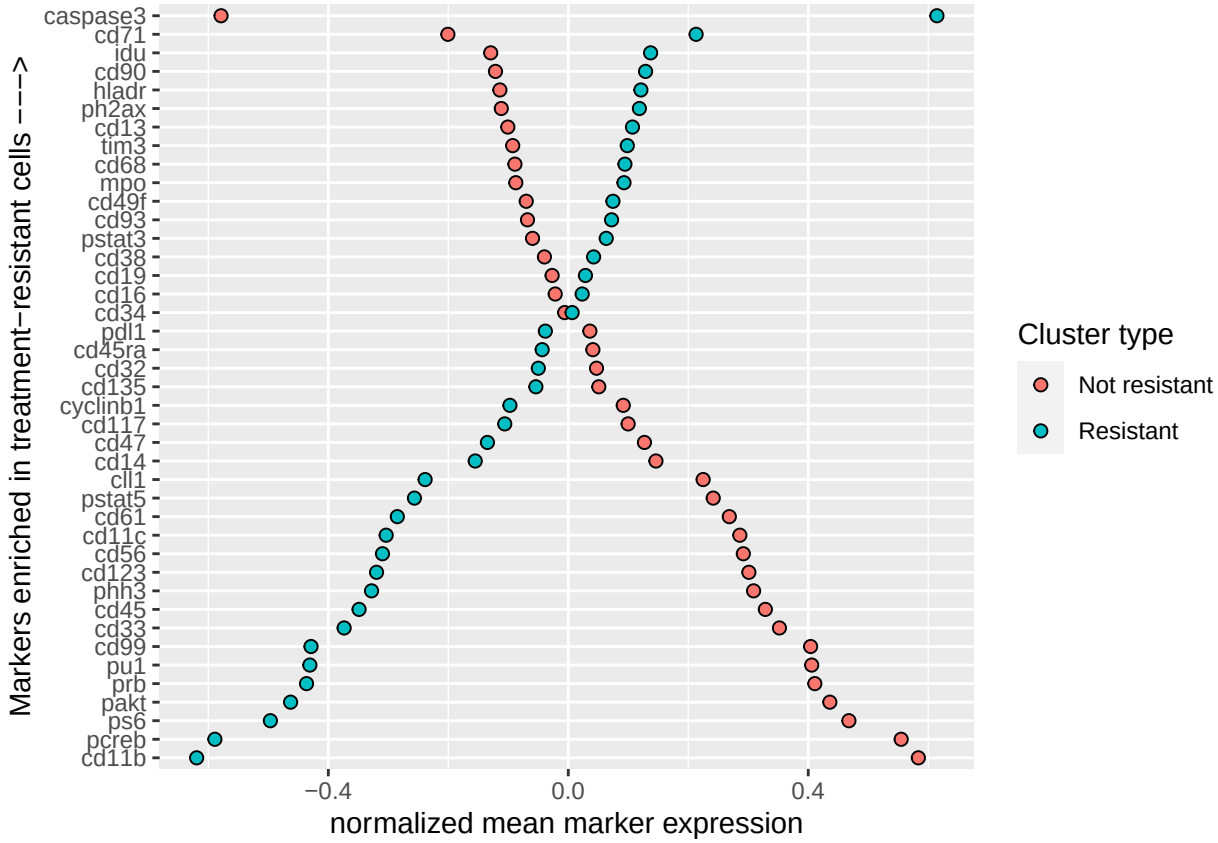
4. Calculate the difference between these averages for all markers and visualize.

```
# find proportion of each cluster at the 0hr timepoint
flowsom_baseline_proportions <-
  jazz_data %>%
  filter(timepoint == "0hr") %>%
  count(patient, flowsom_clusters) %>%
  group_by(patient) %>%
  mutate(total = sum(n)) %>%
  ungroup() %>%
  mutate(
    baseline_prop = n / total,
    baseline_perc = baseline_prop * 100
  ) %>%
  select(-total, -n)

# pull a character vector indicating which clusters decreased
# at the 96hr timepoint relative to the 0hr timepoint.
clusters_that_decrease <-
  jazz_data %>%
  filter(timepoint == "96hr") %>%
  count(patient, flowsom_clusters) %>%
  group_by(patient) %>%
  mutate(
    total = sum(n),
    final_prop = n / total,
    final_perc = final_prop * 100
  ) %>%
  select(-total, -n) %>%
  left_join(flowsom_baseline_proportions) %>%
  group_by(flowsom_clusters) %>%
  summarize(across(where(is.numeric), mean, na.rm = TRUE)) %>%
  filter(final_perc < baseline_perc) %>%
  pull(flowsom_clusters) %>%
  as.character()

# find a tibble of cluster names and whether or not they decreased or increased
# at the 96hr timepoint relative to the 0hr timepoint
flowsom_cluster_pre_post_status <-
  jazz_data %>%
  filter(timepoint != "healthy") %>%
  count(timepoint, flowsom_clusters) %>%
  mutate(
    decrease = if_else(flowsom_clusters %in% clusters_that_decrease, "Not resistant", "Resistant")
  ) %>%
  count(flowsom_clusters, decrease) %>%
  select(-n)

# flowsom_cluster_pre_post_status <-
#   jazz_data %>%
#   filter(timepoint != "healthy") %>%
#   count(timepoint, flowsom_clusters) %>%
```

```
#   mutate(
#     decrease = if_else(flowsom_clusters %in% sig_clusters, "Not resistant", "Resistant")
#   ) %>%
#   count(flowsom_clusters, decrease) %>%
#   select(-n)

# find a tibble summarizing the central tendencies of each cluster
jazz_central_tendencies <-
  jazz_data %>%
  left_join(flowsom_cluster_pre_post_status) %>%
  #group_by(patient) %>%
  select(
    -time, -length, -offset,
    -width, -center, -beaddist,
    -omiqfilter, -viability, -residual,
    -dna1, -dna2
  ) %>%
  # scale all markers to mean 0 and variance 1
  mutate(across(where(is.numeric), scale)) %>%
  # take the mean of the treatment-resistant and
  # sensitive clusters for each marker in each patient
  extract_feature_ct(
    cluster_col = decrease
  ) %>%
  pivot_longer(
    #cols = c(-patient),
    cols = everything(),
    names_to = c("marker", "cell_type"),
    values_to = "measurement",
    names_sep = "@"
  ) %>%
  # group_by(marker) %>%
  # # Scale the cluster means across all patients to get all markers on the same scale
  # mutate(measurement = scale(measurement)) %>%
  # group_by(cell_type, marker) %>%
  # # Find the mean of means across all patients
  # summarize(measurement = mean(measurement)) %>%
  ungroup()
```

```
marker_order <-
  jazz_central_tendencies %>%
  pivot_wider(
    names_from = cell_type,
    values_from = measurement
  ) %>%
  mutate(difference = `Not resistant` - Resistant) %>%
  arrange(-difference) %>%
  pull(marker)

jazz_central_tendencies %>%
  mutate(marker = factor(marker, levels = marker_order)) %>%
  ggplot(aes(x = marker, y = measurement, fill = cell_type)) +
  geom_point(shape = 21, size = 2) +
  coord_flip() +
```

```
labs(
  x = "Markers enriched in treatment-resistant cells --->",
  y = "normalized mean marker expression",
  fill = "Cluster type"
)
```



From the plot above, we can see that there are certain markers that are relatively higher on "treatment-resistant cells" (aka cells that increased in proportion during the course of treatment) relative to Non-resistant cells (aka cells that decreased in proportion over the course of treatment). Some interesting markers among resistant cells are `CD90`, `HLA-DR`, `MPO`, and the most different marker (`Caspase-3`), which could indicate that trx-resistant cells express cellular stress signals without dying moreso than cells that do end up dying from the treatment.

We can also see that several signaling markers (including `pCreb`, `pS6`, and `pSTAT5` seem to be **lower** in treatment-resistant cells than in non-resistant cells (at least in the basal state), suggesting that there might be some underlying signaling program differences between these two categories of cells.

Differential discovery analysis

While the above exploratory visualizations are useful to get a sense of what is present in the data, significance testing would meaningfully pare down which clusters (and cluster-marker pairs) we focus the rest of our analysis on. For this, we can use the diffcyt framework for differential discovery analysis.

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 CyTOF 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 and between wells in the treatment setup (i.e. sample-to-sample variation), we can more reliably detect differences attributable solely to the effect of Vyxeos treatment.

To do this, we can use the `{diffcyt}` package (or manual fitting of a similar procedure) to test for differential abundance of clusters across timepoints 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 sample i and a given sample j p_{ij} according to the following equation:

$$logit(p_{ij}) = log(\frac{p_{ij}}{1-p_{ij}}) = \beta_0 + \alpha_i^{(p)} + \alpha_j^{(s)} + \beta_1 X_{72hr_j} + \beta_2 X_{96hr_j}$$

In the equation above, we use the following definitions:

- p_{ij}: The proportion of cells in a given cluster in patient i and sample j
- $\alpha_i^{(p)}$: 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.
- $\alpha_j^{(s)}$: A random intercept for each sample j in which $\alpha_j^{(s)} \sim N(0, \sigma_s)$, where σ_s is estimated during model fitting. Note that the inclusion of this term allows us to model observation-level random effects, which can be useful for modeling the overdispersion in count data commonly present in biology.
- X_{72hr}: an indicator variable representing whether or not a sample was taken from the 72hr timepoint (1 if yes, 0 otherwise).
- X_{96hr}:an indicator variable representing whether or not a sample was taken from the 96hr timepoint (1 if yes, 0 otherwise).
- 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 and β_2 (under the null hypothesis that $\beta_1 = \beta_2 = 0$): if either β value is significantly different from 0 in the model, we can state that the proportion of cells in our cluster differs significantly between the 0hr timepoint and the 72hr or 96hr timepoints, respectively, while controlling for individual-to-individual and sample-to-sample variation.

We can use a similar procedure to test for differences in mean marker expression across clusters by fitting a linear regression model (thus, an LMM, not a GLMM) for each cluster/marker pair. For this, we implicitly assume the that mean marker expression values in each cluster are distributed normally among patients (which is a large assumption even though this method is state-of-the-art in the CyTOF community). Thus, for differential marker expression, we use the following equation to predict the mean expression value y_{ij} for a given cluster/marker pair in patient **i** and sample **j**:

$$y_{ij} = \beta_0 + \alpha_i^{(p)} + \beta_1 X_{72hr_j} + \beta_2 X_{96hr_j}$$

in which the values in this expression are defined as above and the null-hypothesis significance testing is carried out identically. Importantly, we can correct for multiple comparisons (due to the number of models being fit) by using the `p.adjust` function in base r (which has a variety of methods already implemented).

With this framework in hand, we can then move on to the actual computations.

Set up data structures

First, we must put our data into the kinds of data structures that `diffcyt` uses (eventually I will develop a framework that is a bit easier to work with).

```
# set up experiment information
experiment_info <-
  jazz_data %>%
  filter(timepoint != "healthy") %>%
  distinct(name, patient, timepoint) %>%
  dplyr::rename(sample_id = name)

# set up marker information
my_markers <-
  c(
    "cd45", "cd61", "cd99", "idu", "cd45ra",
    "cll1", "cd19", "prb", "cd117", "cd123",
    "cd93", "cd90", "cyclinb1", "cd34", "cd32",
    "pstat5", "cd11c", "cd13", "pakt", "tim3",
    "cd56", "pu1", "cd33", "pdl1", "cd14",
    "caspase3", "cd38", "pstat3", "cd16", "cd68",
    "mpo", "ph2ax", "cd47", "cd135", "phh3",
    "ps6", "cd49f", "hladr", "cd71", "pcreb",
    "cd11b"
  )

marker_info <-
  tibble(
    marker_name = colnames(jazz_data)
  ) %>%
  filter(marker_name %in% my_markers) %>%
  mutate(
    marker_class =
      if_else(marker_name %in% CLASSIFIER_MARKERS, "type", "state") %>%
      as.factor()
  )

# create formula
my_formula <-
  createFormula(
    experiment_info = experiment_info,
    cols_fixed = c("timepoint"),
    cols_random = c("sample_id", "patient")
  )

#create design matrix
my_design <-
  createDesignMatrix(
    experiment_info = experiment_info,
    cols_design = c("timepoint")
  )

# create contrast matrix
my_contrast <-
  createContrast(attr(my_design, "assign"))
```

Because the flowSOM metaclusters look nice above, we will use them for the differential discovery analysis (although code is available to use the other form of clustering as well, and it is arbitrary which clustering method we choose for the analysis).

```
# configure jazz data into the format diffcyt likes
jazz_data_nested <-
  jazz_data %>%
  filter(timepoint != "healthy") %>%
  group_by(name) %>%
  nest() %>%
  pull(data)

jazz_data_diff <-
  prepareData(
    d_input = jazz_data_nested,
    experiment_info = as.data.frame(experiment_info),
    marker_info = as.data.frame(marker_info),
    cols_to_include =
      (colnames(select(jazz_data, -name)) %in% marker_info$marker_name)
  )

# add flowSOM metaclusters to diffcyt object
temp <-
  jazz_data_diff %>%
  rowData()

temp[,"cluster_id"] <-
  jazz_data %>%
  pull(flowsom_metaclusters) %>%
  as.factor()

rowData(jazz_data_diff) <- temp

# fix some typing issues in the exprs component of the SummarizedExperiment
jazz_exprs <-
  jazz_data_diff %>%
  assays() %>%
  `[[`("exprs")

jazz_colnames <- colnames(jazz_exprs)

jazz_exprs <-
  jazz_exprs %>%
  apply(MARGIN = 2, FUN = as.numeric)

colnames(jazz_exprs) <- jazz_colnames

assays(jazz_data_diff)[["exprs"]] <- jazz_exprs
```

This is the code that we did **not** run, but which can be used to use either the developmental clusters or the raw (not metaclustered) flowSOM clusters instead.

```
# add flowSOM clusters to diffcyt object
temp <-
  jazz_data_diff %>%
```

```
  rowData()

temp[,"cluster_id"] <-
  jazz_data %>%
  pull(flowsom_clusters) %>%
  as.factor()

rowData(jazz_data_diff) <- temp

# add developmental clusters to diffcyt object
temp <-
  jazz_data_diff %>%
  rowData()

temp[,"cluster_id"] <-
  jazz_data %>%
  pull(cluster) %>%
  as.factor()

rowData(jazz_data_diff) <- temp
```

Differential abundance analysis

Now that the right data structures have been created, we can move forward with the actual differential discovery analysis. We start by detecting which flowSOM metaclusters are differentially abundant at different timepoints in the study. Remember that using a GLMM framework allows us to control for the random effects that arise from individual variation between patients and the overdispersion between samples.

For this analysis, we throw away any clusters that don't have at least 100 cells in at least 5 samples because in that case the proportions we're comparing are just not stable enough to be meaningful.

Statistical Testing

```
# only test the null hypothesis that B_1 is different from 0
contrast_1 <- createContrast(c(0, 1, 0))
# only test the null hypothesis that B_2 is different from 0
contrast_2 <- createContrast(c(0, 0, 1))
# test the null hypothesis that either B_1 or B_2 is different from 0
contrast_3 <- createContrast(c(0, 1, 1))

# we choose the third option
my_contrast <- contrast_3

# clusters that don't have at least 100 cells in 5 patients will be ignored
# in the analysis
min_cells <- 100
min_samples <- 5

# test differential abundance
jazz_counts <- calcCounts(jazz_data_diff)

# use the GLMM model
da_results <-
  testDA_GLMM(
```

```
    d_counts = jazz_counts,
    formula = my_formula,
    contrast = my_contrast,
    min_cells = min_cells,
    min_samples = min_samples
  )

# use an edgeR-based model, which uses a negative binomial distribution
# instead of a binomial distribution
da_results_2 <-
  testDA_edgeR(
    d_counts = jazz_counts,
    design = my_design,
    contrast = my_contrast,
    min_cells = min_cells,
    min_samples = min_samples
  )

# use a voom/limma-based model, which I don't really understand.
da_results_3 <-
  testDA_voom(
    d_counts = jazz_counts,
    design = my_design,
    contrast = my_contrast,
    block_id = as.factor(experiment_info$patient),
    min_cells = min_cells,
    min_samples = min_samples
  )

num_sig_1 <-
  da_results %>%
  topTable(all = TRUE) %>%
  as_tibble() %>%
  mutate(significant = if_else(p_adj < 0.05, "*", "")) %>%
  dplyr::count(significant) %>%
  filter(significant == "*") %>%
  pull(n)

num_sig_2 <-
  da_results_2 %>%
  topTable(all = TRUE) %>%
  as_tibble() %>%
  mutate(significant = if_else(p_adj < 0.05, "*", "")) %>%
  dplyr::count(significant) %>%
  filter(significant == "*") %>%
  pull(n)

num_sig_3 <-
  da_results_3 %>%
  topTable(all = TRUE) %>%
  as_tibble() %>%
  mutate(significant = if_else(p_adj < 0.05, "*", "")) %>%
  dplyr::count(significant) %>%
```

```
  filter(significant == "*") %>%
  pull(n)

tibble(
  method = c("GLMM", "edgeR", "limma/voom"),
  num_significant_clusters = c(num_sig_1, num_sig_2, num_sig_3)
) %>%
  knitr::kable()
```

method	num_significant_clusters
GLMM	4
edgeR	1
limma/voom	5

We can see that each of these testing procedures identify a similar number of significantly changed clusters, so we can move forward analyzing the GLMM approach (because it is the most intuitive to me, at least until I read more about the negative binomial distribution... maybe starting with this highly-rated blog post).

So, I'll just pull out the IDs of the significant clusters from the GLMM approach above.

```
sig_clusters_diffcyt <-
  da_results %>%
  topTable(all = TRUE) %>%
  as_tibble() %>%
  mutate(significant = if_else(p_adj < 0.05, "*", "")) %>%
  filter(significant == "*") %>%
  pull(cluster_id) %>%
  as.character()
```

Tim models GLMMs directly

To see if the `diffcyt` GLMM implementation does anything differently than I would do it if I implemented the testing procedure from scratch, I provide a *de novo* implementation here. Note that I have to rely on the `{broomExtra}` package to do the null-hypothesis significance testing of the β values for me.

```
# count the number of cells in each cluster in each patient at each timepoint
fit_data <-
  jazz_data %>%
  dplyr::count(name, patient, timepoint, flowsom_metaclusters, .drop = FALSE) %>%
  group_by(patient, timepoint) %>%
  mutate(
    total_cells = sum(n),
    prop = n / total_cells
  ) %>%
  ungroup()

# remove clusters from analysis that don't have at least 100 cells in 5 samples
clusters_to_remove <-
  fit_data %>%
  dplyr::count(name, flowsom_metaclusters, wt = n) %>%
  mutate(over_min_cells = n > min_cells) %>%
  dplyr::count(flowsom_metaclusters, over_min_cells) %>%
  filter(over_min_cells) %>%
  filter(n < min_samples) %>%
```

```
  pull(flowsom_metaclusters)

fit_data <-
  fit_data %>%
  # remove clusters that have no cells in the jazz patients (were only)
  # present in healthies
  group_by(flowsom_metaclusters) %>%
  filter(sum(n) != 0) %>%
  ungroup() %>%
  filter(!(flowsom_metaclusters %in% clusters_to_remove))

# nest the data so that we can map() over each cluster, fitting a unique
# GLMM for each one
cluster_fit_data <-
  fit_data %>%
  group_by(flowsom_metaclusters) %>%
  nest() %>%
  ungroup()
```

Then I can write some functions to do the actual model fitting (binomial and poisson for comparison - they turn out to be essentially identical, which makes sense).

```
# a function to fit binomial models on our count data
fit_model <- function(data, formula) {
  model_fit <-
    glmer(formula, data, family = "binomial", weights = total_cells)
}

# a function to fit poisson models on our count data
fit_model_poisson <- function(data, formula) {
  model_fit <-
    glmer(formula, data, family = "poisson", offset = log(total_cells))
}
```

Then I can apply the fitting functions to the data

```
cluster_fit_data <-
  cluster_fit_data %>%
  mutate(
    results =
      map(
        .x = data,
        .f = fit_model,
        formula = prop ~ timepoint + (1 | patient) + (1 | name)
      ),
    results = map(.x = results, .f = tidy)
  )

glmm_manual_result <-
  cluster_fit_data %>%
  select(-data) %>%
  unnest(cols = results) %>%
  filter(term != "(Intercept)", !is.na(p.value)) %>%
  mutate(p_adj = p.adjust(p.value, method = "fdr")) %>%
  arrange(p_adj) %>%
```

```
  mutate(significant = if_else(p_adj < 0.05, "*", ""))

# glmm_manual_result %>%
#   dplyr::count(term, significant)

sig_clusters_tim <-
  glmm_manual_result %>%
  dplyr::count(flowsom_metaclusters, significant) %>%
  filter(significant == "*", n == 2) %>%
  pull(flowsom_metaclusters) %>%
  as.character()

sig_clusters_tim
```

```
## [1] "1" "3" "6" "9"
```

And we can see that the significant clusters that we've identified are identical (the set difference between the list of significant clusters in either case is empty):

```
setdiff(sig_clusters_diffcyt, sig_clusters_tim)
```

```
## character(0)
```

```
setdiff(sig_clusters_tim, sig_clusters_diffcyt)
```

```
## character(0)
```

Visualization of differentially abundant clusters

```
sig_clusters <- sig_clusters_tim

glmm_manual_result %>%
  filter(flowsom_metaclusters %in% sig_clusters) %>%
  select(flowsom_metaclusters, term, estimate) %>%
  arrange(flowsom_metaclusters)
```

```
## # A tibble: 8 x 3
##   flowsom_metaclusters term          estimate
##   <fct>                <chr>            <dbl>
## 1 1                    timepoint72hr    0.838
## 2 1                    timepoint96hr    0.714
## 3 3                    timepoint72hr   -1.26
## 4 3                    timepoint96hr   -1.17
## 5 6                    timepoint72hr    1.36
## 6 6                    timepoint96hr    1.23
## 7 9                    timepoint72hr    5.55
## 8 9                    timepoint96hr    5.36
```

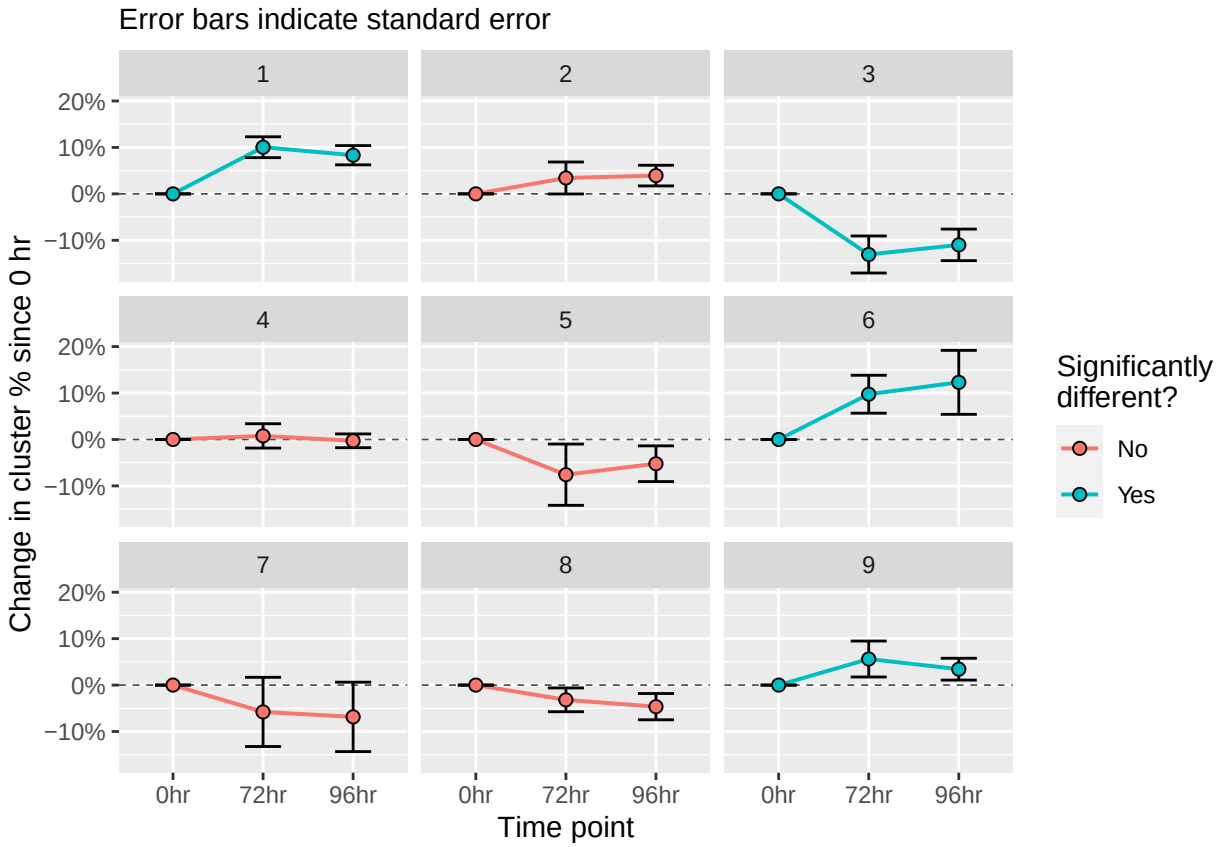
From the results of our GLMM, we can see several flowSOM metaclusters decreased in proportion at both the 72- and 96-hour timepoints relative to pre-treatment and that several metaclusters increased in proportion relative to pre-treatment. We can visualize these changes below:

```
sig_cluster_data <-
  jazz_data %>%
  dplyr::count(
    patient,
    timepoint,
```

```
    flowsom_metaclusters,
    name = "cluster_cells",
    .drop = FALSE
  ) %>%
  group_by(patient, timepoint) %>%
  mutate(
    total_cells = sum(cluster_cells),
    cluster_prop = cluster_cells / total_cells
  ) %>%
  mutate(
    is_sig = flowsom_metaclusters %in% sig_clusters,
    is_sig = if_else(is_sig, "Yes", "No")
  ) %>%
  ungroup()

sig_cluster_data_0hr <-
  sig_cluster_data %>%
  filter(timepoint == "0hr") %>%
  select(-timepoint, -total_cells, -is_sig) %>%
  dplyr::rename(
    baseline_cluster_cells = cluster_cells,
    baseline_cluster_prop = cluster_prop
  )

sig_cluster_data <-
  sig_cluster_data %>%
  left_join(sig_cluster_data_0hr) %>%
  mutate(cluster_prop_diff = cluster_prop - baseline_cluster_prop)
```

```
sig_cluster_plot <-
  sig_cluster_data %>%
  group_by(timepoint, flowsom_metaclusters, is_sig) %>%
  summarize(
    sem = sd(cluster_prop_diff) / sqrt(n()),
    cluster_prop = mean(cluster_prop),
    cluster_prop_diff = mean(cluster_prop_diff)
  ) %>%
  ggplot(aes(x = timepoint, y = cluster_prop_diff, fill = is_sig)) +
  geom_hline(yintercept = 0, linetype = "dashed", size = 0.3, color = "gray30") +
  geom_line(
    aes(color = is_sig, group = flowsom_metaclusters),
    size = 0.7
  ) +
  geom_errorbar(
    aes(ymin = cluster_prop_diff - sem, ymax = cluster_prop_diff + sem),
    width = 0.4
  ) +
  geom_point(shape = 21, size = 2) +
  scale_y_continuous(labels = scales::label_percent(1)) +
  facet_wrap(facets = vars(flowsom_metaclusters)) +
  labs(
    x = "Time point",
    y = "Change in cluster % since 0 hr",
    color = "Significantly\ndifferent?",
```

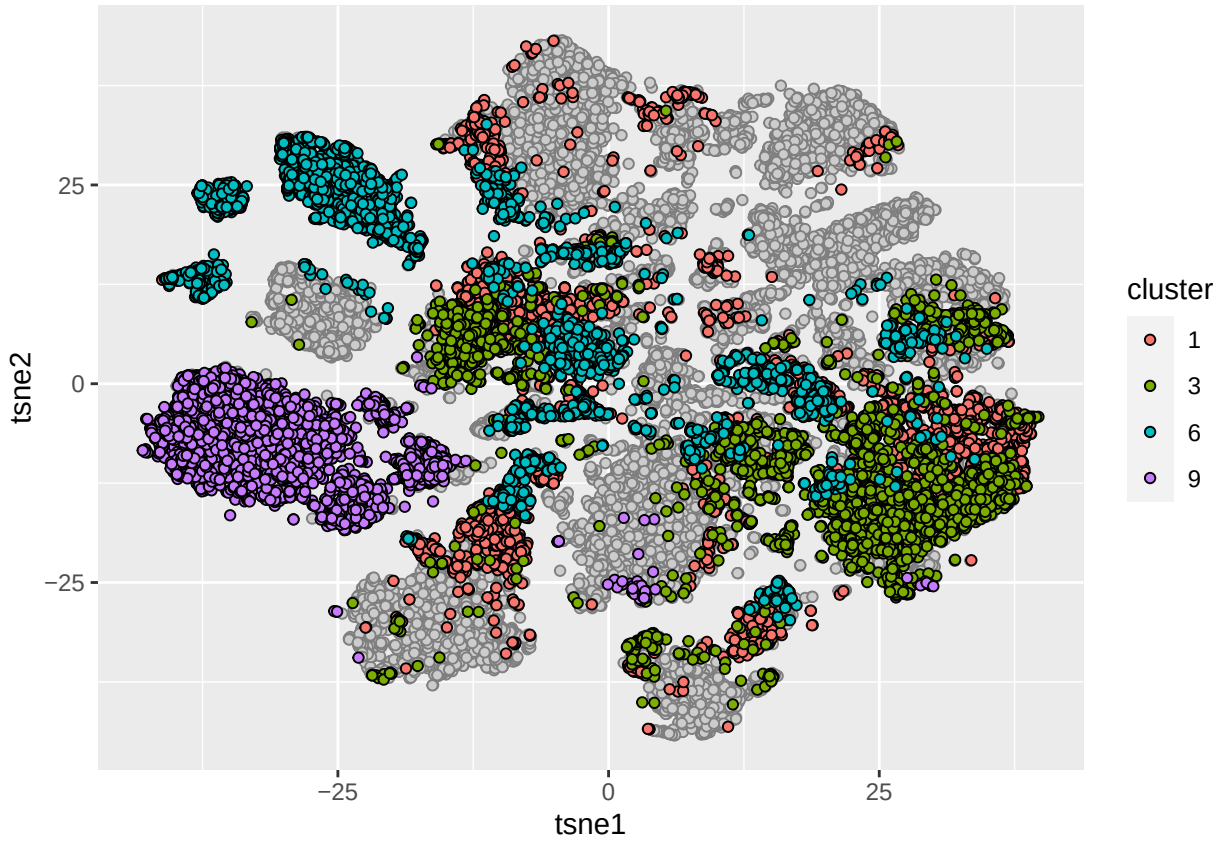
```
    fill = "Significantly\ndifferent?",
    subtitle = "Error bars indicate standard error"
  )

sig_cluster_plot
```



We can also get a sense of where the significantly different clusters organize in phenotypic space relative to other clusters using tSNE:

```
sampled_data <-
  jazz_data %>%
  mutate(is_sig = flowsom_metaclusters %in% sig_clusters) %>%
  group_by(flowsom_metaclusters) %>%
  slice_sample(n = 3000) %>%
  ungroup()

tsne_coordinates <-
  sampled_data %>%
  select(one_of(my_markers)) %>%
  Rtsne(X = .) %>%
  pluck("Y") %>%
  as_tibble() %>%
  dplyr::rename(tsne1 = V1, tsne2 = V2)

sampled_data <-
  sampled_data %>%
  bind_cols(tsne_coordinates)
```

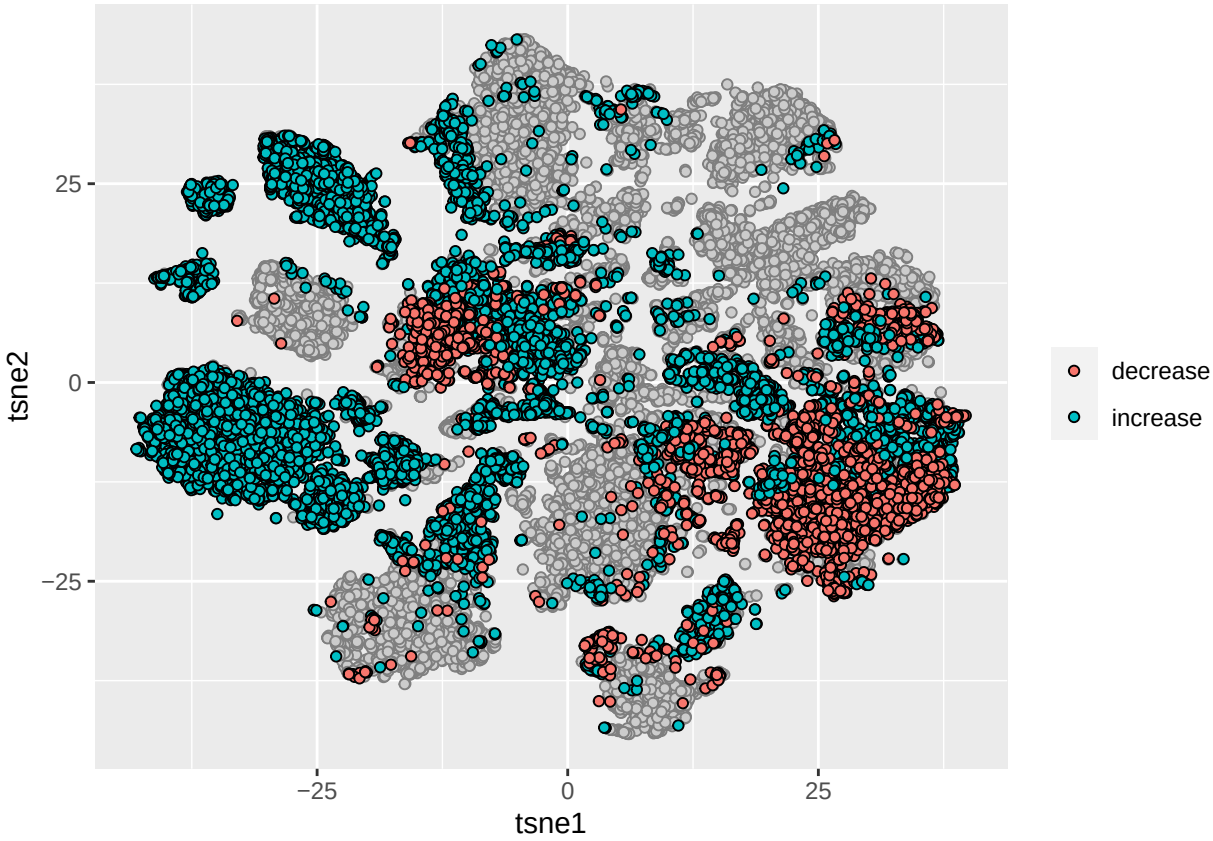
```
sig_cluster_tsne <-
  sampled_data %>%
  arrange(-is_sig) %>%
  filter(!(flowsom_metaclusters %in% sig_clusters)) %>%
  ggplot(aes(x = tsne1, y = tsne2)) +
  geom_point(shape = 21, fill = "gray80", color = "gray50") +
  geom_point(
    aes(x = tsne1, y = tsne2, fill = flowsom_metaclusters),
    data = filter(sampled_data, flowsom_metaclusters %in% sig_clusters),
    shape = 21
  ) +
  labs(fill = "cluster")

sig_cluster_tsne
```



From this tSNE plot, we can see that the clusters that change significantly in proportion (in either the positive or negative direction) preferentially aggregate in certain parts of the phenotypic space (i.e. are not distributed randomly across the phenotypical space). This is what we're hoping to see, as this suggests that there are specific phenotypic features (marker expression levels) among our clusters-of-interest that could be used to distinguish between them.

```
inc_or_dec_tibble <-
  sig_cluster_data %>%
  group_by(timepoint, flowsom_metaclusters, is_sig) %>%
  summarize(
    sem = sd(cluster_prop_diff) / sqrt(n()),
    cluster_prop = mean(cluster_prop),
    cluster_prop_diff = mean(cluster_prop_diff)
```

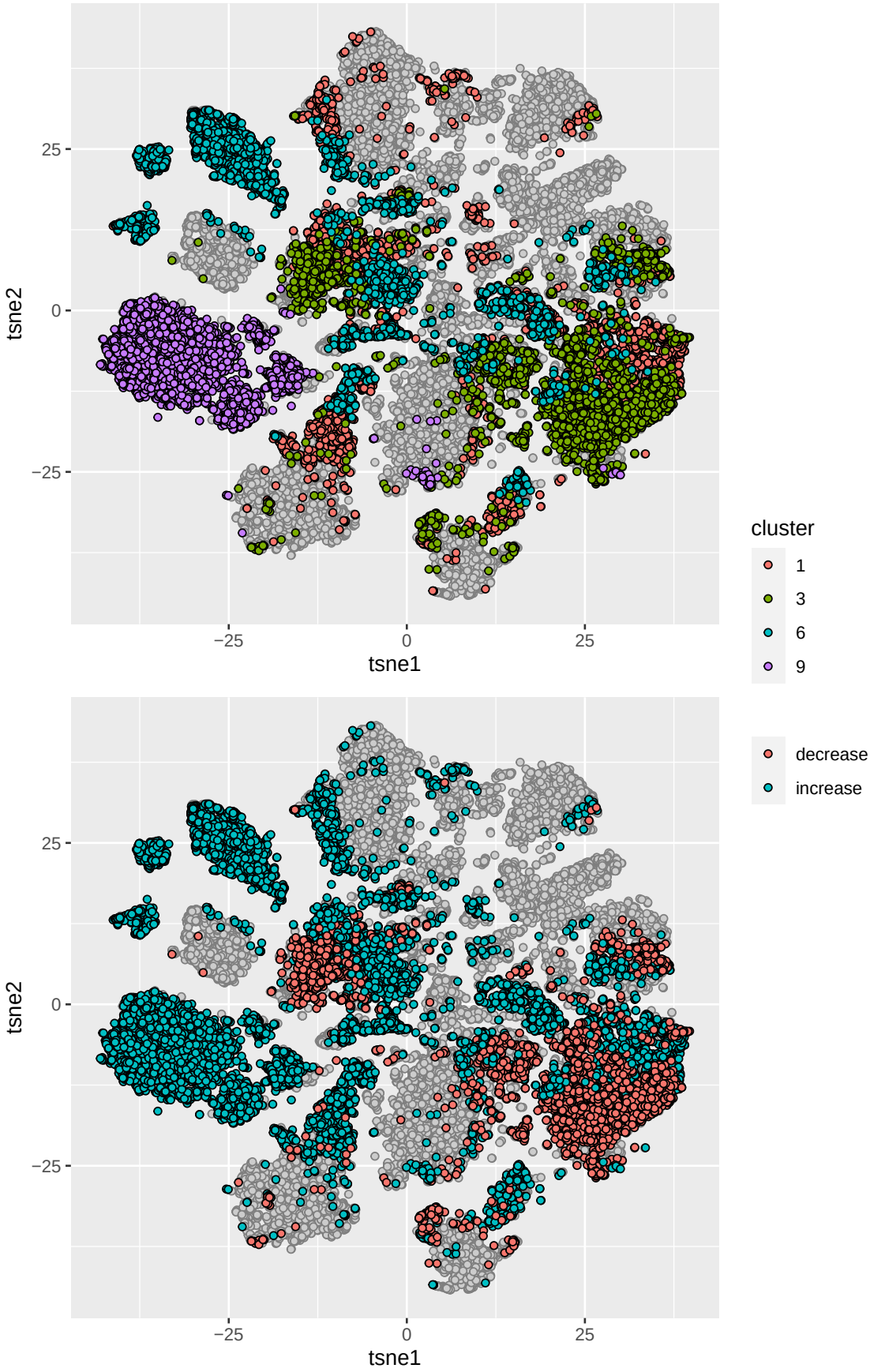
```
  ) %>%
  ungroup() %>%
  filter(is_sig == "Yes") %>%
  filter(timepoint != "0hr") %>%
  mutate(inc_or_dec = if_else(cluster_prop_diff > 0, "increase", "decrease")) %>%
  count(flowsom_metaclusters, inc_or_dec) %>%
  select(-n)

inc_cluster_tsne <-
  sampled_data %>%
  arrange(-is_sig) %>%
  filter(!(flowsom_metaclusters %in% sig_clusters)) %>%
  ggplot(aes(x = tsne1, y = tsne2)) +
  geom_point(shape = 21, fill = "gray80", color = "gray50") +
  geom_point(
    aes(x = tsne1, y = tsne2, fill = inc_or_dec),
    data =
      sampled_data %>%
      filter(flowsom_metaclusters %in% sig_clusters) %>%
      left_join(inc_or_dec_tibble),
    shape = 21
  ) +
  labs(fill = NULL)

inc_cluster_tsne
```



From this plot, we can see that the metaclusters that decrease or increase over the course of Vyxeos treatment

tend to associate with one another in phenotypic space.

```
sig_cluster_tsne +
  inc_cluster_tsne +
  plot_layout(guides = "collect", nrow = 2)
```


25
0
−25
tsne2
−25
0
25
tsne1
cluster
1
3
6
9
decrease
increase

Differential Expression Analysis

Now that the differential abundance analysis has been done, we can also do differential expression analysis across timepoints in the study to see how marker expression changes among clusters at the different timepoints at which we collected them.

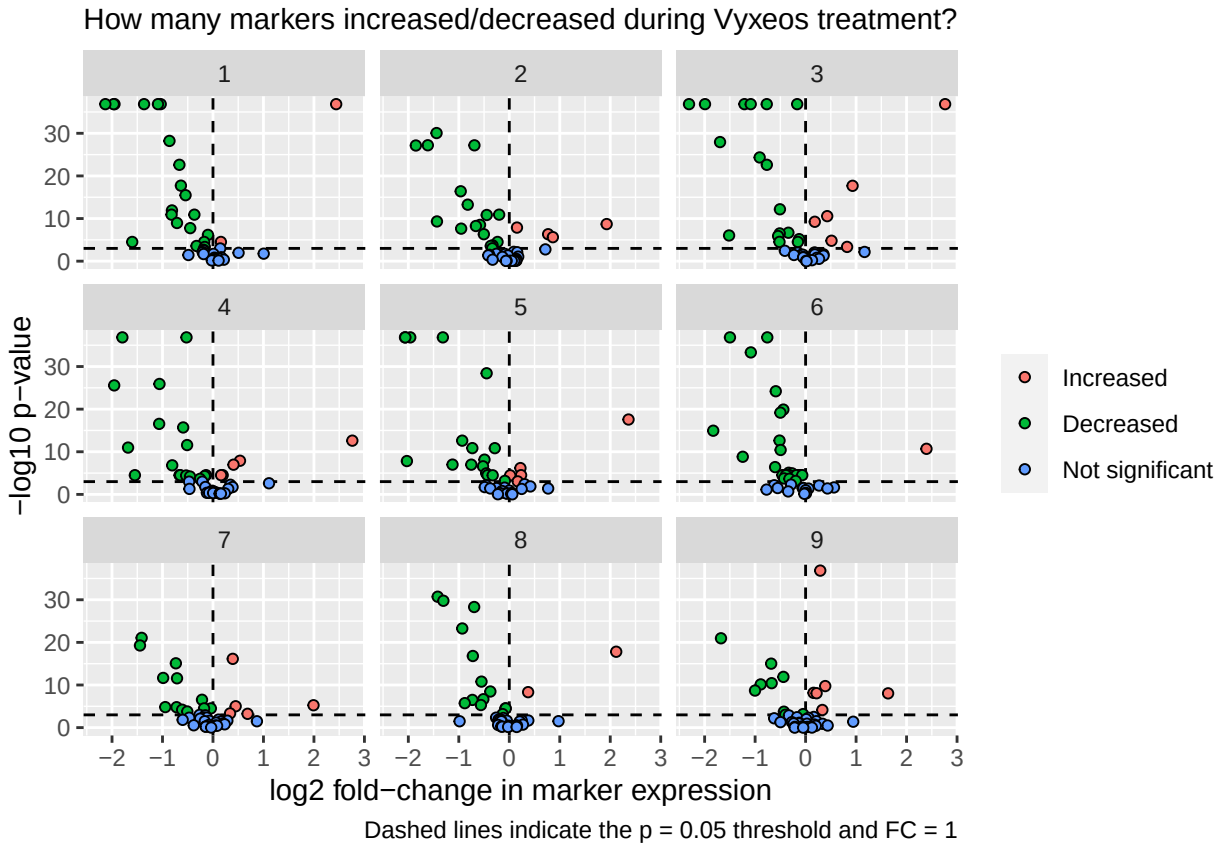
Statistical Testing

```
# test differential abundance
jazz_medians <- calcMedians(jazz_data_diff)

# fix formula
my_formula$formula <- y ~ timepoint + (1 | patient)

ds_results <-
  testDS_LMM(
    d_counts = jazz_counts,
    d_medians = jazz_medians,
    formula = my_formula,
    contrast = my_contrast,
    min_cells = min_cells,
    min_samples = min_samples,
    markers_to_test = rep(TRUE, nrow(marker_info)),
    weights = FALSE
  )

ds_results_2 <-
  testDS_limma(
    d_counts = jazz_counts,
    d_medians = jazz_medians,
    design = my_design,
    contrast = my_contrast,
    block_id = as.factor(experiment_info$patient),
    min_cells = min_cells,
    min_samples = min_samples,
    markers_to_test = rep(TRUE, nrow(marker_info))
  )
```

```
my_markers <-
  ds_results %>%
  topTable(all = TRUE) %>%
  as_tibble() %>%
  pull(marker_id) %>%
  unique() %>%
  as.character()

# find cluster-marker expression differences between 92 and 0hr timepoints
diff_tibble <-
  jazz_data %>%
  group_by(patient, timepoint, flowsom_metaclusters) %>%
  select(any_of(my_markers)) %>%
  summarize(across(everything(), mean, na.rm = TRUE)) %>%
  pivot_longer(
    cols = any_of(my_markers),
    names_to = "marker",
```

```
    values_to = "value"
  ) %>%
  pivot_wider(
    names_from = timepoint,
    values_from = value
  ) %>%
  mutate(
    diff = `96hr` - `0hr`,
    fc = `96hr` / `0hr`,
    log2_fc = log(fc, base = 2)
  ) %>%
  group_by(marker, flowsom_metaclusters) %>%
  summarize(across(everything(), mean, na.rm = TRUE)) %>%
  select(-patient, cluster_id = flowsom_metaclusters) %>%
  ungroup() %>%
  rename(marker_id = marker)
```

```
volcano_tibble <-
  ds_results %>%
  topTable(all = TRUE) %>%
  as_tibble() %>%
  left_join(diff_tibble) %>%
  mutate(
    p_adj = pmax(p_adj, 1e-16),
    neg_log10_pval = -log(p_adj),
    marker_id = fct_reorder(marker_id, neg_log10_pval),
    marker_type =
      case_when(
        p_adj >= 0.05   ~   "Not significant",
        log2_fc > 0     ~   "Increased",
        TRUE            ~   "Decreased"
      )
  )

volcano_tibble %>%
  mutate(
    marker_type =
      factor(marker_type, levels = c("Increased", "Decreased", "Not significant"))
  ) %>%
  ggplot(aes(y = neg_log10_pval, x = log2_fc, fill = marker_type)) +
  geom_vline(xintercept = 0, linetype = "dashed", color = "black") +
  geom_hline(yintercept = -log(0.05), linetype = "dashed", color = "black") +
  geom_point(shape = 21) +
  #scale_fill_discrete(labels = c("Up", "Down", "Not significant")) +
  facet_wrap(facets = vars(cluster_id)) +
  labs(
    subtitle = "How many markers increased/decreased during Vyxeos treatment?",
    x = "log2 fold-change in marker expression",
    y = "-log10 p-value",
    fill = NULL,
    caption = "Dashed lines indicate the p = 0.05 threshold and FC = 1"
  )
```



From these volcano plots, we can see that there are several markers that significantly increased and decreased in each cluster during Vyxeos treatment. In general, we see that there are more markers that significantly decreased within clusters than that increased (which is an interesting finding and might suggest that many of the markers in our panel, which is focused on myeloid markers, are downregulated as resistant cells lose their lineage-specific traits).

We can explicitly identify the markers that increased or decreased in expression for each cluster ranked by significance below:

Markers that increased during Vyxeos treatment Note that markers are arranged from most significant to least significant within each cluster.

```
increased_markers <-
  volcano_tibble %>%
  filter(marker_type == "Increased") %>%
  pull(marker_id) %>%
  unique() %>%
  as.character()

volcano_tibble %>%
  filter(marker_type == "Increased") %>%
  select(cluster_id, marker_id, p_adj, fold_change = fc) %>%
  arrange(as.numeric(cluster_id), p_adj) %>%
  knitr::kable()
```

cluster_id	marker_id	p_adj	fold_change
1	caspase3	0.0000000	5.540775

cluster_id	marker_id	p_adj	fold_change
1	cd16	0.0109942	1.123269
2	caspase3	0.0001664	4.277863
2	hladr	0.0003774	1.114258
2	cd13	0.0017951	1.892309
2	cd71	0.0035972	2.051372
3	caspase3	0.0000000	6.927222
3	ph2ax	0.0000000	1.991717
3	mpo	0.0000259	1.371115
3	cd47	0.0000966	1.138091
3	cd13	0.0083093	1.450354
3	cd71	0.0353411	1.944061
4	caspase3	0.0000033	7.030624
4	mpo	0.0003728	1.473218
4	ph2ax	0.0009385	1.451766
4	cd16	0.0109942	1.157008
4	cd49f	0.0109942	1.187075
5	caspase3	0.0000000	5.442385
5	mpo	0.0020961	1.180057
5	cd16	0.0109942	1.186352
5	ph2ax	0.0115319	1.068745
5	cd13	0.0474078	1.159367
6	caspase3	0.0000227	5.714551
7	cd13	0.0000001	1.322778
7	caspase3	0.0052506	4.641595
7	ph2ax	0.0066699	1.416707
7	cd68	0.0356223	1.322444
7	cd71	0.0391065	1.841226
8	caspase3	0.0000000	4.512990
8	ph2ax	0.0002432	1.330399
9	cd14	0.0000000	1.223351
9	cd13	0.0000583	1.331328
9	cd99	0.0002871	1.120139
9	hladr	0.0003119	1.169791
9	cd71	0.0003231	3.319221
9	cd32	0.0158540	1.299243

```
decreased_markers <-
  volcano_tibble %>%
  filter(marker_type == "Decreased") %>%
  pull(marker_id) %>%
  unique() %>%
  as.character()

volcano_tibble %>%
  filter(marker_type == "Decreased") %>%
  select(cluster_id, marker_id, p_adj, fold_change = fc) %>%
  arrange(as.numeric(cluster_id), p_adj) %>%
  knitr::kable()
```

Markers that decreased during Vyxeos treatment

cluster_id	marker_id	p_adj	fold_change
1	prb	0.0000000	0.2694541
1	pakt	0.0000000	0.3991233
1	pu1	0.0000000	0.5119551
1	ps6	0.0000000	0.2667041
1	pcreb	0.0000000	0.2368061
1	cd11b	0.0000000	0.4800383
1	cd99	0.0000000	0.5680674
1	cd61	0.0000000	0.6358708
1	cd56	0.0000000	0.6607853
1	pstat5	0.0000002	0.6977363
1	cd33	0.0000067	0.6121728
1	cd123	0.0000182	0.6095842
1	cd38	0.0000182	0.7905225
1	cll1	0.0001290	0.6397181
1	pstat3	0.0004321	0.7450391
1	cd135	0.0020949	0.9601592
1	cyclinb1	0.0109942	0.8876437
1	phh3	0.0109942	0.3691349
1	cd14	0.0281288	0.8239631
1	cd45	0.0357449	0.9031399
2	ps6	0.0000000	0.4259326
2	pcreb	0.0000000	0.3626222
2	cd61	0.0000000	0.6271536
2	prb	0.0000000	0.2935510
2	pakt	0.0000001	0.5487705
2	cd123	0.0000017	0.5887458
2	cd135	0.0000182	0.8917220
2	cd56	0.0000197	0.7557244
2	phh3	0.0000894	0.4015089
2	cd33	0.0002002	0.7048324
2	cll1	0.0002610	0.6739175
2	pu1	0.0004845	0.5737900
2	cd99	0.0017951	0.7322451
2	cyclinb1	0.0109942	0.8521806
2	pstat5	0.0245723	0.8087711
2	cd38	0.0280452	0.8065417
2	pstat3	0.0492738	0.8157851
3	cd11c	0.0000000	0.8910378
3	pakt	0.0000000	0.4373998
3	pu1	0.0000000	0.4870312
3	ps6	0.0000000	0.2722777
3	pcreb	0.0000000	0.2113792
3	cd11b	0.0000000	0.5928194
3	prb	0.0000000	0.3173710
3	cd99	0.0000000	0.5503466
3	cd61	0.0000000	0.5892559
3	pstat5	0.0000052	0.7089667
3	cd56	0.0012625	0.7982395
3	cd123	0.0014891	0.7420138
3	phh3	0.0024131	0.3866897
3	cll1	0.0027587	0.7085506
3	cd135	0.0056930	0.9447000

cluster_id	marker_id	p_adj	fold_change
3	cd33	0.0108742	0.7345219
3	cyclinb1	0.0109942	0.9004128
4	cd61	0.0000000	0.7106583
4	ps6	0.0000000	0.3296668
4	cd11b	0.0000000	0.4941531
4	pcreb	0.0000000	0.3014197
4	pakt	0.0000001	0.5276484
4	cd99	0.0000002	0.6714899
4	cd123	0.0000094	0.7344305
4	prb	0.0000170	0.3617324
4	cd11c	0.0010933	0.7693461
4	cyclinb1	0.0109942	0.9097348
4	pu1	0.0109942	0.6818497
4	phh3	0.0109942	0.3765789
4	cd56	0.0110688	0.6516113
4	cd135	0.0118467	0.7216943
4	cd34	0.0137706	0.8942118
4	cll1	0.0151263	0.8400193
4	pstat5	0.0261598	0.8702565
5	prb	0.0000000	0.2895562
5	pakt	0.0000000	0.4080606
5	ps6	0.0000000	0.2778649
5	pcreb	0.0000000	0.2701361
5	cd99	0.0000000	0.7366727
5	cd11b	0.0000033	0.5503988
5	cd61	0.0000193	0.6113503
5	cd34	0.0000195	0.8283668
5	pstat5	0.0002876	0.7232623
5	phh3	0.0004011	0.2657546
5	cd117	0.0009207	0.5025676
5	pu1	0.0009330	0.6329128
5	cd123	0.0013868	0.7230328
5	cd33	0.0071931	0.7429179
5	cyclinb1	0.0109942	0.7543849
5	cd135	0.0113516	0.8503255
5	cd47	0.0422327	0.9429810
6	pcreb	0.0000000	0.3732726
6	cd11b	0.0000000	0.5987491
6	pakt	0.0000000	0.4799255
6	cd99	0.0000000	0.6732822
6	pstat5	0.0000000	0.7408221
6	cd33	0.0000000	0.7143903
6	prb	0.0000003	0.3066100
6	pu1	0.0000033	0.7269198
6	cll1	0.0000298	0.7208549
6	ps6	0.0001461	0.4394030
6	cd56	0.0016540	0.6807722
6	cd45	0.0062441	0.8126916
6	cd47	0.0071556	0.8468594
6	cd61	0.0109942	0.7318952
6	cd117	0.0109942	0.8088542
6	cyclinb1	0.0109942	0.8961938

cluster_id	marker_id	p_adj	fold_change
6	tim3	0.0109942	0.9165834
6	cd14	0.0109942	0.7685169
6	pstat3	0.0109942	0.9299429
6	cd16	0.0109942	0.9107319
6	cd135	0.0109942	0.9052560
6	cd49f	0.0109942	0.9587505
6	cd71	0.0237297	0.7606501
6	cd68	0.0257159	0.8227371
6	pdl1	0.0426958	0.9174811
7	ps6	0.0000000	0.4304668
7	pcreb	0.0000000	0.4346285
7	cd61	0.0000003	0.6181592
7	prb	0.0000086	0.5309926
7	pakt	0.0000094	0.6517267
7	cd56	0.0014496	0.8720576
7	phh3	0.0081140	0.5444770
7	cd123	0.0083093	0.6312904
7	cyclinb1	0.0109942	0.9726681
7	cd49f	0.0109942	0.9028813
7	cll1	0.0150527	0.7117253
7	cd33	0.0227028	0.7458049
8	ps6	0.0000000	0.3854171
8	pcreb	0.0000000	0.4365773
8	cd11b	0.0000000	0.6228893
8	pakt	0.0000000	0.5582877
8	cd32	0.0000001	0.6260562
8	cd11c	0.0000205	0.6970029
8	pstat5	0.0002081	0.7877558
8	cd61	0.0012771	0.7112256
8	prb	0.0015048	0.6512278
8	phh3	0.0032074	0.5655515
8	cll1	0.0050007	0.7331483
8	cyclinb1	0.0109942	0.9493048
8	cd49f	0.0109942	0.9599650
8	tim3	0.0420276	0.9386191
9	cll1	0.0000000	0.3385044
9	cd19	0.0000003	0.6297724
9	pcreb	0.0000067	0.7415198
9	cd33	0.0000295	0.6518667
9	ps6	0.0000394	0.5649400
9	cd123	0.0001668	0.5012616
9	cd93	0.0237297	0.8907733
9	cd45	0.0420276	0.9664275
9	phh3	0.0486783	0.8415771

Visualization

We can visualize some of these marker differences on tSNE plots as well (though there might be better ways to do so...)

```
sampled_data_2 <-
  jazz_data %>%
  group_by(flowsom_metaclusters, timepoint) %>%
```

```
  slice_sample(n = 500) %>%
  ungroup() %>%
  mutate(across(all_of(c(my_markers, "hladr")), scale))

sampled_tsne <-
  sampled_data_2 %>%
  select(one_of(str_to_lower(ALL_MARKERS))) %>%
  Rtsne(X = .) %>%
  pluck("Y") %>%
  as_tibble() %>%
  dplyr::rename(tsne1 = V1, tsne2 = V2)

sampled_data_2 <-
  sampled_data_2 %>%
  bind_cols(sampled_tsne)
```

```
max_min_values <-
  sampled_data_2 %>%
  select(all_of(c(my_markers, "hladr"))) %>%
  pivot_longer(
    cols = everything(),
    names_to = "marker",
    values_to = "value"
  ) %>%
  summarize(marker_max = quantile(value, 0.99), marker_min = quantile(value, 0.01))

marker_max <- max_min_values$marker_max
marker_min <- max_min_values$marker_min
```

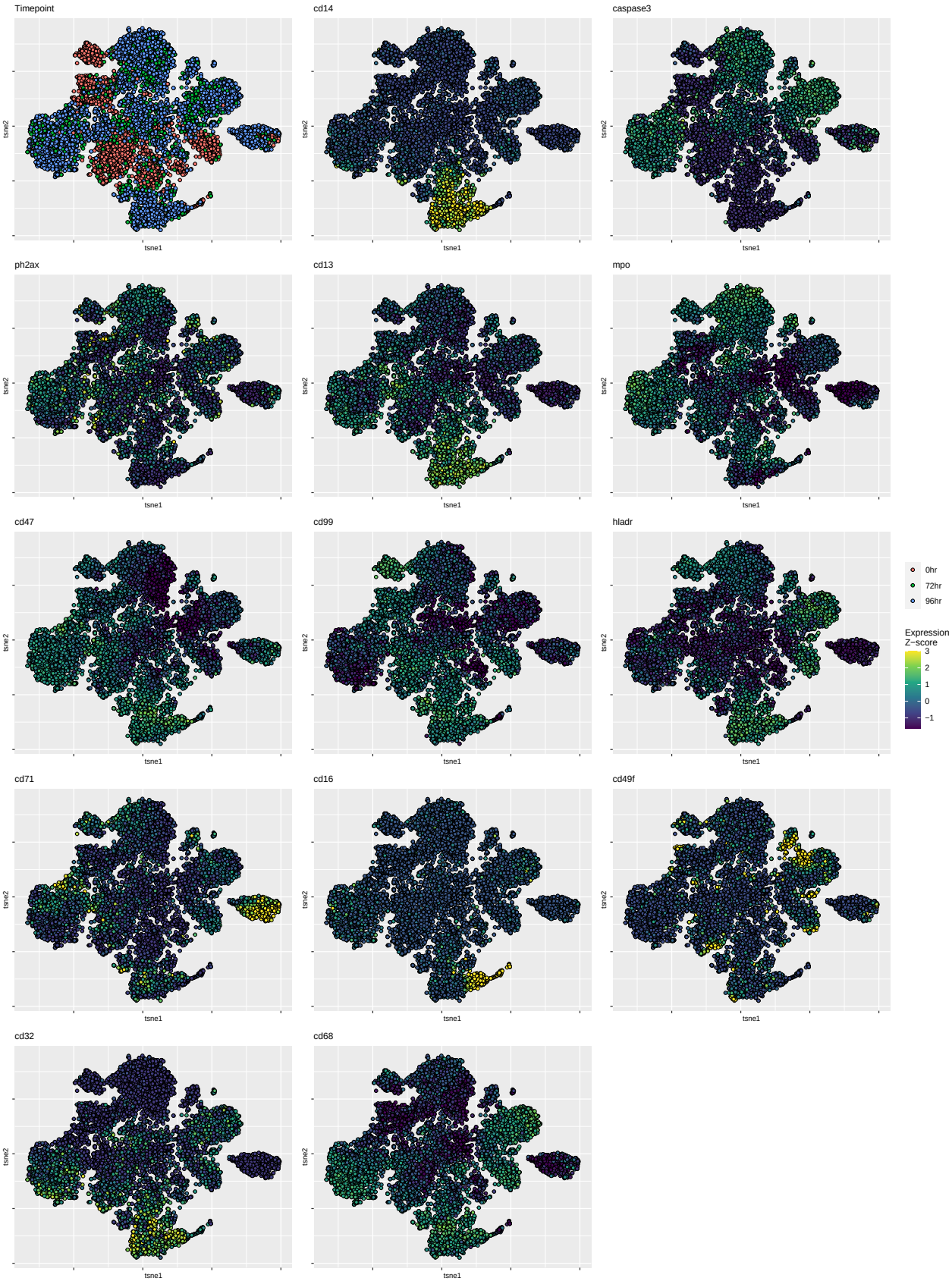
```
metacluster_tsne_plot <-
  sampled_data_2 %>%
  ggplot(aes(x = tsne1, y = tsne2, fill = flowsom_metaclusters)) +
  geom_point(shape = 21) +
  labs(fill = NULL)

timepoint_tsne_plot <-
  sampled_data_2 %>%
  ggplot(aes(x = tsne1, y = tsne2, fill = timepoint)) +
  geom_point(shape = 21) +
  labs(subtitle = "Timepoint", fill = NULL) +
  theme(
    axis.title = element_text(size = 8),
    axis.text = element_blank(),
    plot.subtitle = element_text(size = 10)
  )
```

```
plot_sig_cluster_markers <- function(marker) {

  sampled_data_2 %>%
    ggplot(aes_string(x = "tsne1", y = "tsne2", fill = marker)) +
    geom_point(shape = 21) +
    scale_fill_viridis_c(
      limits = c(marker_min, marker_max),
      oob = scales::oob_squish#,
```

```
      #guide =
        #guide_colorbar(direction = "horizontal", title.position = "top", barheight = 0.7)
    ) +
    labs(subtitle = marker, fill = "Expression\nZ-score") +
    theme(
      axis.title = element_text(size = 8),
      axis.text = element_blank(),
      plot.subtitle = element_text(size = 10),
      legend.title = element_text(size = 10)
    )
}

increased_plot_list <-
  map(
    .x = increased_markers,
    .f = plot_sig_cluster_markers
  )

decreased_plot_list <-
  map(
    .x = decreased_markers,
    .f = plot_sig_cluster_markers
  )
```

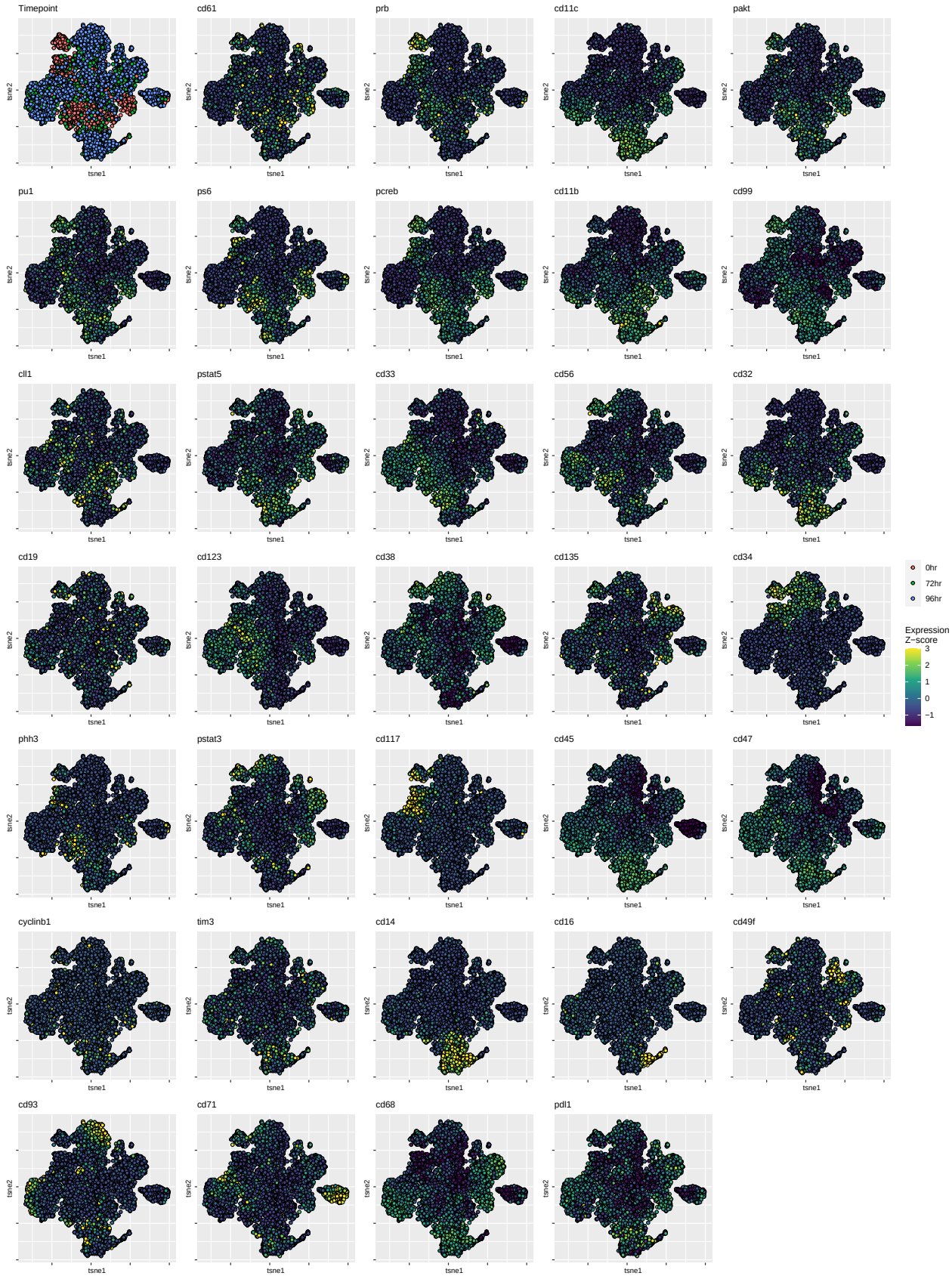
We can start by visualizing the markers that **increased** over the course of Vyxeos treatment.

```
increased_marker_plot <-
  reduce(c(list(timepoint_tsne_plot), increased_plot_list), `+`) +
  #timepoint_tsne_plot +
  #guide_area() +
  plot_layout(ncol = 3, guides = "collect")

increased_marker_plot
```


Timepoint
cd14
caspase3
ph2ax
cd13
mpo
cd47
cd99
hladr
cd71
cd16
cd49f
cd32
cd68
tsne1
tsne2
0hr
72hr
96hr
Expression
Z−score
3
2
1
0
−1

And then we can visualize the markers that **decreased** over the course of Vyxeos treatment.

```
decreased_marker_plot <-
  reduce(c(list(timepoint_tsne_plot), decreased_plot_list), `+`) +
  plot_layout(ncol = 5, guides = "collect")

decreased_marker_plot
```


Timepoint
cd61
prb
cd11c
pakt
pu1
ps6
pcreb
cd11b
cd99
cll1
pstat5
cd33
cd56
cd32
cd19
cd123
cd38
cd135
cd34
phh3
pstat3
cd117
cd45
cd47
cyclinb1
tim3
cd14
cd16
cd49f
cd93
cd71
cd68
pdl1
tsne1
tsne2
0hr
72hr
96hr
Expression
Z−score
3
2
1
0
−1