Tuberculosis Bulk RNA-seq Analysis

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1 Overview

Singhania et al. profiled whole-blood gene expression from humans with active tuberculosis, latent TB infection (LTBI), and uninfected controls across multiple cohorts, using microarrays and RNA-seq. Their main goal was to identify an optimal reduced gene set to serve as a diagnostic biomarker for active TB that did not pick up other diseases.

The Leicester RNA-seq cohort (GSE107994) used here includes longitudinal whole-blood samples from a low-incidence setting, and consists of active TB patients (n = 53) and recent close contacts (n = 108). The close contacts were categorized as either Interferon-Gamma Release Assay (IGRA) negative (n = 50) or IGRA positive (n = 49), indicating the absence or presence of *Mycobacterium tuberculosis*, respectively. A subset of the initial close contact subjects were identified as having active TB during the longitudinal assessment (n = 9) based on microbiological confirmation.

The authors used a modular analysis framework to group co-expressed genes into immune "modules" (e.g. type I interferon–inducible, B-cell, and T-cell modules). They showed that active TB is characterized by overabundance of type I interferon-inducible transcripts and underabundance of IFNG/TBX21 and B-/T-cell–associated genes, and that some individuals with LTBI display "outlier" active-TB-like signatures, suggesting phenotypic heterogeneity and possible higher progression risk.

For this project intended to familiarize myself with bulk RNA-seq data, I focus first on a cross-sectional baseline comparison between active TB, LTBI, and controls to reproduce key aspects of the transcriptional signature (e.g., interferon-related changes), and then extend to longitudinal dynamics and heterogeneity within the LTBI and LTBI-progressor groups.

Singhania A, Verma R, Graham CM, Lee J et al. A modular transcriptional signature identifies phenotypic heterogeneity of human tuberculosis infection. Nat Commun 2018 Jun 19;9(1):2308. PMID: 29921861

2 The Data

The first step is to load some initial R packages to make managing data objects easier (e.g. tidyverse, readxl) and to specifically handle data from the Gene Expression Omnibus (GEO; e.g. GEOquery, Biobase). The code chunk below also establishes a directory to store the data.

```
library(tidyverse) # dplyr, tidyr, tibble, readr, ggplot2
library(readxl)
library(Biobase)
library(GEOquery)

dir.create(file.path("data_raw"), showWarnings = FALSE) # establish directory to raw data downloads

data_dir <- file.path("data_raw", "GSE107994") # establish path to specific study directory
```

2.1 Download data from GSE

Accessing GEO can be time-intensive, so the first section of this code chunk checks if the necessary files are already downloaded before attempting to access GEO. The following code then converts the Excel file into data frame.

```
if (!dir.exists(data_dir) || length(list.files(data_dir)) == 0) { # checks for supplemental files fro
  getGEOSuppFiles("GSE107994", baseDir = "data_raw") # accesses GEO for supplemental files (gene cour
}

counts_file <- list.files( # grabs the specific file name for the raw gene counts data
  path = data_dir,
  pattern = "Raw_counts.*Leicester.*\\.xlsx$",
  full.names = TRUE
)

counts <- readxl::read_xlsx(counts_file) # function to import .xlsx file as data table
```

This section accesses GEO again to retrieve the series matrix, which contains the metadata (e.g. patient info, phenotypes) for the RNA-seq profiles.

```
gse <- getGEO("GSE107994", GSEMatrix = TRUE) # grabs the series matrix from GEO
phenotypes <- Biobase::pData(gse[[1]]) |> as_tibble() # formats GSE download as tabular metadata
```

2.2 Inspection and storage

The counts object contains raw expression counts for 58,051 genes from 175 whole-blood profiles. Also listed are the gene names and general category (e.g. protein-coding, sRNA, rRNA, non-coding).

```
# A tibble: 58,051 x 178
   Genes    Gene_name Gene_biotype Leicester_with_progr~1 Leicester_with_progr~2
   <chr>    <chr>     <chr>                         <dbl>                  <dbl>
 1 ENSG000~ TSPAN6    protein_cod~                      1                     16
 2 ENSG000~ TNMD      protein_cod~                      0                      0
 3 ENSG000~ DPM1      protein_cod~                    215                    263
 4 ENSG000~ SCYL3     protein_cod~                    233                    333
 5 ENSG000~ C1orf112  protein_cod~                     54                     57
 6 ENSG000~ FGR       protein_cod~                  21694                  18130
 7 ENSG000~ CFH       protein_cod~                     46                     44
 8 ENSG000~ FUCA2     protein_cod~                    604                    428
 9 ENSG000~ GCLC      protein_cod~                     69                    153
10 ENSG000~ NFYA      protein_cod~                    310                    420
# i 58,041 more rows
# i abbreviated names: 1: Leicester_with_progressor_longitudinal_Sample1,
#   2: Leicester_with_progressor_longitudinal_Sample2
# i 173 more variables: Leicester_with_progressor_longitudinal_Sample3 <dbl>,
#   Leicester_with_progressor_longitudinal_Sample4 <dbl>,
#   Leicester_with_progressor_longitudinal_Sample5 <dbl>,
#   Leicester_with_progressor_longitudinal_Sample6 <dbl>, ...
```

The phenotypes object contains all the information regarding the RNA-seq profiles, including patient info and RNA sequencing steps.

```
# A tibble: 175 x 60
   title             geo_accession status submission_date last_update_date type
   <chr>             <chr>         <chr>  <chr>           <chr>            <chr>
 1 Leicester_with_p~ GSM2886274    Publi~ Dec 12 2017     May 15 2019      SRA
 2 Leicester_with_p~ GSM2886275    Publi~ Dec 12 2017     May 15 2019      SRA
 3 Leicester_with_p~ GSM2886276    Publi~ Dec 12 2017     May 15 2019      SRA
 4 Leicester_with_p~ GSM2886277    Publi~ Dec 12 2017     May 15 2019      SRA
 5 Leicester_with_p~ GSM2886278    Publi~ Dec 12 2017     May 15 2019      SRA
 6 Leicester_with_p~ GSM2886279    Publi~ Dec 12 2017     May 15 2019      SRA
 7 Leicester_with_p~ GSM2886280    Publi~ Dec 12 2017     May 15 2019      SRA
 8 Leicester_with_p~ GSM2886281    Publi~ Dec 12 2017     May 15 2019      SRA
 9 Leicester_with_p~ GSM2886282    Publi~ Dec 12 2017     May 15 2019      SRA
10 Leicester_with_p~ GSM2886283    Publi~ Dec 12 2017     May 15 2019      SRA
# i 165 more rows
# i 54 more variables: channel_count <chr>, source_name_ch1 <chr>,
#   organism_ch1 <chr>, characteristics_ch1 <chr>, characteristics_ch1.1 <chr>,
#   characteristics_ch1.2 <chr>, characteristics_ch1.3 <chr>,
#   characteristics_ch1.4 <chr>, characteristics_ch1.5 <chr>,
#   characteristics_ch1.6 <chr>, characteristics_ch1.7 <chr>,
#   characteristics_ch1.8 <chr>, characteristics_ch1.9 <chr>, ...
```

Before moving on to analysis, the data are saved to an intermediate folder for easy referencing later.

```
dir.create("data_processed", showWarnings = FALSE) # create data_processed directory folder if it doe

write_tsv(phenotypes, file.path("data_processed", "GSE107994_phenotypes.tsv")) # save phenotypes loca
write_tsv(counts, file.path("data_processed", "GSE107994_counts_raw.tsv")) # save counts locally
```

3 Analysis 1 - Baseline Cross-Section

The first analysis will be focused on differences in expression profiles between active TB cases and recent contacts, and on recovering the type I IFN signatures determined by Singhania et al. The data are subset to baseline profiles and outliers previously determined by the authors are removed ahead of analysis.

The code below selects columns of interest from the metadata and renames them for easier calling. Then, it establishes some factor levels to be used in subsequent analyses and visualizations before filtering down to the desired samples. The counts object is also reformatted into a simple matrix for compatability with various modeling functions.

```
phenotypes_baseline <- phenotypes |>
  select( # subset phenotypes to the columns of interest
    geo_accession,
    patient_id = 'patient_id:ch1',
    group = 'group:ch1',
    tb_disease_type = 'tb_disease_type:ch1',
    smear_result = 'smear_result:ch1',
    outlier = 'outlier:ch1',
    gender = 'gender:ch1',
    ethnicity = 'ethnicity:ch1',
    birthplace = 'birth_place:ch1',
    age_baseline = 'age_at_baseline_visit:ch1',
    timepoint_months = 'timepoint_months:ch1',
    visit_date = 'visit_date:ch1',
    title
) |>
  mutate( # apply some factor levels to multiple variables of interest
    group = factor(group, levels = c("Control", "Active_TB", "LTBI", "LTBI_Progressor")),
    tb_disease_type = factor(tb_disease_type),
    smear_result = factor(smear_result, levels = c("Negative", "Positive")),
    gender = factor(gender)
  ) |>
  dplyr::filter(
    outlier == "No",
    timepoint_months == "Baseline"
  )
    # filter the data to remove predetermined outliers
    # and focus on the baseline visit profiles

write_tsv(phenotypes_baseline, file = file.path("data_processed", "phenotypes_baseline.tsv"))

counts_mat <- counts |> # need to convert counts to a simple matrix
  select(-Gene_name, -Gene_biotype) |>
  column_to_rownames("Genes") |>
  as.matrix()

colnames(counts_mat) <- phenotypes$geo_accession[match(colnames(counts_mat), phenotypes$title)]
  # replace with shorter names

counts_baseline <- counts_mat[, phenotypes_baseline$geo_accession]
  # select only baseline samples
```

3.1 Preprocessing

The edgeR package is loaded for some of its functions used to prepare the data for modeling, and it calls the limma package as a dependency. One such function, filterByExpr(), marks genes for retention that have a minimum count of reads in a worthwhile number of samples. These cutoffs are determined based on each profile's library size, and the process is based on a filtering strategy outlined in Chen et al. (2016).

Chen Y, Lun ATL, and Smyth, GK (2016). From reads to genes to pathways: differential expression analysis of RNA-Seq experiments using Rsubread and the edgeR quasi-likelihood pipeline. *F1000Research* 5, 1438. doi:10.12688/f1000research.8987.2 https://doi.org/10.12688/f1000research.8987.2

```
library(edgeR) # limma loaded as dependency

dge <- DGEList(counts = counts_baseline) # create DGEList object from raw counts matrix

keep_genes <- filterByExpr(dge, group = phenotypes_baseline$group)
  # Determine which genes have sufficient counts to be retained in a statistical analysis

dge <- dge[keep_genes, ] # retains only the genes determined to have sufficiently large counts

dge <- calcNormFactors( # Calculate scaling factors for library sizes
  dge,
  method = "TMM" # trimmed mean of M-values
) # the data are not normalized yet. The factors are just stored in dge$samples$norm.factors
```

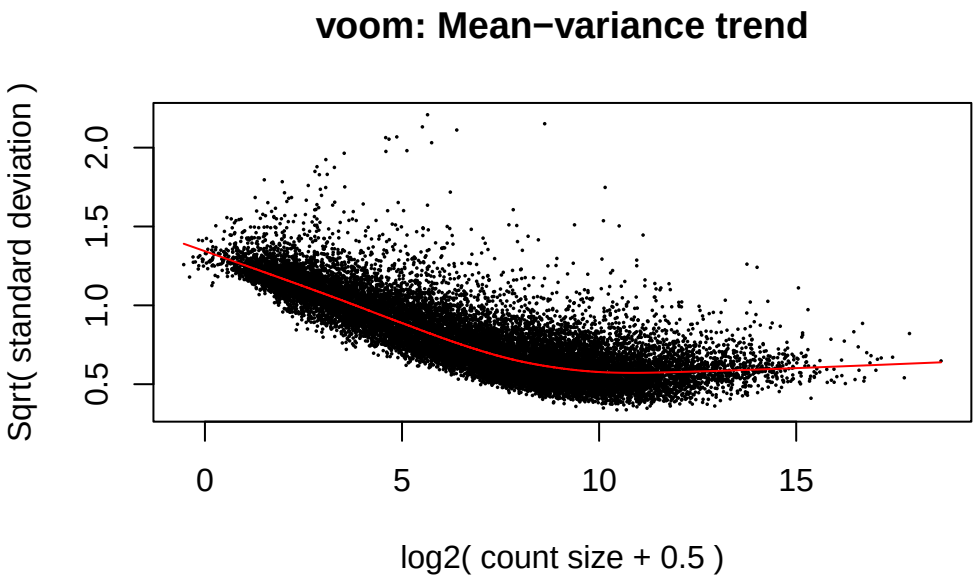
The DGE object contains the expression values, sample information, and calculated normalization factors. These factors are not applied to the data yet, but are stored in the object for later use.

```
           group lib.size norm.factors
GSM2886274     1 18956705    0.8145288
GSM2886275     1 21023702    1.1360703
GSM2886279     1 21229317    0.9785857
GSM2886280     1 14304650    0.8690486
GSM2886281     1 12560609    0.8428912
GSM2886282     1 20234496    0.9660870
```

Importantly, only 18,994 genes were retained by filterByExpr() of the initial 58,051. The analyses below are only taking into account 32.72% of the measured genes, just those determined to have large enough counts to potentially exhibit a signal against background noise.

Next, a design matrix is prepared from the levels in $group to guide the modeling. The voom() function from limma transforms the raw counts into log2 counts-per-million (log2CPM) so that the data are usable to limma's workflow.

```
design_mat <- model.matrix(~ 0 + group, data = phenotypes_baseline) # prepare the design

colnames(design_mat) <- levels(phenotypes_baseline$group) # adjust names

voom_res <- voom(counts = dge, design = design_mat, plot = TRUE)
```




```
  # transform count data to log2 counts-per-million (log2CPM)
  # incorporates the calc norm factors
```

Specifically, this is what voom is doing:

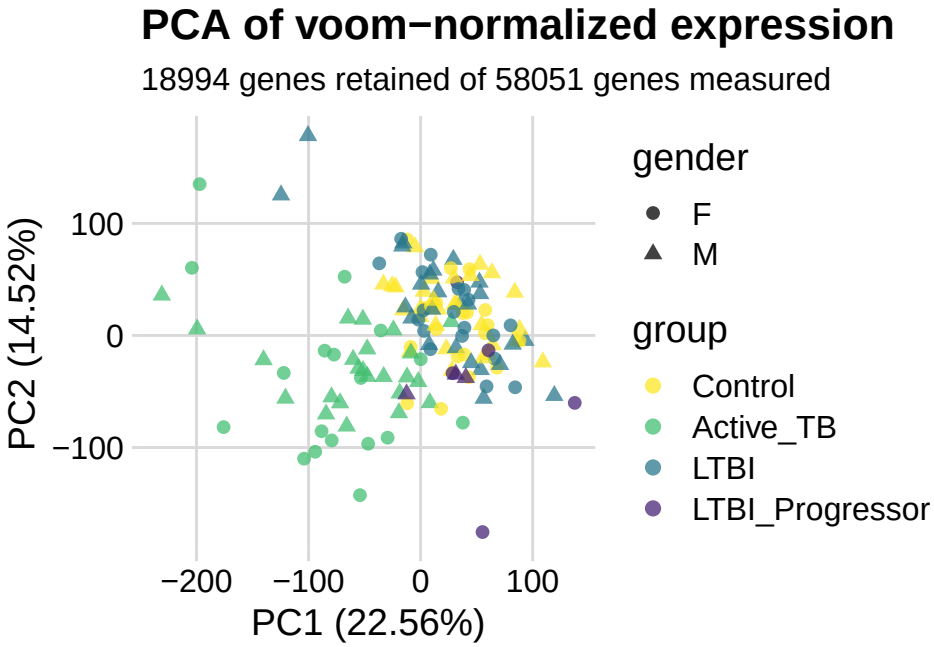
1. Counts are transformed to log2 counts per million reads (CPM), based on the normalization factors calculated earlier.
2. A linear model is fitted to each gene, and the residuals are calculated.
3. A smoothed curve is fitted to the sqrt(residual standard deviation) by average expression.
4. Weights for each gene and sample are calculated from the smoothed curve and stored for use in limma along with the log2 CPMs.

3.2 Principal Components Analysis

With the preprocessing complete, Principal Components Analysis (PCA) can be used to provide an overview of the sequencing profiles and general variance in the data.

```
pca <- prcomp( # perform PCA
  x = t(voom_res$E), # must transpose data to samples x variables format
  center = TRUE,
  scale. = TRUE
)

pca$rotation |> # save the PCA loadings
  as.data.frame() |>
  rownames_to_column("gene") |>
  write_csv(file = file.path("data_processed", "analysis1_pca_loadings.csv"))

pca_df <- pca$x |> # prepare the principal components
  as.data.frame() |>
  rownames_to_column("case")

write_csv(pca_df, file = file.path("data_processed", "analysis1_pca_scores.csv"))

prop_var <- pca$sdev^2 / sum(pca$sdev^2) * 100 # calculaute proportion of variance per PC

dir.create(file.path("output", "plots"), showWarnings = FALSE, recursive = TRUE)

library(cowplot)

pca_plot <- pca_df |> # plot the principal components, coloring by group
  left_join(y = phenotypes_baseline, by = c("case" = "geo_accession")) |>
  ggplot(aes(x = PC1, y = PC2, color = group, shape = gender)) +
    geom_point(alpha = 0.75, size = 2) +
    theme_minimal_grid() +
    coord_fixed(ratio = 1) +
    scale_color_viridis_d(direction = -1, begin = 0.1, end = 1) +
    labs(
      title = "PCA of voom-normalized expression",
      x = paste0("PC1 (", round(prop_var[1], 2), "%)"),
      y = paste0("PC2 (", round(prop_var[2], 2), "%)"),
      subtitle = paste0(nrow(voom_res), " genes retained of ", nrow(counts), " genes measured")
    )

save_plot(
  filename = file.path("output", "plots", "analysis1_pca.png"),
  plot = pca_plot,
  bg = "white"
)
```

While the PCA does not cleanly discriminate all groups, PC1 generally shows a contrast between the active TB cases and the recent contact pool of cases. No apparent trend is captured by PC2, and notably, the profiles do not demonstrate any gender-dependent clustering.



3.3 Fitting linear models

The next sensible step is to look into which features drive the differences observed in the PCA and to explore other contrasts of interest. In the following code chunk, the voom-normalized expression values and design matrix are submitted to limma's lmFit() function. This function fits a linear model for each gene in a series.

```
fit <- lmFit(
  object = voom_res,
  design = design_mat
)

print(head(coef(fit)))
```

```
                  Control  Active_TB       LTBI LTBI_Progressor
ENSG00000000003 -2.4447037 -3.7693669 -2.3521709       -1.642404
ENSG00000000419  3.6259457  3.4583260  3.6513872        3.972407
ENSG00000000457  3.9059472  3.9985405  3.8422006        4.017250
ENSG00000000460  1.4440985  1.4855627  1.4428381        1.630376
ENSG00000000938  9.9293754 10.4531684  9.8231253        9.991104
ENSG00000000971  0.7392169  0.8703221  0.9992953        1.047232
```

The coefficients of the fitted models describe the differences between the RNA sources hybridized to the arrays. To actually test the differences between groups, first, the group comparisons are specified in a contrasts matrix. This produces coefficients indicating whether a given gene is enriched in one group versus another.

```
contrasts_mat <- makeContrasts( # specifies group contrasts
  ATB_vs_allContacts = Active_TB - (Control + LTBI + LTBI_Progressor)/3,
  ATB_vs_Controls = Active_TB - Control,
  ATB_vs_LTBI = Active_TB - LTBI,
  Progressors_vs_LTBI = LTBI_Progressor - LTBI,
  levels = design_mat
)

fit2 <- contrasts.fit(fit, contrasts_mat) |> # compute coefficients and standard errors
  eBayes() # empirical Bayes moderation of the standard errors towards a global value

print(head(coef(fit2)))
```

```
                Contrasts
                 ATB_vs_allContacts ATB_vs_Controls ATB_vs_LTBI
  ENSG00000000003        -1.62294081     -1.32466319 -1.41719599
  ENSG00000000419        -0.29158719     -0.16761968 -0.19306118
  ENSG00000000457         0.07674126      0.09259326  0.15633992
  ENSG00000000460        -0.02020818      0.04146429  0.04272468
  ENSG00000000938         0.53863351      0.52379306  0.63004309
  ENSG00000000971        -0.05825919      0.13110521 -0.12897321
                Contrasts
                 Progressors_vs_LTBI
  ENSG00000000003          0.70976725
  ENSG00000000419          0.32101953
  ENSG00000000457          0.17504932
```

ENSG00000000460	0.18753820
ENSG00000000938	0.16797869
ENSG00000000971	0.04793635

3.4 Differentially expressed genes

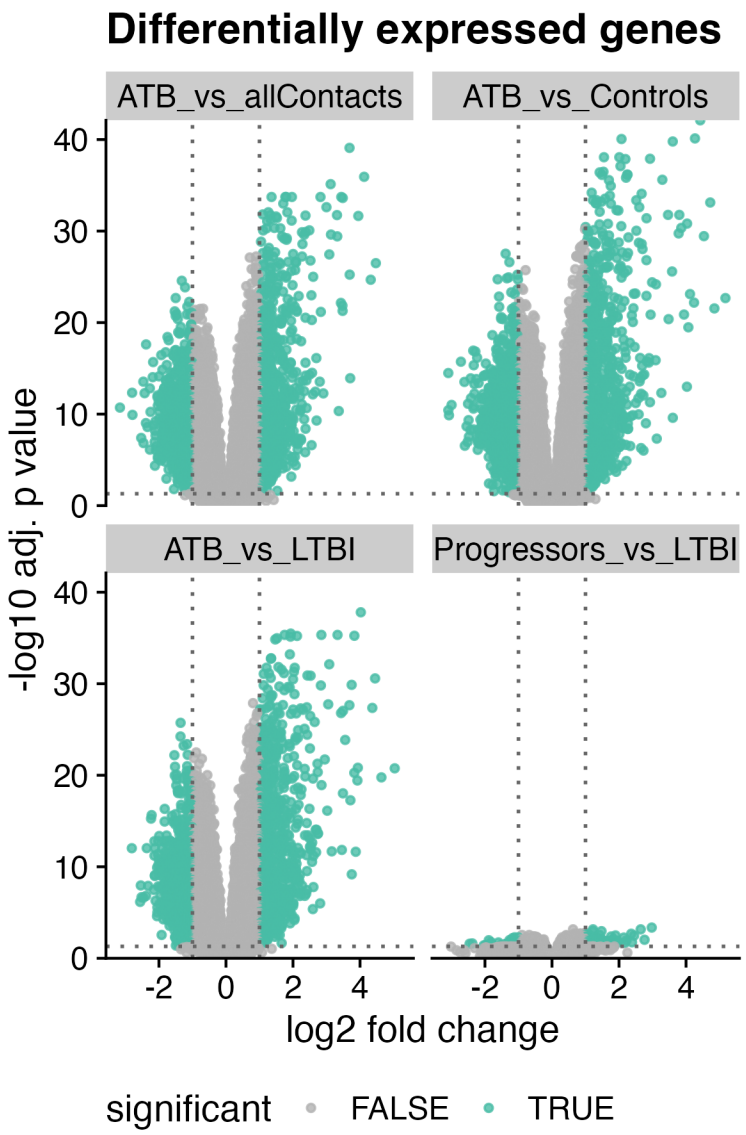
The for loop below cycles through each contrast to extract the differentially abundant genes from the linear model fit. The p values are adjusted for multiple comparisons using the Benjamini-Hochberg method.

```
dir.create(file.path("output", "tables"), showWarnings = FALSE, recursive = TRUE)

DE_genes <- list()

for (i in seq_along(colnames(contrasts_mat))) {

  top_table <- topTable(
    fit = fit2,
    coef = i,
    number = Inf,
    adjust.method = "BH",
    sort.by = "P"
  ) |>
    rownames_to_column("gene")

  top_table |>
    dplyr::filter(adj.P.Val < .05) |> # only export significant results
    write_csv(
      file = file.path(
        "output", "tables",
        paste0("analysis1_limma_", colnames(contrasts_mat)[i], ".csv")
      )
    )

  DE_genes[[colnames(contrasts_mat)[i]]] <- top_table # gather results in a list

}
```

This next section collapses the list of differentially-expressed genes into a dataframe for plotting each contrast in a facet of the figure.

```
DE_genes_df <- bind_rows(DE_genes, .id = "contrast") # collapse list to data frame

DE_genes_plot <- DE_genes_df |>
  mutate(significant = adj.P.Val < .05 & abs(logFC) > 1) |>
  ggplot(aes(x = logFC, y = -log10(adj.P.Val), color = significant)) +
    geom_point(size = 1, alpha = 0.8) +
    facet_wrap(~ contrast) +
    theme_half_open() +
    theme(legend.position = "bottom") +
    scale_color_manual(values = c("FALSE" = "grey70", "TRUE" = "#48bda6")) +
    scale_y_continuous(expand = c(0,0), limits = c(0,NA)) +
    geom_hline(yintercept = -log10(0.05), linetype = "dotted", color = "grey40") +
    geom_vline(xintercept = 1, linetype = "dotted", color = "grey40") +
    geom_vline(xintercept = -1, linetype = "dotted", color = "grey40") +
    labs(
      x = "log2 fold change",
      y = "-log10 adj. p value",
      title = "Differentially expressed genes"
    )

cowplot::save_plot(
  filename = file.path("output", "plots", "analysis1_limma_contrasts.png"),
  plot = DE_genes_plot,
  bg = "white",
  base_height = 6,
  base_width = 4
)
```

These volcano plots corroborate the group differences observed in the PCA, where active TB cases show distinct expression profiles compared to the recent contact cases.



For these active TB contrasts, over half of the retained features were found to be differentially expressed. The LTBI_Progressor vs LTBI profiles still exhibited over 2,000 differentially expressed genes.

```
DE_genes_df |> filter(adj.P.Val < .05) |> count(contrast)
```

```
             contrast     n
1     ATB_vs_Controls 12203
2         ATB_vs_LTBI 11886
3  ATB_vs_allContacts 12269
4 Progressors_vs_LTBI  2270
```

(What next for Analysis 1? Checking gene identities? re-PCA from a subset of only the DE genes?)

4 Analysis 2 — LTBI longitudinal view

(look at the longitudinal view within LTBI)