In vivo statistical analysis Figure 3

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Contents

Loading and preprocessing

Tumor-growth data were analyzed using a linear mixed-effects model (http://dx.doi.org/10.1007/b98882) fitted by Restricted Maximum Likelihood (REML) to evaluate the treatment effects over time. Tumor volumes were normalized relative to baseline measurements to correct for initial tumor-size differences and then log-transformed to model the natural exponential growth of tumor. The model incorporated fixed effects for the time, the interaction between time and treatment/cell line as well as the interaction between the three to assess differential temporal dynamics between groups. Individual animal variability was accounted by adding a random intercept for each mouse. The significance level was set at 0.05. All statistical analyses were performed using R (version 4.3.2), using the nlme package (version 3.1-164) to fit the mixed-effect model. After the models were fitted, the slopes of the tumor growth curves and their respectives confidence intervals were computed using the emmeans package (version 1Lenth R (2025). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.11.1-00001.10.2,).

```
data <- read_excel("/Users/racheleniccolai/Desktop/Statistics_in_vivo/LSS_invivo_2.xlsx", sheet = "tumo
data_surv <- read_excel("/Users/racheleniccolai/Desktop/Statistics_in_vivo/LSS_invivo_2.xlsx", sheet = "
```

```
data2 <-data %>% separate(
    Group,
    into = c("cell_line", "treatment"),
    sep = "\\s*[._-]?\\s*(?=(?:vehicule|vehicle|GSK)$)",
    remove = FALSE,
    extra = "merge",
    fill = "right"
  ) %>%
```

```r
  mutate(treatment = ifelse(treatment == "vehicule", "vehicle", treatment))
data2$treatment[is.na(data2$treatment)] <- "vehicle"

data2$cell_line <- as.factor(data2$cell_line)
data2$ID <- as.factor(data2$ID)
data2$treatment <- as.factor(data2$treatment)

data2 <- data2 %>% group_by(ID) %>%                       # Group by mouse ID
  arrange(Day) %>%                              # Ensure data is ordered by date for each mouse
  mutate(first_volume = first(Size),           # Get the first tumor volume for each mouse
         normalized_volume = Size / first_volume)    # Normalize the tumor volume

data2$cell_line <- sub("^\\s*\\d+\\.\\s*", "", data2$cell_line)

data2 <- droplevels(data2)
table(data2$cell_line, data2$treatment)
```

```
##
##            GSK vehicle
##   LSS KO    81      60
##   Oci-Ly1   46      53
```

```r
data2$treatment <- factor(data2$treatment, levels = c("vehicle", "GSK"))  # <- and here
data2$cell_line <- factor(data2$cell_line, levels = c("LSS KO", "Oci-Ly1"))  # <- and here
```
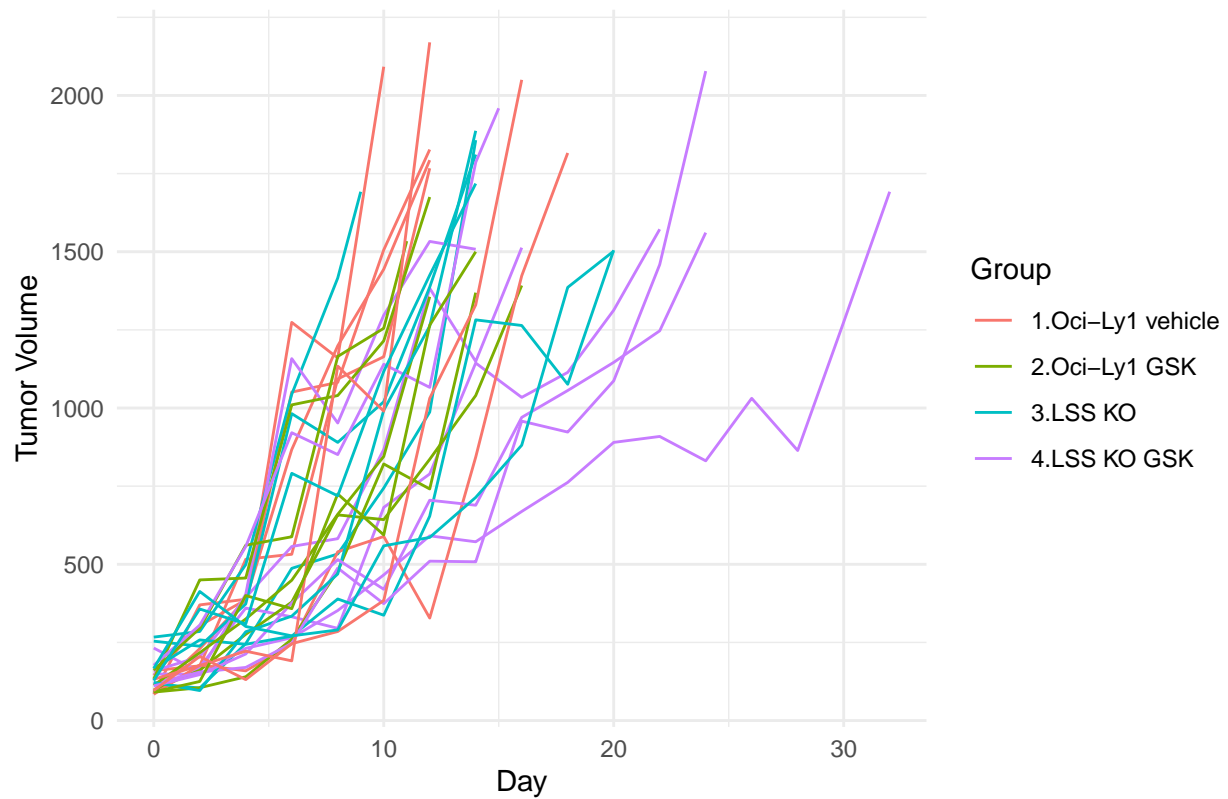
EDA

```r
p_1 <- ggplot(data2, aes(x = Day, y = Size, group = ID, color = Group)) +
  geom_line() +
  labs(title="Tumor Volume per Animal by Group",
       x="Day", y="Tumor Volume") +
  theme_minimal() + theme(legend.position="right")

# plotly::ggplotly(p_1)
p_1
```
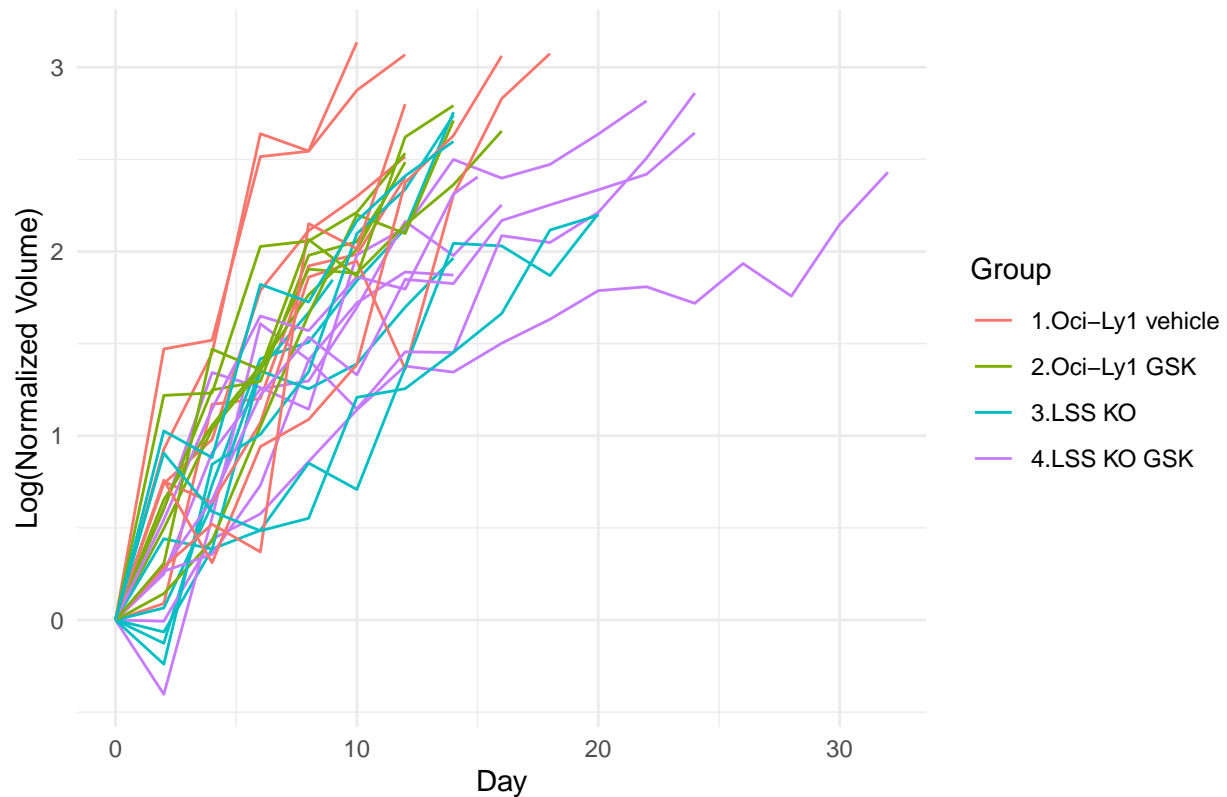
Tumor Volume per Animal by Group


```
p_2 <- ggplot(data2, aes(x = Day, y = log(normalized_volume), group = ID, color = Group)) +
  geom_line() +
  labs(title="Normalized Tumor Volume per Animal by Group",
       x="Day", y="Log(Normalized Volume)") +
  theme_minimal() + theme(legend.position="right")

# plotly::ggplotly(p_2)
p_2
```

Normalized Tumor Volume per Animal by Group


```r
fit <- lme(
  fixed = log(normalized_volume) ~ 1 + Day + Day:treatment + Day:cell_line + Day:treatment:cell_line,
  random = ~ 1 | ID,
  data   = data2,
  method = "REML"
)
```

```r
table <- summary(fit)$tTable
# DT::datatable(round(table, 4))
print(round(table, 4))
```

```
##                                   Value Std.Error  DF t-value p-value
## (Intercept)                      0.2294    0.0709 209  3.2360  0.0014
## Day                              0.1392    0.0075 209 18.4911  0.0000
## Day:treatmentGSK                -0.0377    0.0087 209 -4.3549  0.0000
## Day:cell_lineOci-Ly1             0.0585    0.0114 209  5.1128  0.0000
## Day:treatmentGSK:cell_lineOci-Ly1 0.0253   0.0157 209  1.6141  0.1080
```

Interpretations:

1) In the control group AND cell-line LSS KO, the tumor increases on average by 16.5% per day $(\exp(0.1392) = 1.149)$.

2) In the control group AND cell-line Oci_Ly1, the tumor increases on average by 21.7% per day $(\exp(0.1392 + 0.0585) = 1.219)$.

3) In the treated group AND cell-line LSS KO, the tumor increases on average by 10.6% per day (exp(0.1392 - 0.0377) = 1.107).
4) In the treated group AND cell-line Oci_Ly1, the tumor increases on average by 20.2% per day (exp(0.1392 - 0.0377 + 0.0585 + 0.0253) = 1.204).

```r
# --------------------------------------------------------------------
# Build a combined "Group" = treatment × cell_line for plotting & coloring
# --------------------------------------------------------------------
data2$treatment  <- droplevels(factor(data2$treatment))
data2$cell_line  <- droplevels(factor(data2$cell_line))
data2$Group      <- interaction(data2$treatment, data2$cell_line, sep = " • ", drop = TRUE)

# --- base spaghetti plot (individual animals) ---
p_2 <- ggplot(data2, aes(x = Day,
                         y = log(normalized_volume),
                         group = ID,                # random-effect unit
                         color = Group)) +
  geom_line(alpha = 0.35) +
  labs(title = "Normalized Tumor Volume per Animal",
       subtitle = "Colored by treatment × cell line",
       x = "Day", y = "Log(Normalized Volume)") +
  theme_minimal() +
  theme(legend.position = "right")


# ----------------------------------------------------------
# Population (fixed-effects) lines from the mixed model
# ----------------------------------------------------------
# prediction grid over Day × treatment × cell_line
Day_seq <- seq(min(data2$Day, na.rm = TRUE),
               max(data2$Day, na.rm = TRUE),
               length.out = 100)

tlev <- levels(data2$treatment)
clev <- levels(data2$cell_line)

pred_df <- expand.grid(Day = Day_seq,
                       treatment = tlev,
                       cell_line = clev,
                       KEEP.OUT.ATTRS = FALSE,
                       stringsAsFactors = FALSE)

# create Group in pred_df to match plot mapping
pred_df$treatment <- factor(pred_df$treatment, levels = tlev)
pred_df$cell_line <- factor(pred_df$cell_line, levels = clev)
pred_df$Group     <- interaction(pred_df$treatment, pred_df$cell_line, sep = " • ", drop = TRUE)

# population-level predictions (no random effects)
pred_df$fit <- as.numeric(predict(fit, newdata = pred_df, level = 0))  # nlme::lme

# --- overlay the lines ---
p_2_lines <- p_2 +
  geom_line(data = pred_df,
            aes(x = Day, y = fit, color = Group, group = Group),
            linewidth = 1.3,
```

```
            inherit.aes = FALSE) +
  labs(subtitle = "Solid lines = mixed-model population fits per treatment × cell line")

# show it
# p_2_lines
# Optional interactive:
# plotly::ggplotly(p_2_lines)
p_2_lines
```

```
## Warning in grid.Call(C_textBounds, as.graphicsAnnot(x$label), x$x, x$y, :
## conversion failure on 'vehicle • LSS KO' in 'mbcsToSbcs': dot substituted for
## <e2>
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## Warning in grid.Call.graphics(C_text, as.graphicsAnnot(x$label), x$x, x$y, :
## conversion failure on 'vehicle • LSS KO' in 'mbcsToSbcs': dot substituted for
## <e2>

## Warning in grid.Call.graphics(C_text, as.graphicsAnnot(x$label), x$x, x$y, :
## conversion failure on 'vehicle • LSS KO' in 'mbcsToSbcs': dot substituted for
## <80>
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## conversion failure on 'GSK • Oci-Ly1' in 'mbcsToSbcs': dot substituted for <a2>
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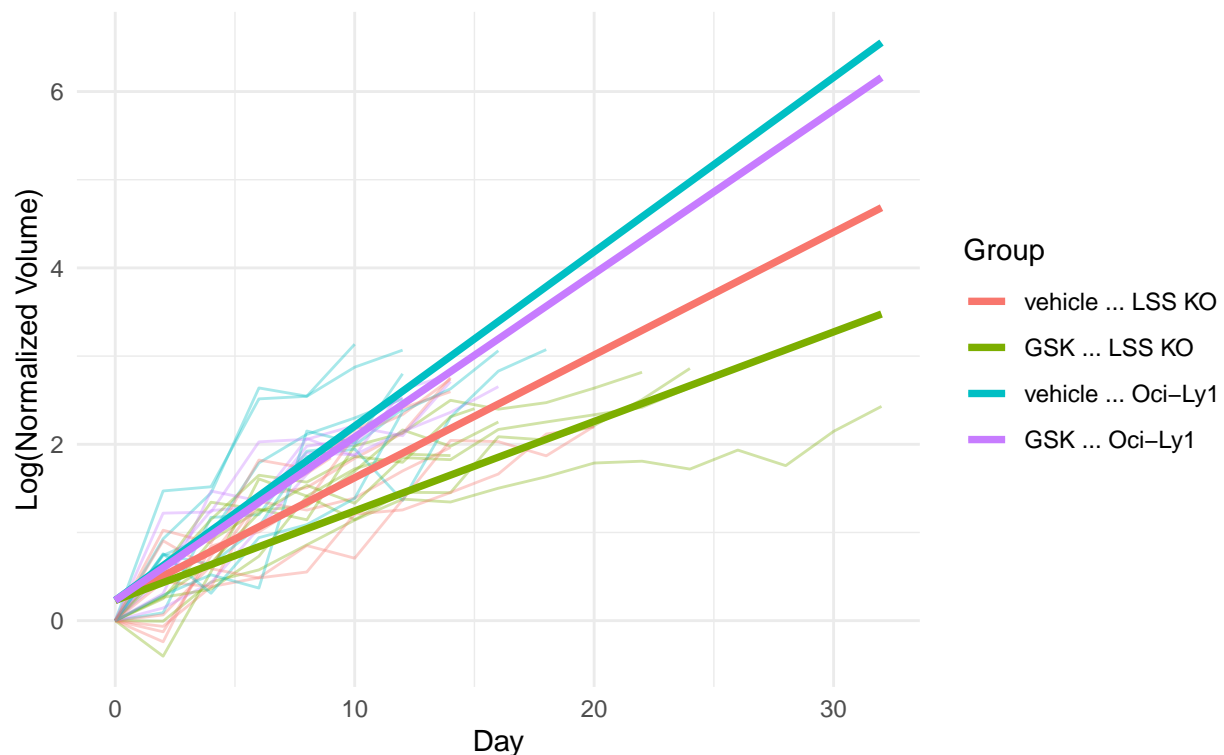
Normalized Tumor Volume per Animal
Solid lines = mixed−model population fits per treatment × cell line

Trend pairwise comparison

```r
# 1) Day slopes by treatment × cell_line + pairwise tests (Bonferroni)
trends <- emtrends(fit, specs = ~ treatment * cell_line, var = "Day")
pairs_trends <- pairs(trends, adjust = "bonferroni") |> as.data.frame()

pairs_trends[,c(2:6)] <- round(pairs_trends[,c(2:6)], 3)

# DT::datatable(pairs_trends)
print(pairs_trends)
```

```
## contrast                          estimate    SE  df t.ratio p.value
## vehicle LSS KO - GSK LSS KO          0.038 0.009 209   4.355 <.0001
## vehicle LSS KO - (vehicle Oci-Ly1)  -0.059 0.011 209  -5.113 <.0001
## vehicle LSS KO - (GSK Oci-Ly1)      -0.046 0.012 209  -3.810 0.0010
## GSK LSS KO - (vehicle Oci-Ly1)      -0.096 0.010 209  -9.618 <.0001
## GSK LSS KO - (GSK Oci-Ly1)          -0.084 0.011 209  -7.789 <.0001
## (vehicle Oci-Ly1) - (GSK Oci-Ly1)    0.012 0.013 209   0.949 1.0000
##
## Degrees-of-freedom method: containment
## P value adjustment: bonferroni method for 6 tests
```

Survival

```r
# one row per subject
surv_df <- data2 %>%
  group_by(ID) %>%
  slice_tail(n = 1) %>%
  ungroup() %>%
  mutate(
    treatment = droplevels(factor(treatment)),
    cell_line = droplevels(factor(cell_line)),
    Group = interaction(treatment, cell_line, sep = " • ", drop = TRUE),
    status = 1L                        # all events occurred
  ) %>%
  dplyr::select(ID, treatment, cell_line, Day, status, Group)

# Kaplan-Meier (will drop to 0 eventually; no censor ticks)
surv_obj <- Surv(time = surv_df$Day, event = surv_df$status)

# Cox PH with interaction
cox_fit <- coxph(surv_obj ~ treatment * cell_line, data = surv_df)
summary(cox_fit)
```

```
## Call:
## coxph(formula = surv_obj ~ treatment * cell_line, data = surv_df)
##
##   n= 27, number of events= 27
##
##                                  coef exp(coef) se(coef)      z Pr(>|z|)
## treatmentGSK                  -1.4513    0.2343   0.6989 -2.077   0.0378 *
## cell_lineOci-Ly1               0.5881    1.8006   0.5570  1.056   0.2911
## treatmentGSK:cell_lineOci-Ly1  1.5275    4.6065   0.9037  1.690   0.0910 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
##                               exp(coef) exp(-coef) lower .95 upper .95
## treatmentGSK                     0.2343     4.2686   0.05954    0.9217
## cell_lineOci-Ly1                 1.8006     0.5554   0.60431    5.3651
## treatmentGSK:cell_lineOci-Ly1    4.6065     0.2171   0.78361   27.0800
##
## Concordance= 0.719  (se = 0.055 )
## Likelihood ratio test= 12.51  on 3 df,   p=0.006
## Wald test            = 9.25  on 3 df,   p=0.03
## Score (logrank) test = 11.48  on 3 df,   p=0.009
```

```r
exp(cbind(HR = coef(cox_fit), confint(cox_fit)))
```

```
##                                      HR       2.5 %      97.5 %
## treatmentGSK                  0.2342695 0.05954482   0.9216955
## cell_lineOci-Ly1              1.8005946 0.60430675   5.3650582
## treatmentGSK:cell_lineOci-Ly1 4.6065395 0.78361296  27.0799581
```

10

```r
cox.zph(cox_fit)
```

```
##                   chisq df    p
## treatment         0.244  1 0.62
## cell_line         0.109  1 0.74
## treatment:cell_line 0.200 1 0.65
## GLOBAL            0.504  3 0.92
```

```r
km_fit <- survfit(surv_obj ~ Group, data = surv_df)


# KM (no built-in median lines)
km_plot <- ggsurvplot(
  km_fit, data = surv_df, risk.table = TRUE, conf.int = F,
  pval = TRUE, ggtheme = theme_minimal(),
  xlab = "Time (days)", ylab = "Survival probability",
  surv.median.line = "none"
)

# Per-group medians
med <- survminer::surv_median(km_fit)  # columns: strata, median, etc.

km_plot$plot <- km_plot$plot +
  geom_segment(data = med,
               aes(x = 0, xend = median, y = 0.5, yend = 0.5, color = strata)) +
  geom_segment(data = med,
               aes(x = median, xend = median, y = 0.5, yend = 0, color = strata),
               linetype = "dashed")

print(km_plot)
```

```
## Warning in grid.Call(C_textBounds, as.graphicsAnnot(x$label), x$x, x$y, :
## conversion failure on 'Group=vehicle • LSS KO' in 'mbcsToSbcs': dot substituted
## for <e2>
```

```
## Warning in grid.Call(C_textBounds, as.graphicsAnnot(x$label), x$x, x$y, :
## conversion failure on 'Group=vehicle • LSS KO' in 'mbcsToSbcs': dot substituted
## for <80>
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## <a2>

## Warning in grid.Call(C_textBounds, as.graphicsAnnot(x$label), x$x, x$y, :
## conversion failure on 'Group=vehicle • Oci-Ly1' in 'mbcsToSbcs': dot
## substituted for <e2>

## Warning in grid.Call(C_textBounds, as.graphicsAnnot(x$label), x$x, x$y, :
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## conversion failure on '•' in 'mbcsToSbcs': dot substituted for <a2>


## Warning in grid.Call.graphics(C_text, as.graphicsAnnot(x$label), x$x, x$y, :
## conversion failure on 'Group=vehicle • LSS KO' in 'mbcsToSbcs': dot substituted
## for <e2>


## Warning in grid.Call.graphics(C_text, as.graphicsAnnot(x$label), x$x, x$y, :
## conversion failure on 'Group=vehicle • LSS KO' in 'mbcsToSbcs': dot substituted
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```
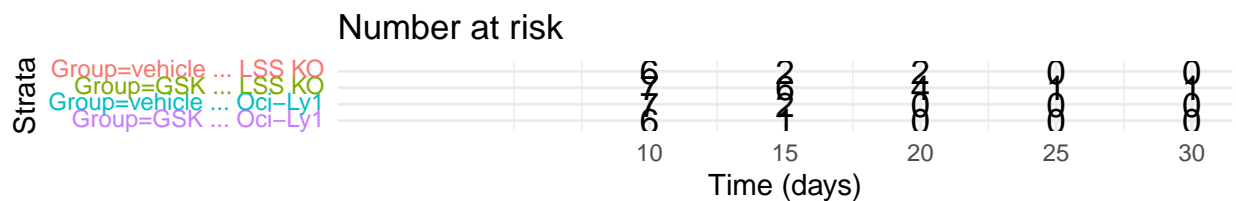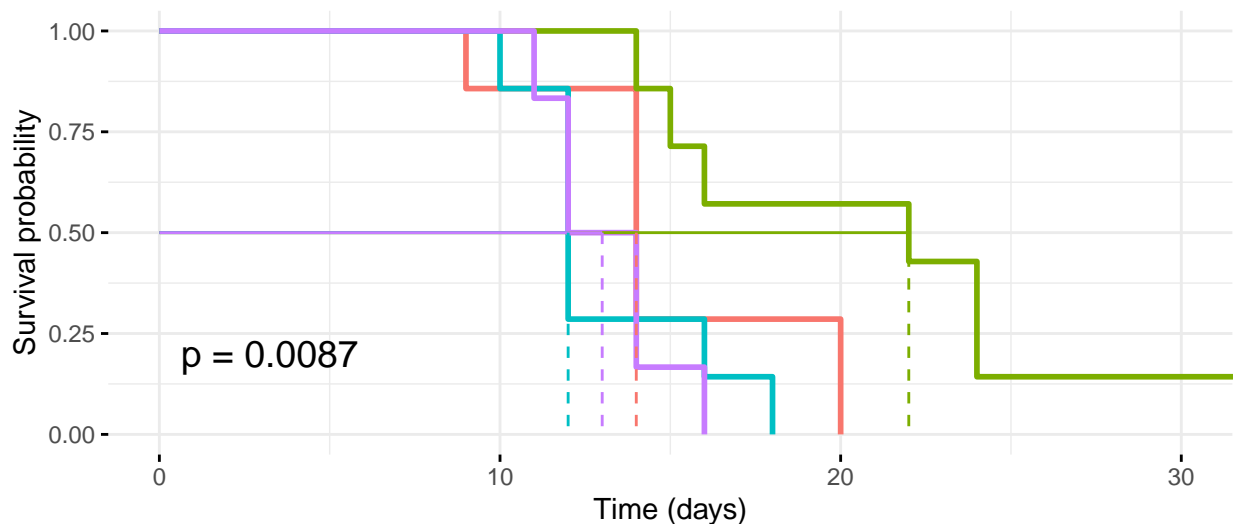
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```



```r
# 1) Pairwise log-rank tests (BH-adjusted p-values)
pw_lr <- pairwise_survdiff(Surv(Day, status) ~ Group,
                          data = surv_df, p.adjust.method = "bonferroni")
pw_lr$p.value  # matrix of adjusted p-values
```

```
##                 vehicle • LSS KO GSK • LSS KO vehicle • Oci-Ly1
## GSK • LSS KO         0.1614206          NA                   NA
## vehicle • Oci-Ly1    0.8667438   0.07385723                  NA
## GSK • Oci-Ly1        0.9852517   0.04215760                   1
```

Conclusions:

1) The overall tumor volume increases on average by 16.5% per day.
2) The cell-line without LSS knocked out have a significantly faster tumor's volume increases.
3) The treatment have a significant impact ONLY in the LSS KO group. Indeed Vehicle + Oci-Ly1 and GSK + Oci-Ly1 have similar trends.
4) None of the tested variable hav a significant impact on survival. However, overall comparison show that the 4 groups are significantly different. In particular the pairwise comparison indicates that GSK • Oci-Ly1 differs from GSK • LSS KO. Also, vehicle • Oci-Ly1 and GSK • LSS KO are borderline significant.