

Bioinformatics Analysis in R

Advanced Gene Expression: Analysis of Cancer Genome Atlas

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Summary

1. Obtain data from cancer patients from TCGA
2. Pre-process and analysis of RNA-seq data
3. Use machine learning to build a classifier for personalised medicine
4. Use interesting markers for survival analysis

The Cancer Genome Atlas

- TCGA is a NCI (US) funded project to generate cohorts of cancers:
 - Currently 33 cancers with 80-780 patients
- Comprehensive data from tissues:
 - Histology, clinical, gene expression profiling, copy number variation, DNA methylation using arrays or sequencing
- Data is publicly available upon generation and deposited in a portal (portal.gdc.cancer.gov)

The Cancer Genome Atlas - Portal

Harmonized Cancer Datasets

Genomic Data Commons Data Portal

Get Started by Exploring:

- Projects
- Exploration
- Analysis
- Repository

Q e.g. BRAF, Breast, TCGA-BLCA, TCGA-A5-A0G2

Data Portal Summary

Data Release 13.0 - September 27, 2018

PROJECTS

43

PRIMARY SITES

69

CASES

33,096

FILES

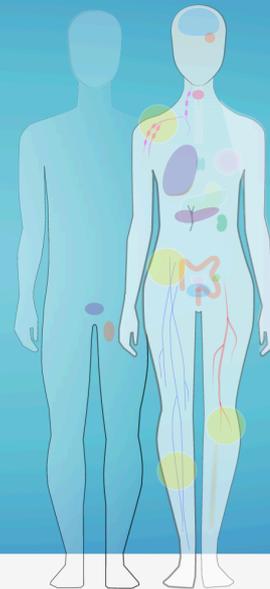
358,092

GENES

22,147

MUTATIONS

3,142,246

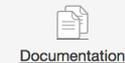
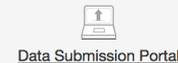


Cases by Major Primary Site



GDC Applications

The GDC Data Portal is a robust data-driven platform that allows cancer researchers and bioinformaticians to search and download cancer data for analysis. The GDC applications include:



The Cancer Genome Atlas - Portal

NIH NATIONAL CANCER INSTITUTE GDC Data Portal

Home Projects Exploration Analysis Repository

Quick Search Manage Sets Login Cart 0 GDC Apps

Harmonized Cancer Datasets

Genomic Data Commons Data Portal

Get Started by Exploring:

Projects Exploration Analysis Repository

Q e.g. BRAF, Breast, TCGA-BLCA, TCGA-A5-A0G2

Data Portal Summary

Data Release 13.0 - September 27, 2018

PROJECTS 43	PRIMARY SITES 69	CASES 33,096
FILES 358,092	GENES 22,147	MUTATIONS 3,142,246

Cases by Major Primary Site

Adrenal Gland	100
Bile Duct	100
Bladder	100
Blood	100
Bone	100
Bone Marrow	100
Brain	100
Breast	3500
Cervix	100
Colorectal	2500
Esophagus	100
Eye	100
Head and Neck	100
Kidney	100
Liver	100
Lung	4500
Lymph Nodes	100
Nervous System	2000
Ovary	100
Pancreas	100
Pleura	100
Prostate	100
Skin	100
Soft Tissue	100
Stomach	100
Testis	100
Thymus	100
Thyroid	100
Uterus	100

GDC Applications

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Data Portal Website Data Transfer Tool API Data Submission Portal Documentation Legacy Archive

Check a gene or cancer type!
I will try liver

LIHC - Liver Hepatocellular Carcinoma

Explore Project Data

↓ Biospecimen

↓ Clinical

↓ Manifest

Summary

Project ID	TCGA-LIHC
Project Name	Liver Hepatocellular Carcinoma
Disease Type	Adenomas and Adenocarcinomas
Primary Site	Liver and intrahepatic bile ducts
Program	TCGA

CASES

[377](#)



FILES

[10,814](#)



ANNOTATIONS

[28](#)



Cases and File Counts by Data Category

Data Category	Cases (n=377)	Files (n=10,814)
Raw Sequencing Data	377	1,637
Transcriptome Profiling	376	2,122
Simple Nucleotide Variation	375	3,032
Copy Number Variation	376	1,536
DNA Methylation	377	430
Clinical	377	423
Biospecimen	377	1,634

Cases and File Counts by Experimental Strategy

Experimental Strategy	Cases (n=377)	Files (n=10,814)
Diagnostic Slide	365	379
Tissue Slide	377	491
WXS	376	3,820
RNA-Seq	371	1,696
miRNA-Seq	373	1,275
Genotyping Array	376	1,536
Methylation Array	377	430

LIHC - Liver Hepatocellular Carcinoma

Explore Project Data

Biospecimen

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Gene expression data!

LIHC - Liver Hepatocellular Carcinoma

Files Cases

Add a File Filter

File

Q e.g. 142682.bam, 4f6e2e7a-b...

Data Category

- Simple Nucleotide Variation 3,032
- Transcriptome Profiling 2,122
- Raw Sequencing Data 1,637
- Biospecimen 1,634
- Copy Number Variation 1,536

2 More...

Data Type

- Gene Expression Quantification 1,272
- Isoform Expression Quantification 425
- miRNA Expression Quantification 425

Experimental Strategy

- RNA-Seq 1,272
- miRNA-Seq 860

Workflow Type

- BCGSC miRNA Profiling 860
- HTSeq - Counts 424
- HTSeq - FPKM 424
- HTSeq - FPKM-UQ 424

Data Format

- TXT 2,122

Platform

No data for this field

Access

- open 2,122

Clear
Project Id
IS
TCGA-LIHC
AND
Data Category
IS
Transcriptome Profiling

Add All Files to Cart
Manifest
View 376 Cases in Exploration
View Images

Files (2,122)

Cases (376)

Primary Site


Project


Data Category


[Show More](#)

Showing 1 - 20 of 2,122 files

Access	File Name	Cases	Project
open	7085ee3a-b604-4a12-a877-63eef2d905e8.htseq.counts.gz	1	TCGA-LIHC
open	acf3d05a-0ca4-4fee-8f07-44b93017b5fd.mirbase21.isoforms.quantification.txt	1	TCGA-LIHC
open	13240f8b-ae36-4f5f-8e95-2c9d0c83e58c.FPKM-UQ.txt.gz	1	TCGA-LIHC
open	77e29a20-68d3-4881-a3ac-a564359bcc05.FPKM-UQ.txt.gz	1	TCGA-LIHC
open	103b1320-8c4e-44ea-9449-fdcb6b405f94.htseq.counts.gz	1	TCGA-LIHC
open	466776cb-6906-4da2-b788-a05a154decf3.mirbase21.mirnas.quantification.txt	1	TCGA-LIHC
open	e4c90512-0e06-4517-95fe-c10b999f5f81.mirbase21.mirnas.quantification.txt	1	TCGA-LIHC
open	5f94c33f-588b-4b6a-9c13-4505b0f94403.htseq.counts.gz	1	TCGA-LIHC
open	6ce06871-a6a4-4a4a-bd08-0c448914dfcf.FPKM.txt.gz	1	TCGA-LIHC
open	a762a98f-9041-47e2-8561-46fae396f12.htseq.counts.gz	1	TCGA-LIHC
open	61ec8919-8b12-43d7-b127-8b68a66bd033.mirbase21.mirnas.quantification.txt	1	TCGA-LIHC
open	f3e152ef-5048-4157-a195-d13ed8851170.htseq.counts.gz	1	TCGA-LIHC
open	ca28f37f-d686-41f9-90fb-9da55fec40cb.mirbase21.isoforms.quantification.txt	1	TCGA-LIHC
open	13240f8b-ae36-4f5f-8e95-2c9d0c83e58c.FPKM.txt.gz	1	TCGA-LIHC
open	e035a46e-6114-4a64-b5ae-9e6209223493.FPKM.txt.gz	1	TCGA-LIHC
open	a96f2f6c-38e0-453c-961d-aa83b92652da.mirbase21.mirnas.quantification.txt	1	TCGA-LIHC
open	a0c56eec-568a-46b0-88db-f14d64a3942b.FPKM.txt.gz	1	TCGA-LIHC
open	9c644f65-0ebb-4862-98a9-308b81c8fb26.mirbase21.mirnas.quantification.txt	1	TCGA-LIHC
open	ad114591-0409-4bc5-8f0b-dbb44a5ad0eb.mirbase21.isoforms.quantification.txt	1	TCGA-LIHC
open	3edd413e-831d-442a-be8d-70b2f49e9d67.FPKM.txt.gz	1	TCGA-LIHC

Show 20 entries

LIHC - Liver Hepatocellular Carcinoma

Files Cases

Clear Project Id IS TCGA-LIHC AND Data Category IS Transcriptome Profiling

Add All Files to Cart Manifest View 376 Cases in Exploration View Images

Files (2,122) Cases (376)

Primary Site Project Data Category

Show More

Showing 1 - 20 of 2,122 files

Access	File Name	Cases	Project
open	7086e931-f8m-412-a1m-67e62905081ts.fcocm.s.gz	1	TCGA-LIHC
open	acf0003a-0ca4-4fec-bf07-44b9001b5fd.mirbase21.isoforms.quantification.txt	1	TCGA-LIHC
open	13240f8b-ae36-4f5f-8e95-2c9d0c83e58c.FPKM-UQ.txt.gz	1	TCGA-LIHC
open	77e29a20-68d3-4881-a3ac-a564359bcc05.FPKM-UQ.txt.gz	1	TCGA-LIHC
open	103b1320-8c4e-44ea-9449-fdcb6b405f94.htseq.counts.gz	1	TCGA-LIHC
open	466776cb-6906-4da2-b788-a05a154decf3.mirbase21.mirnas.quantification.txt	1	TCGA-LIHC
open	e4c90512-0e06-4517-95fe-c10b999f5f81.mirbase21.mirnas.quantification.txt	1	TCGA-LIHC
open	5f94c33f-588b-4b6a-9c13-4505b0f94403.htseq.counts.gz	1	TCGA-LIHC
open	6ce06871-a6a4-4a4a-bd08-0c448914dfcf.FPKM.txt.gz	1	TCGA-LIHC
open	a762a98f-9041-47e2-8561-46fae396f12.htseq.counts.gz	1	TCGA-LIHC
open	61ec819-b12-43d7-b127-8b68a661d033.mirbase21.mirnas.quantification.txt	1	TCGA-LIHC
open	f3e15af-5f4c-415c-495-d11e18851170.htseq.counts.gz	1	TCGA-LIHC
open	ca28f37f-d686-41f9-90fb-9da55fec40cb.mirbase21.isoforms.quantification.txt	1	TCGA-LIHC
open	13240f8b-ae36-4f5f-8e95-2c9d0c83e58c.FPKM.txt.gz	1	TCGA-LIHC
open	e035a46e-6114-4a64-b5ae-9e6209223493.FPKM.txt.gz	1	TCGA-LIHC
open	a96f2f6c-38e0-453c-961d-aa83b92652da.mirbase21.mirnas.quantification.txt	1	TCGA-LIHC
open	a0c56eec-568a-46b0-88db-f14d64a3942b.FPKM.txt.gz	1	TCGA-LIHC
open	9c644f65-0ebb-4862-98a9-308b81c8fb26.mirbase21.mirnas.quantification.txt	1	TCGA-LIHC
open	ad114591-0409-4bc5-8f0b-dbb44a5ad0eb.mirbase21.isoforms.quantification.txt	1	TCGA-LIHC
open	3edd413e-831d-442a-be8d-70b2f49e9d67.FPKM.txt.gz	1	TCGA-LIHC

Show 20 entries

Distinct ways to represent transcripts

Distinct ways to count gene expression.

Data Category

- Simple Nucleotide Variation (3,032)
- Transcriptome Profiling (2,122)**
- Raw Sequencing Data (1,637)
- Biospecimen (1,634)
- Copy Number Variation (1,536)

Data Type

- Gene Expression Quantification (1,272)
- Isoform Expression Quantification (425)
- miRNA Expression Quantification (425)

Experimental Strategy

- RNA-Seq (1,272)
- miRNA-Seq (860)

Workflow Type

- BCGSC miRNA Profiling (860)
- HTSeq - Counts (424)
- HTSeq - FPKM (424)
- HTSeq - FPKM-UQ (424)

Data Format

- TEXT (2,122)

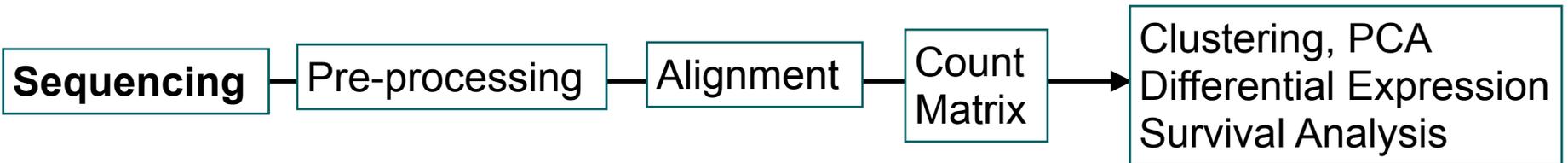
Platform

No data for this field

Access

- open (2,122)

Bioinformatics Pipeline / RNA-seq



Bioinformatics Pipeline / RNA-seq



Sequencing

Pre-processing

Alignment

Count
Matrix

Clustering, PCA
Differential Expression
Survival Analysis

Practical part not covered!

Bioinformatics Pipeline / RNA-seq



Sequencing

Pre-processing

Alignment

Count Matrix

Clustering, PCA
Differential Expression
Survival Analysis

Next Generation Sequencing

- ▶ NGS take advantage of **parallelization**
 - ▶ reads millions/billions of reads per run
 - ▶ short reads (50-100 bps)
 - ▶ error rates (0.1-1%)
- ▶ commercial products:
 - ▶ 454
 - ▶ SOLiD
 - ▶ **Solexa (Illumina)**



Read Types

Fragment DNA:

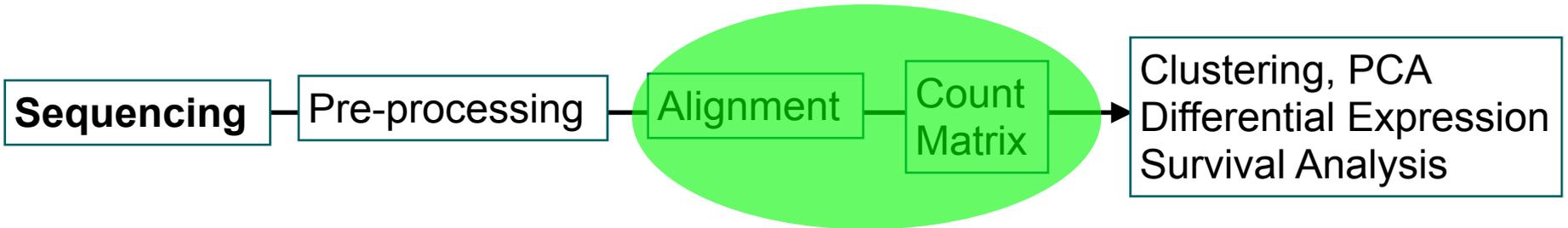


Single end



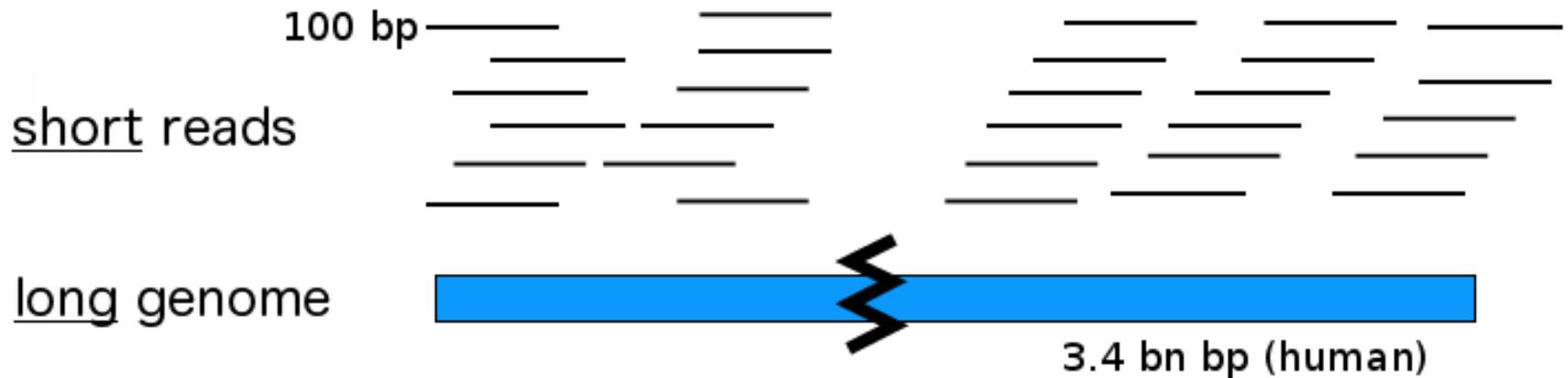
Paired end
Ins: 200-800 bp

Bioinformatics Pipeline / RNA-seq

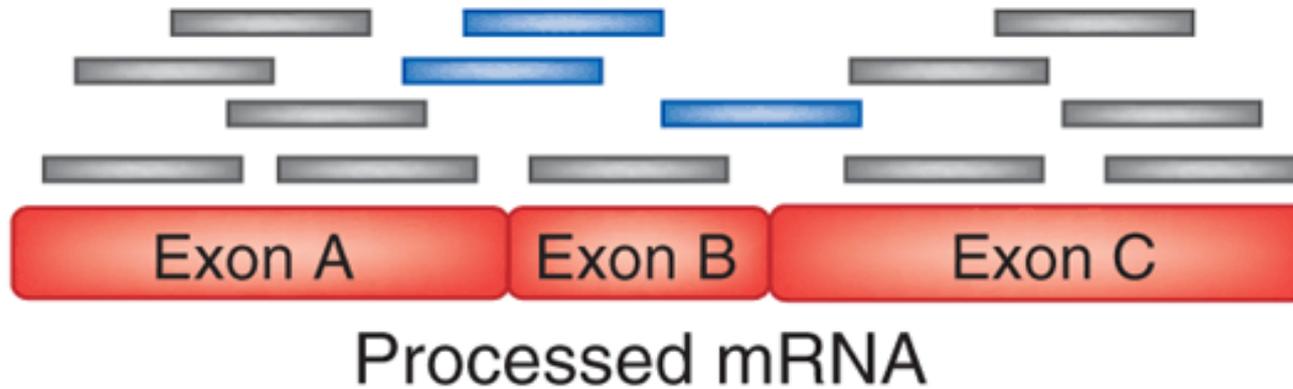


Alignment

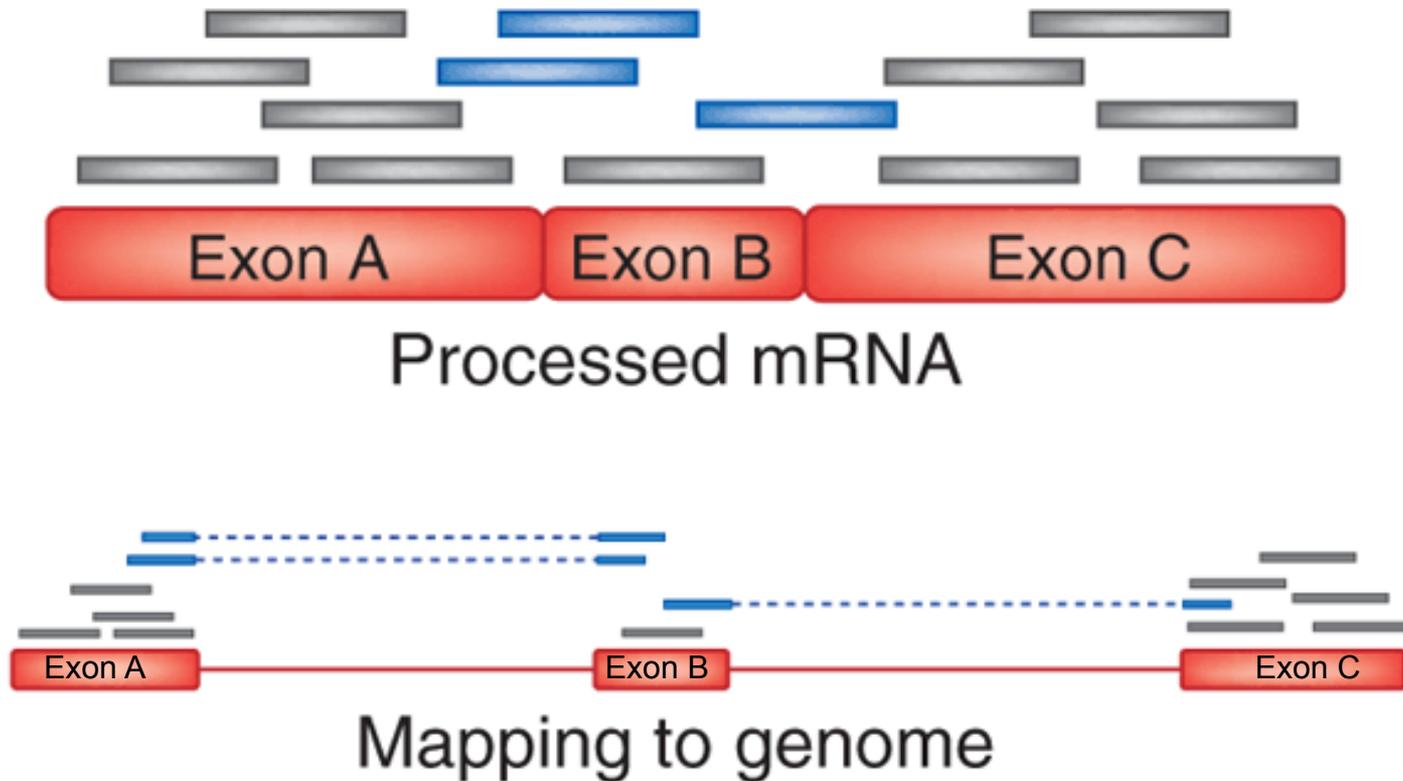
- a large reference sequence is given (genome)
 - up to billions of base pairs
- short reads (<200bps)
- find most probable position of the read in the genome (by inexact string matching)



Alignment - Split Read Mapping (RNA-Seq)



Alignment - Split Read Mapping (RNA-Seq)



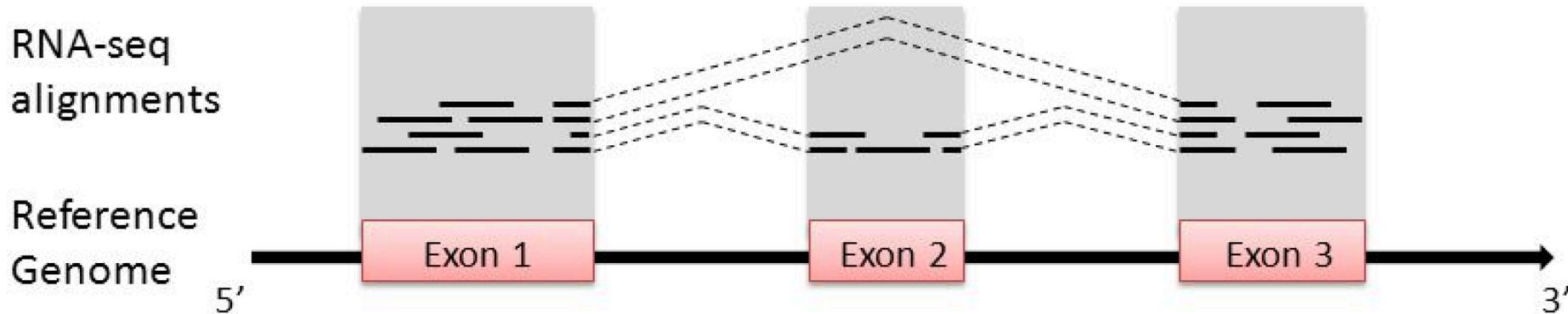
- reads are split within introns when mapped to genome
- aligners use transcript information or try to find splice events (STAR & TOPHAT)

Reference based aligners - Overview

	<i>Time</i>	<i>Precision</i>	<i>Pairs</i>	<i>GAPS</i>	<i>Phred</i>	<i>Memory</i>	<i>Application (Comments)</i>
BOWTIE	+		+	-	-	5GB	General <i>(max. 3 mismatches)</i>
BWA	+		+	+	+	8GB	General <i>(max of 200bps reads)</i>
NOVOALIGN		+	+	+	+	8GB	General <i>(commercial license)</i>
STAR	+		+	-	+	32GB	RNA-Seq <i>(allow split-maps)</i>
BISMARCK	+		+	+	+	10GB	Bisulfite/reduced sequencing

Computers need large memory and a few hours of computation per experiment!

Quantification (Count Matrix)



Simple Counting Approaches

Gene Level - 17 reads

Exon level - exon 1 (8 reads), exon 2 (3 reads), exon 3 (6 reads)

Transcript Level - Exons 1,2 & 3 (10 reads) and exon 1 & 3 (7 reads) *

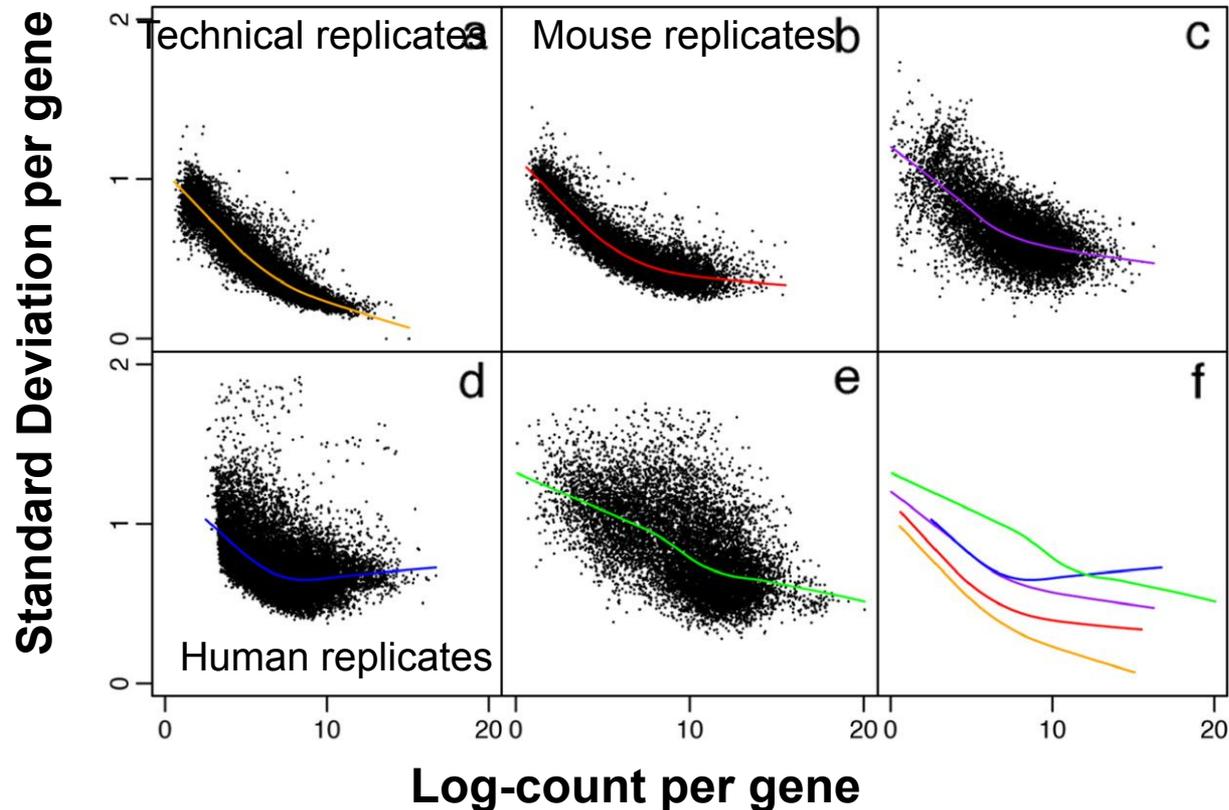
* complex computational methods required (RSe, or TopHAT needed for this)

Fragments per Kilobase (FPKM)

- normalize counts by read size (kb) and RNA-seq library size (mb)

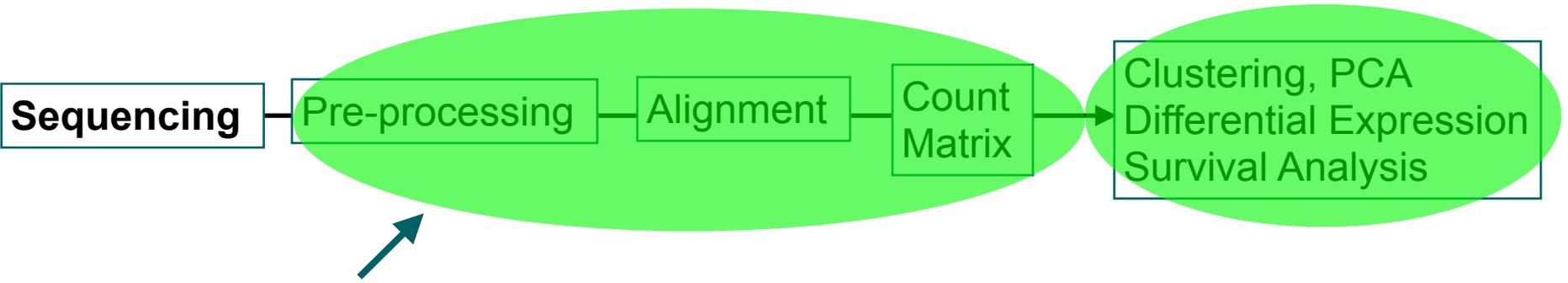
RNA-seq and Differential Analysis

Arrays and RNA-seq have distinct distributions



VOOM analysis is necessary to make variance similar to arrays.

Bioinformatics Pipeline / RNA-seq



Provided by TGCA or your Core Facility!

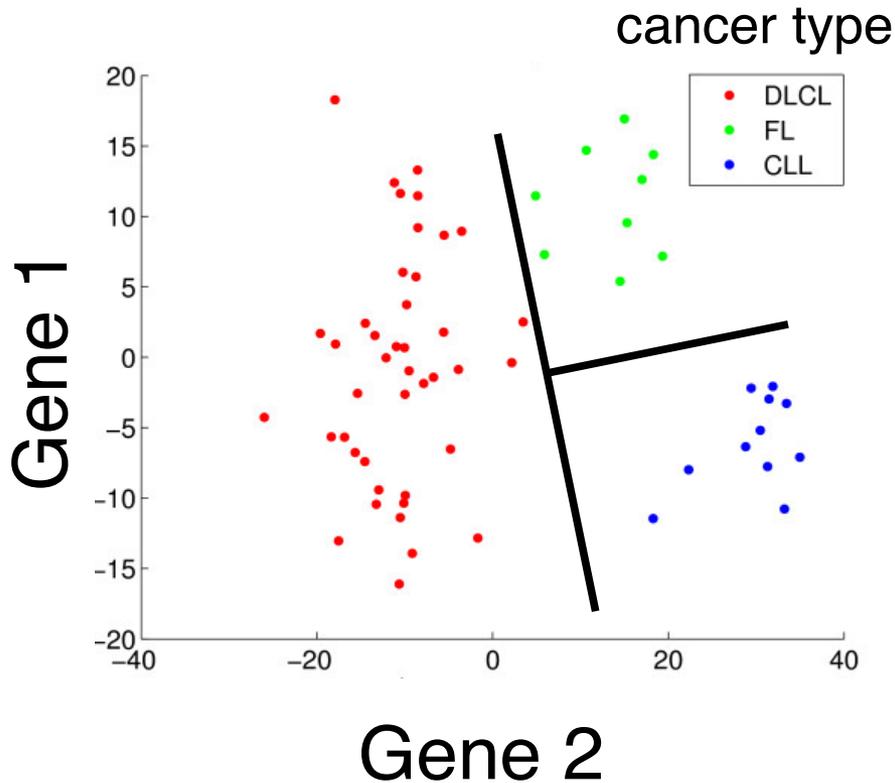
Personalized Medicine

Diagnosis and treatment choices is mostly carried on macromolecular features:

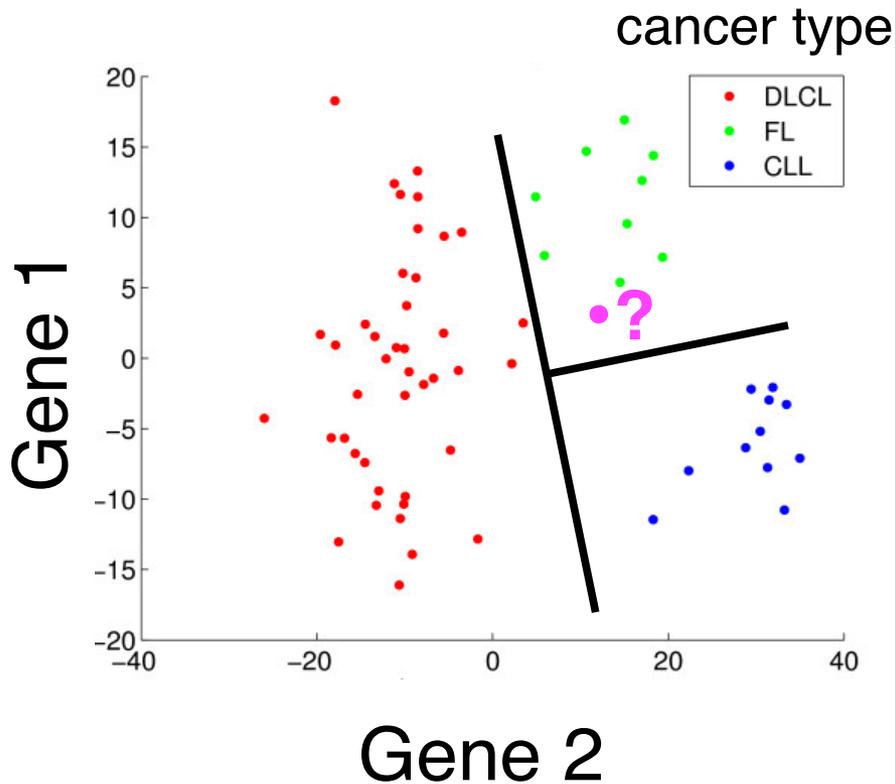
- morphology of tumours (image), symptoms, blood levels

Challenges: use molecular markers (expression or genetics) for diagnosis or treatment selection.

Machine Learning - Classifier



Machine Learning - Classifier



Data

Expression matrix X
(genes vs samples)
classification vector Y
(diagnosis)

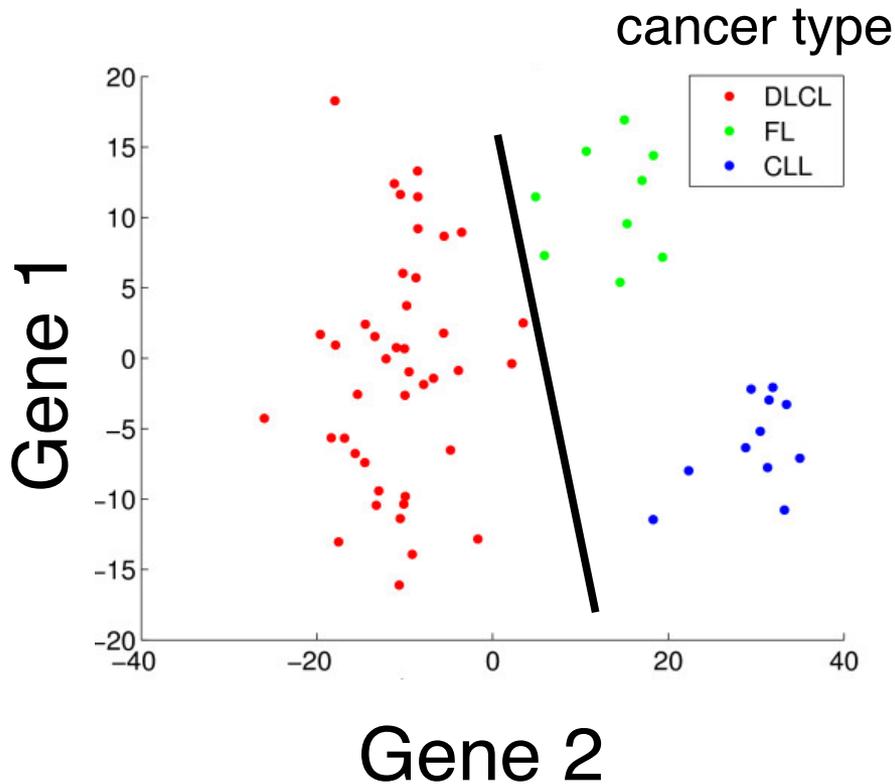
Find a function:

$$f(x) \rightarrow y$$

For new patients X' :

$$f(x') \rightarrow y'$$

Linear Classifier



Linear Function:

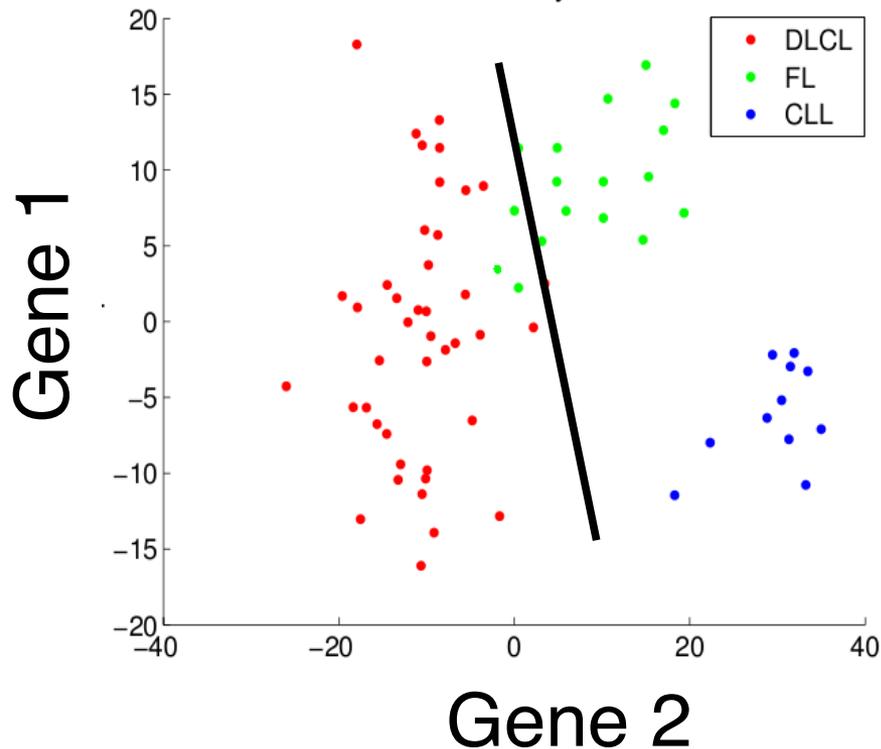
$$f(x, A) = a_0 + a_1x_1 + \dots + a_Lx_L$$

$$f(x, A) > 0 \Rightarrow \text{classe A}$$

$$f(x, A) \leq 0 \Rightarrow \text{classe B}$$

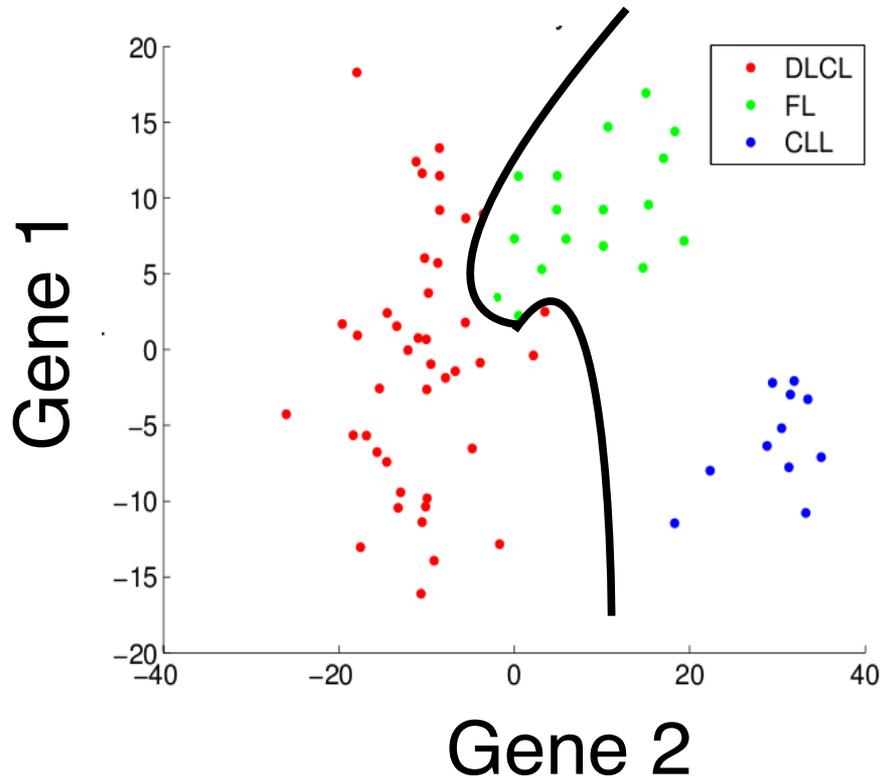
- Works for 2 classes only
 - Train a function for each cancer type
- Find coefficients A
 - estimated with neural networks or support vector machines

Linear Classifier - Problems



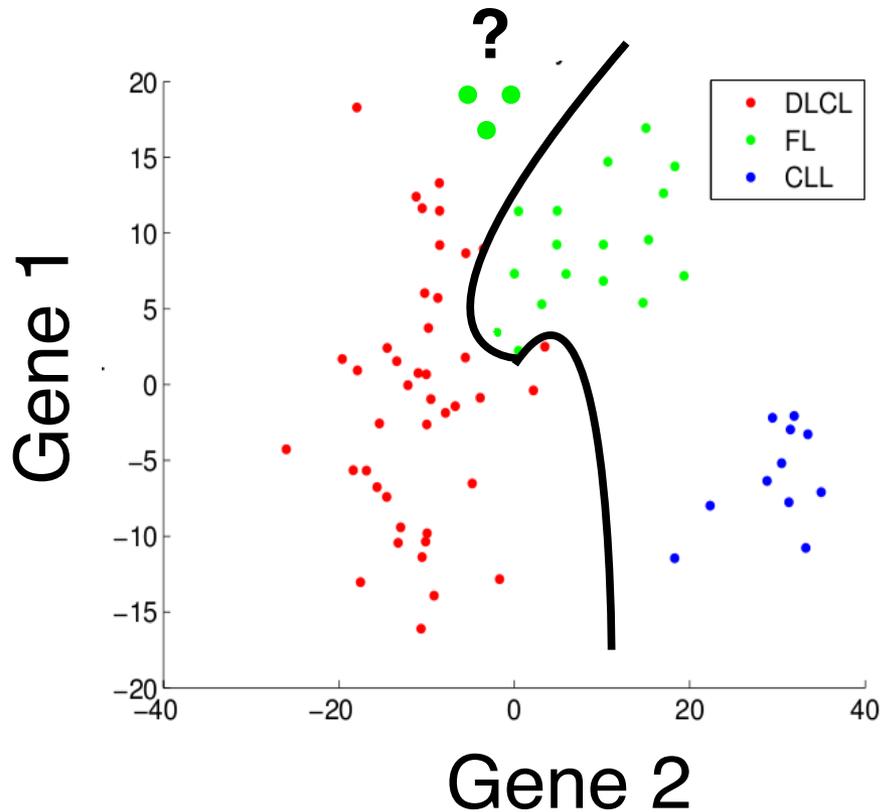
- Most real world problems are not linearly separable!
- There will be always some error!
- Solution: non-linear functions

Nonlinear Classifier - Problems



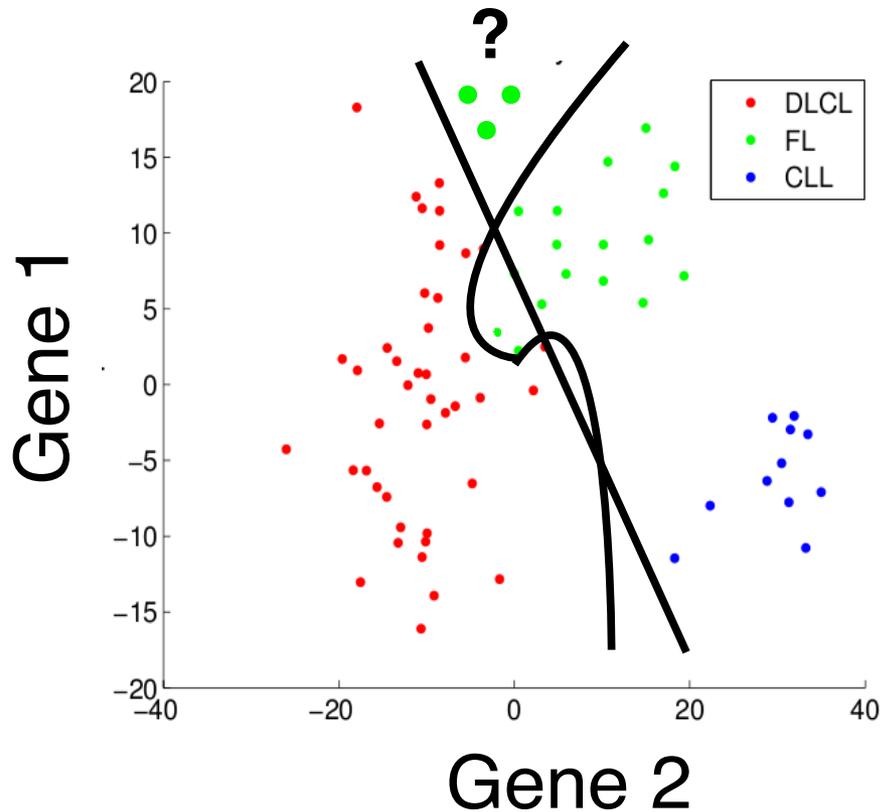
- Polynomial Function
- $f(x, A) = a_0 + a_{11}x^3_1 + \dots + a_{L1}x^3_L$
 $a_{12}x^2_1 + \dots + a_{L2}x^2_L$
 $a_{12}x_1 + \dots + a_{L2}x_L$
- Third order polynomial
- Problem: overfitting

Nonlinear Classifier - Problems



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Nonlinear Classifier - Problems



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Curse of Dimensionality

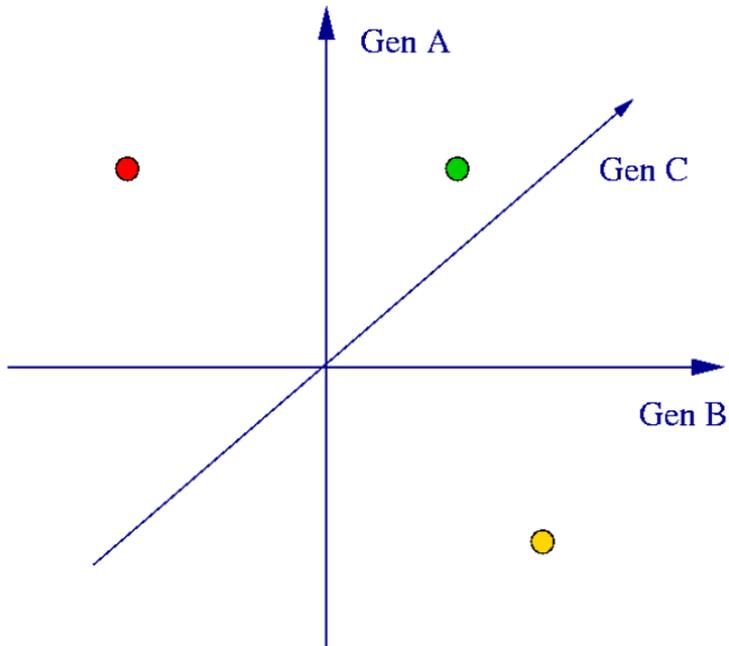
Size of a Euclidean space grows with dimension (number of genes)

Dots (patients) are sparsely distributed in space

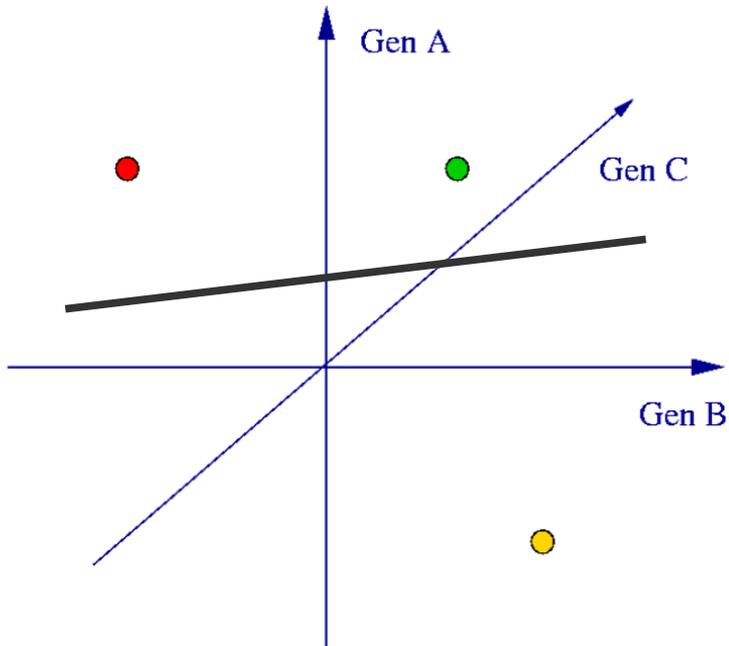
Curse of Dimensionality : Example

Sparse data

- three genes
- 2 patients with known cancer (red vs yellow)
- 1 unknown (green)



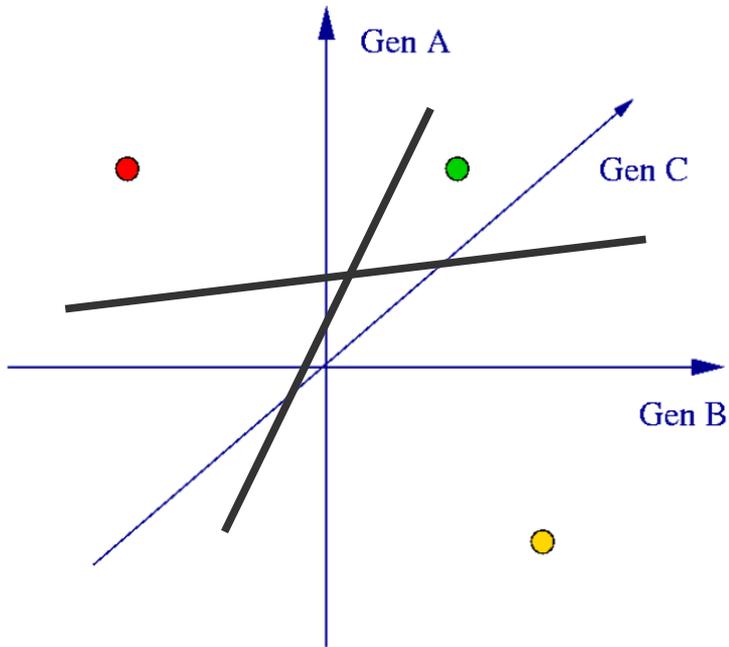
Curse of Dimensionality : Example



- Sparse data
 - three genes
 - 2 patients with known cancer (red vs yellow)
 - 1 unknown (green)

Perfect classifier (on training)

Curse of Dimensionality : Example

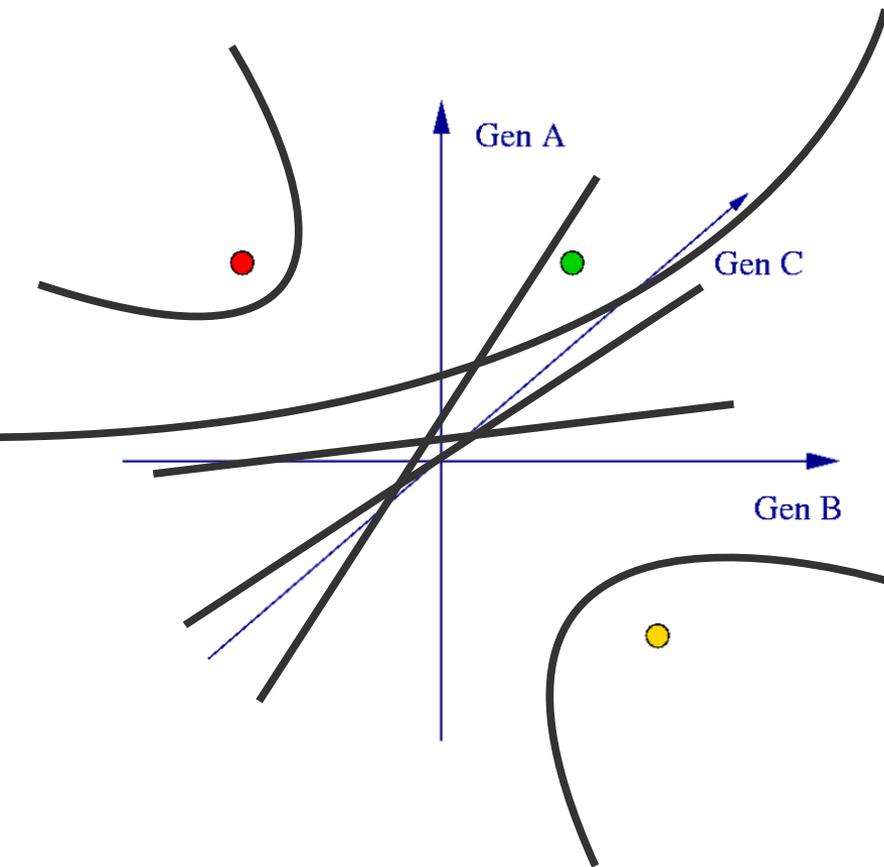


- Sparse data
 - three genes
 - 2 patients with known cancer (red vs yellow)
 - 1 unknown (green)

Both are perfect classifiers
(on training)

Hard to generalise!

Curse of Dimensionality : Example



- There are millions of perfect linear classifiers
- And even more non-linear classifiers!

Dealing with Curse of Dimensionality

- Have a proper training / test evaluation procedure
- Use classifiers which are as simple as possible
- Reduce the dimension of your data (feature selection or PCA)

Classifier Evaluation

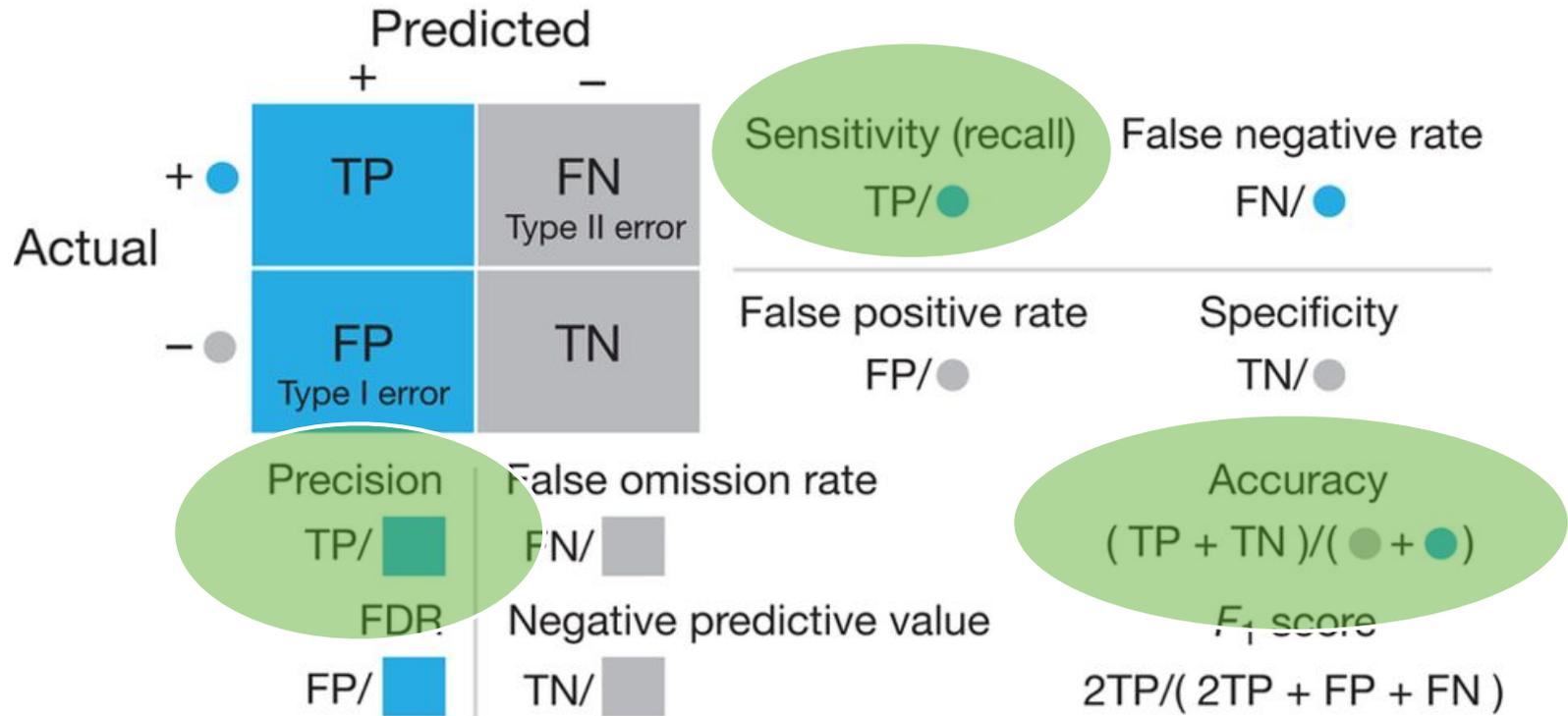
Measures for a two class problem (cancer + vs. non-cancer -)

		Predicted	
		+	-
Actual	+ ●	TP	FN Type II error
	- ●	FP Type I error	TN

Source: Lever et al., Nat. Methods (2016)

Classifier Evaluation

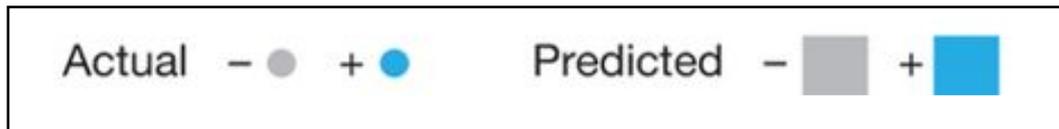
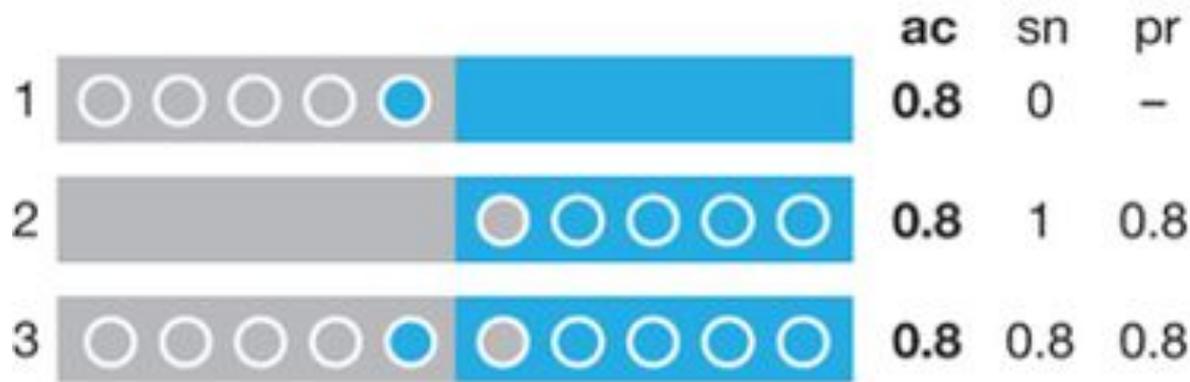
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Classifier Evaluation

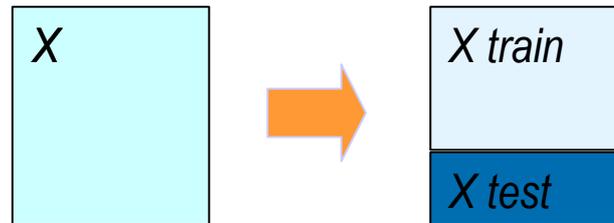
Measures for a two class problem (cancer + vs. non-cancer -)



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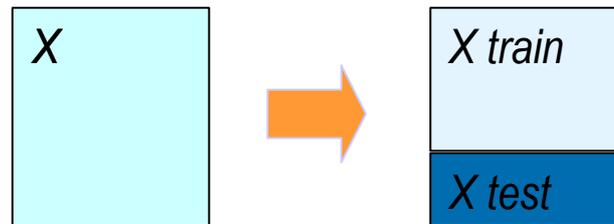
Classifier Evaluation

- The performance of your classifier needs to be evaluated at your test data:
 - an independent "validation cohort"
 - or a large (1/3 of samples) and have similar distribution of classes as train data



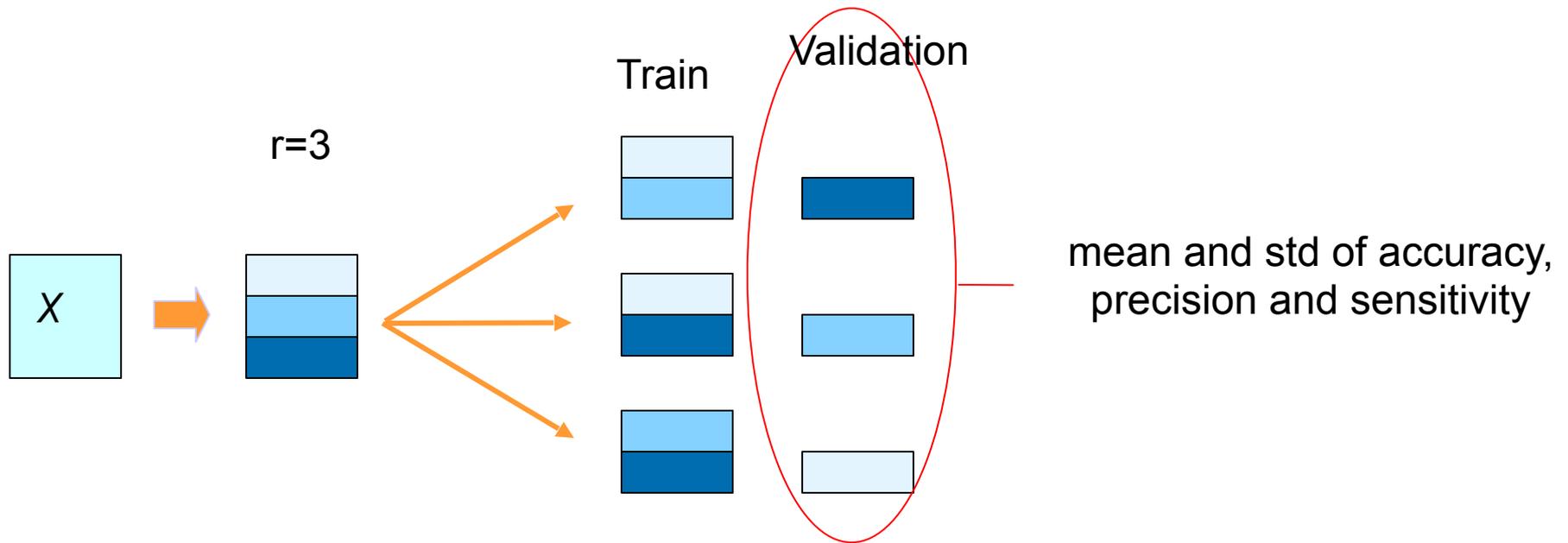
Classifier Evaluation

- The performance of your classifier needs to be evaluated at your test data:
 - an independent "validation cohort"
 - or a large (1/3 of samples) and have similar distribution of classes as train data



- Never use test data to improve classification (choose a better classifier or marker gene)
 - For this you need to establish validation data (or cross validation)

Cross-validation



Elastic Net

Is based on a linear function:

$$f(x, A) = a_0 + a_1x_1 + \dots + a_Lx_L$$

$$f(x, A) > 0 \Rightarrow \text{classe A}$$

$$f(x, A) \leq 0 \Rightarrow \text{classe B}$$

- Find coefficients A , *while most of them have 0*.
 - A shrinkage factor (λ) controls the number of genes selected.
 - Shrinkage factor can be automatically identified with cross-validation.

Break time !

Survival Analysis

- Can be used to evaluate if characteristics of a patients indicates an increase/decrease risk of survival
- clinical: tumour type, gender
 - Molecular: expression of a gene, mutation

Common Survival Tests:

- Cox proportional hazards regression (not seen here)
 - Compares survival with a numeric variable
- Kaplan-Meier graph / Log-rank test
 - compares the survival of groups of individuals

Kaplan-Meier graph / Log-rank test

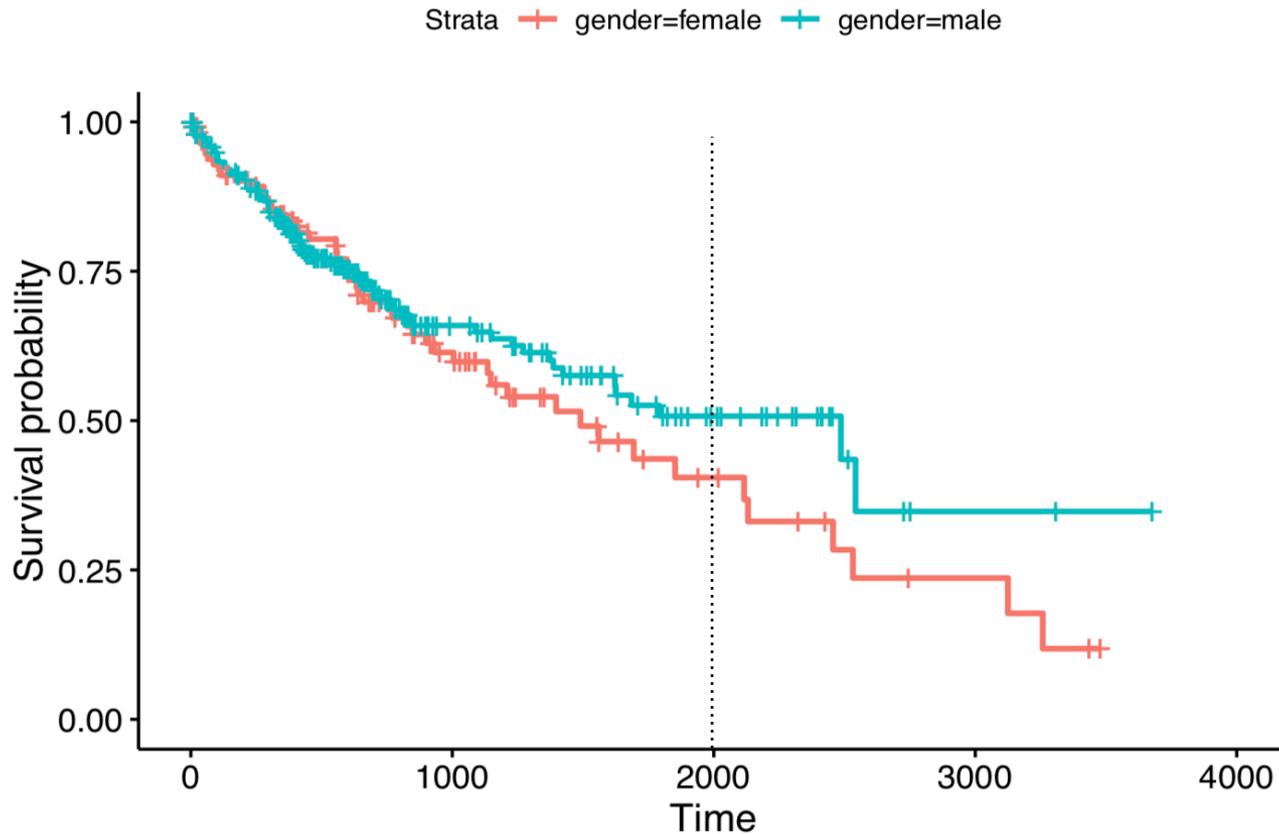
Data:

- **Event:** death / alive
- **Time:** period between first and last observation.
- **Characteristics:** sex, tumor grade

<i>Patient</i>	<i>Status</i>	<i>Time</i>	<i>Sex</i>
<i>1</i>	<i>Dead</i>	<i>343</i>	<i>Male</i>
<i>2</i>	<i>Alive</i>	<i>20</i>	<i>Male</i>
<i>3</i>	<i>Alive</i>	<i>300</i>	<i>Female</i>
<i>4</i>	<i>Dead</i>	<i>200</i>	<i>Male</i>

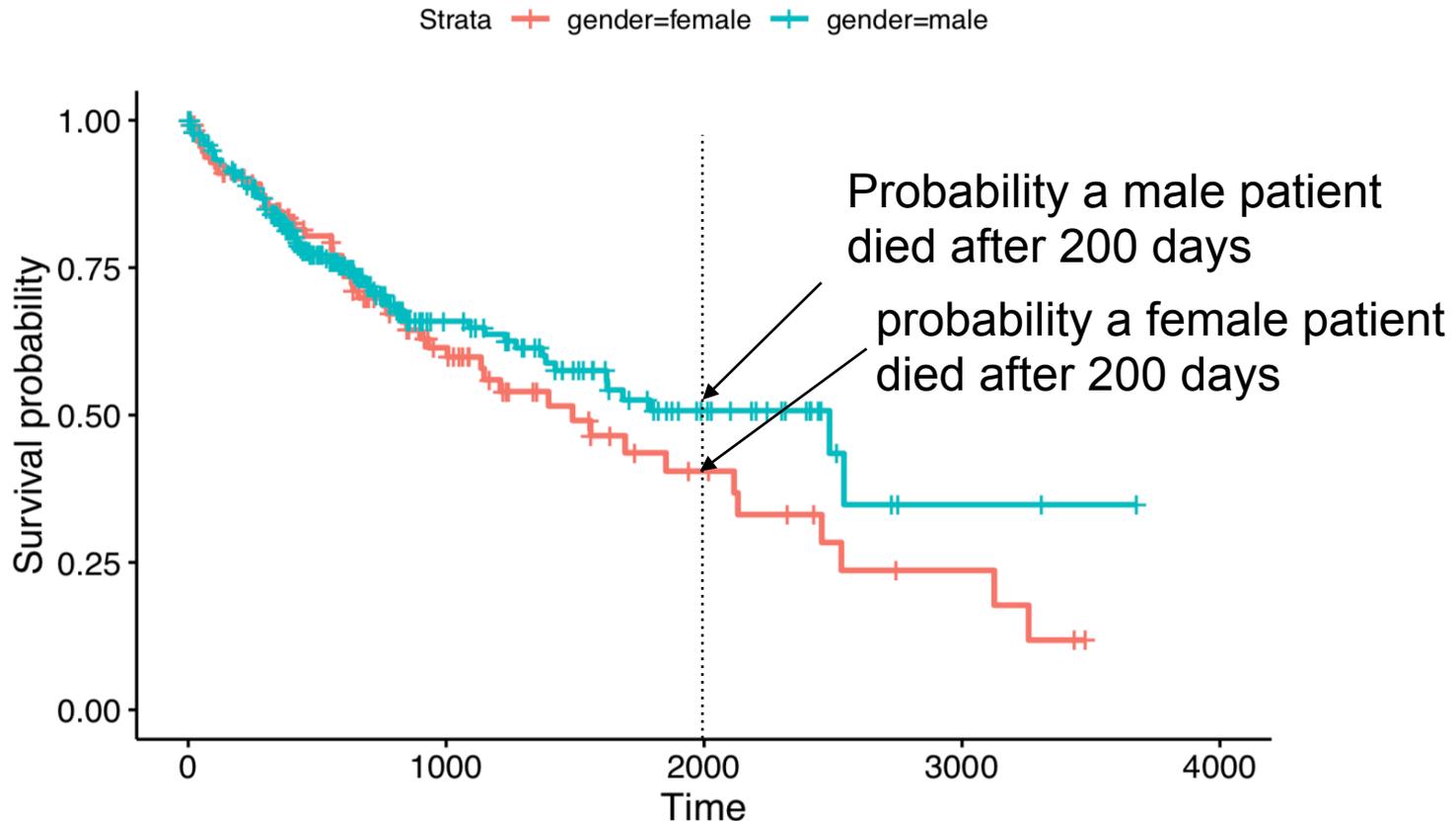
Kaplan-Meier plot

Survival of LHC patients - male vs. Female



Kaplan-Meier plot

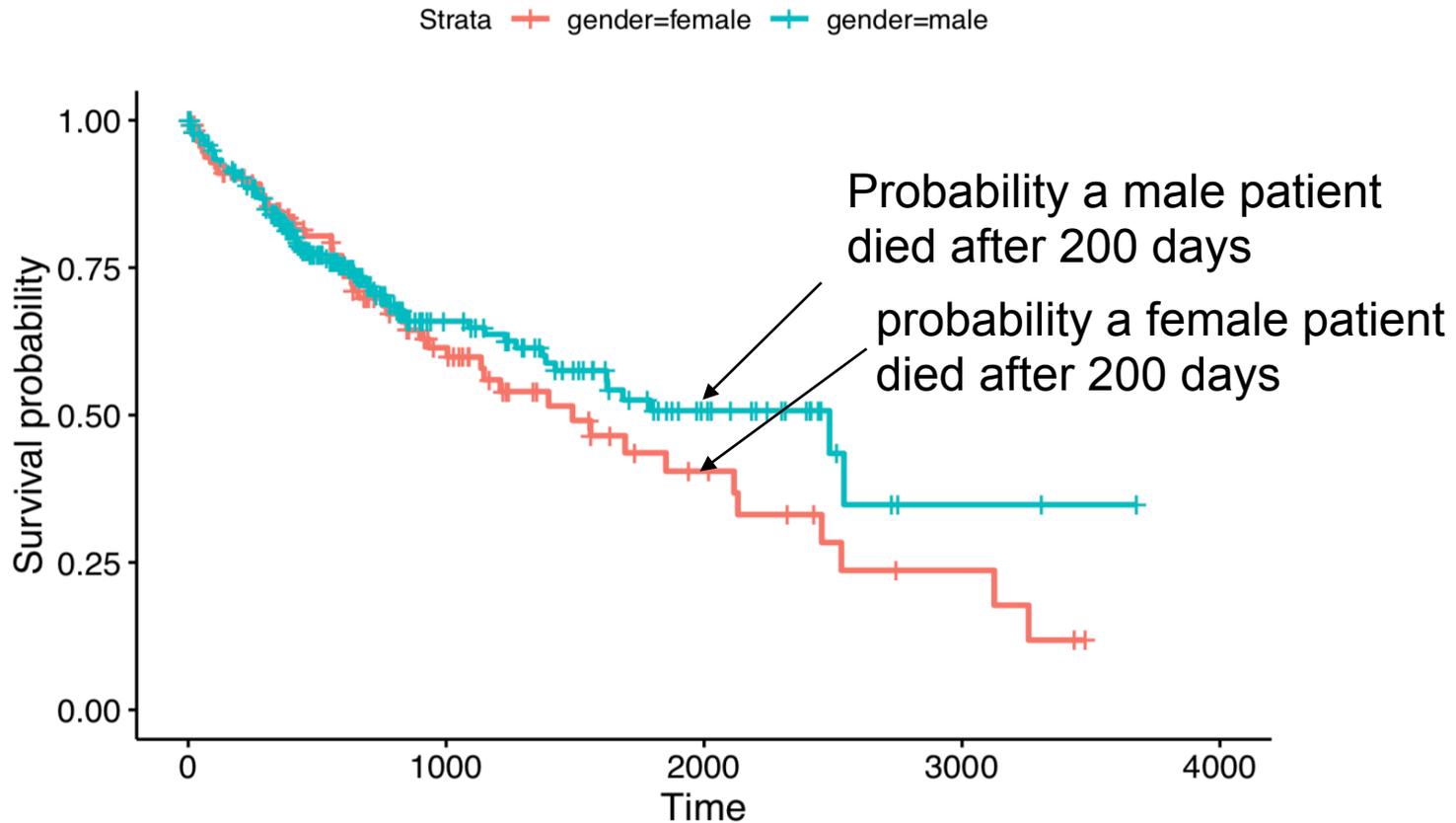
Survival of LHC patients - male vs. Female



$$\text{Probability (X days)} = \frac{\# \text{ cases alive after X days}}{\# \text{ cases measured after X days}}$$

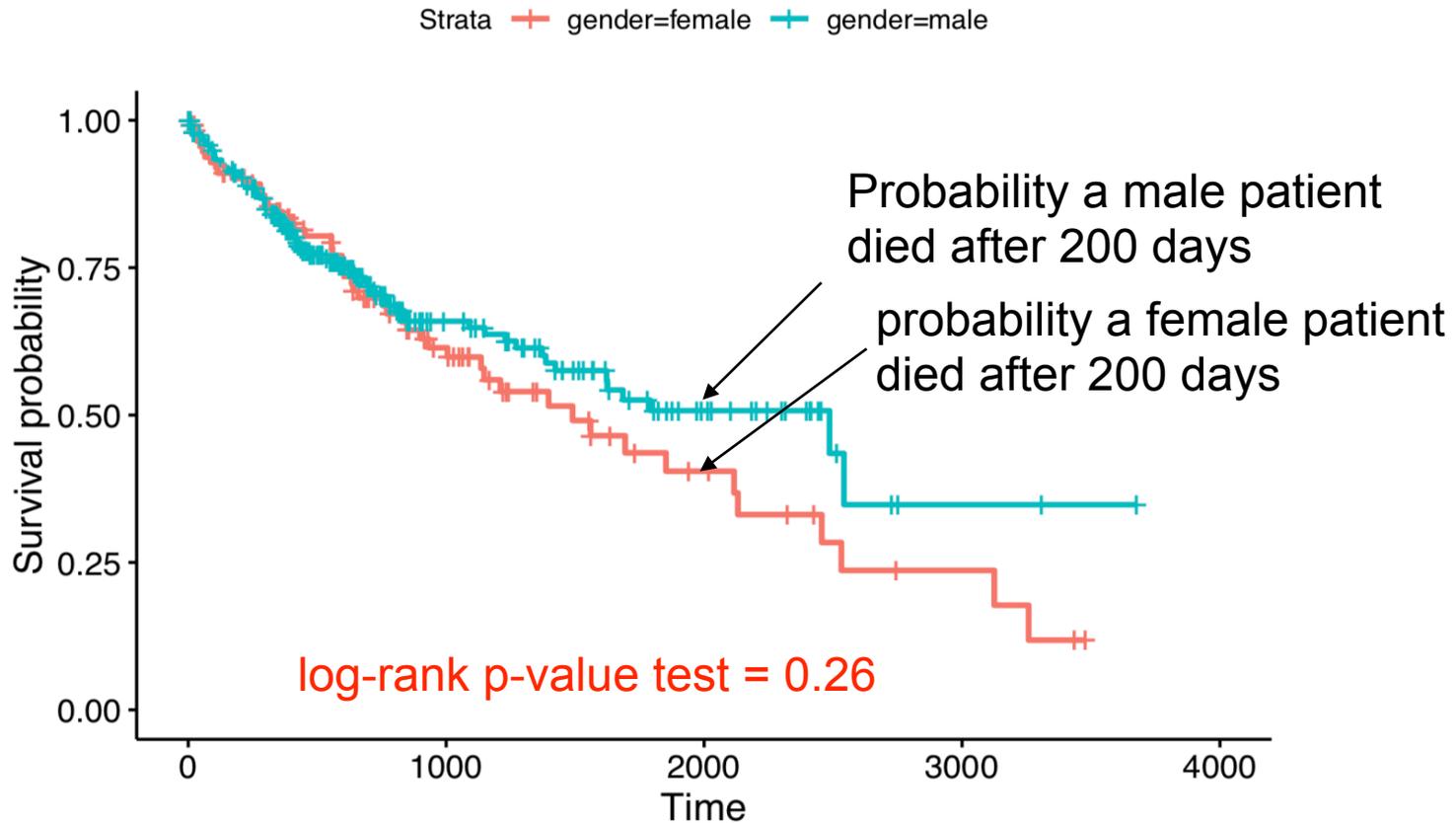
Log-rank test

Is the survival difference significant?

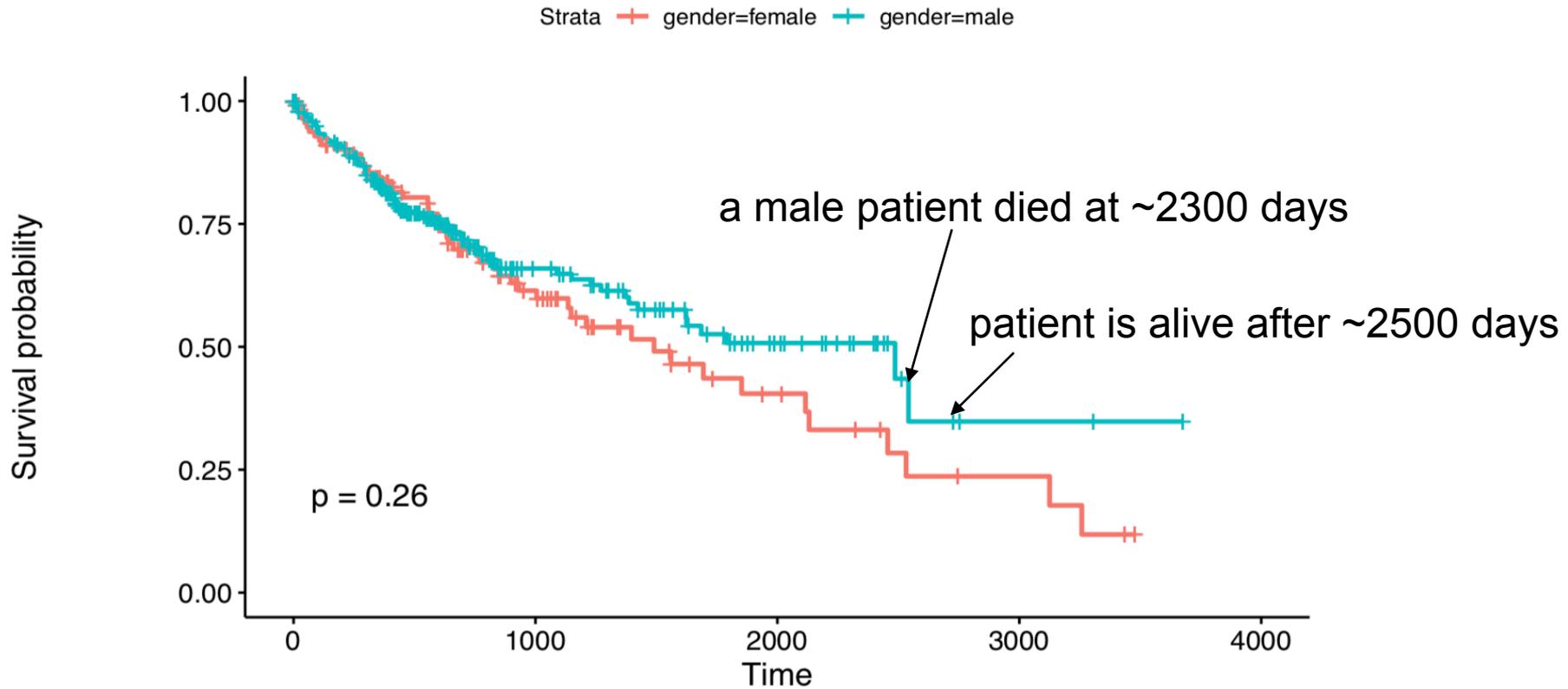


Log-rank test

Is the survival difference significant?



Kaplan-Meier plot

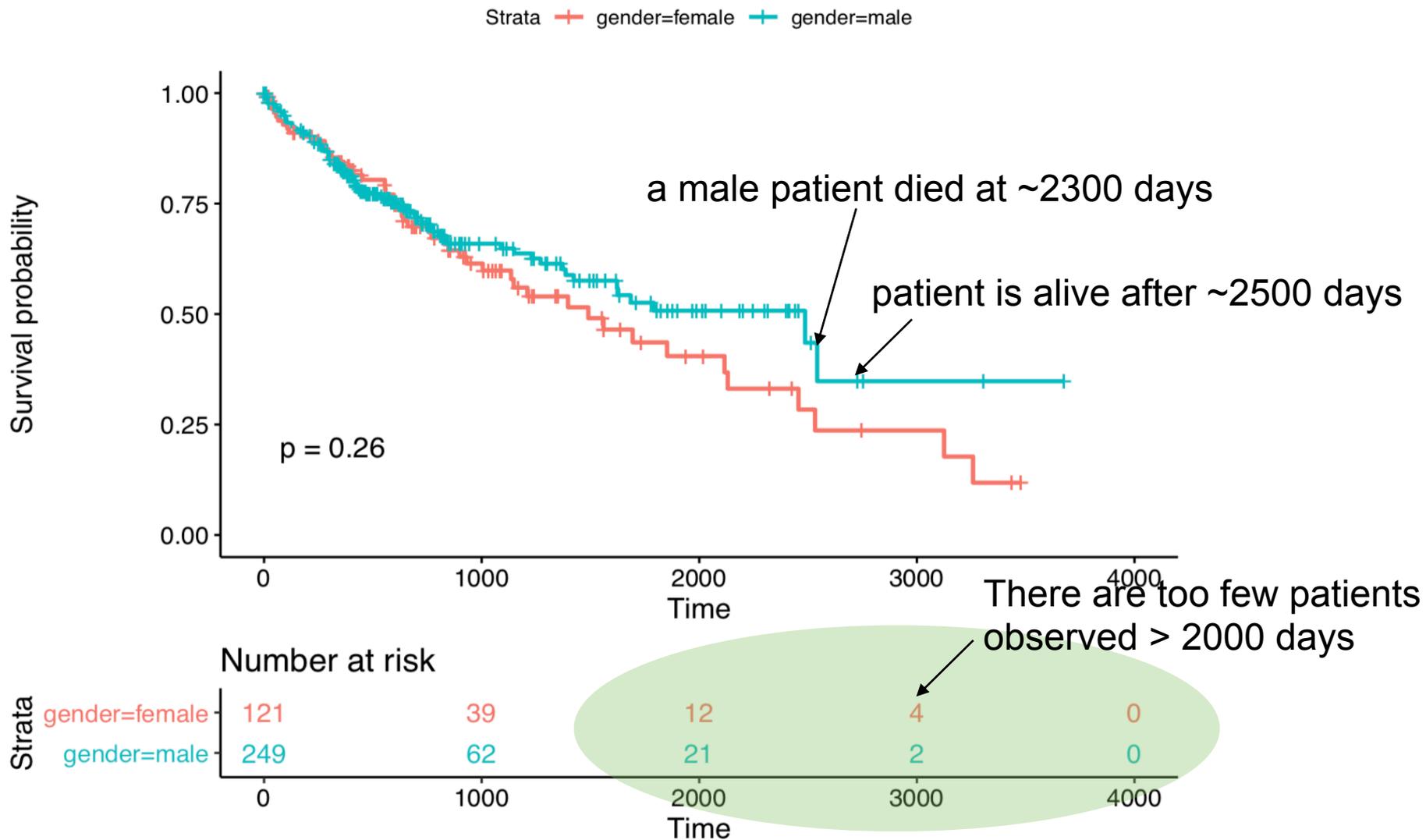


Number at risk

Strata	0	1000	2000	3000	4000
gender=female	121	39	12	4	0
gender=male	249	62	21	2	0

Time

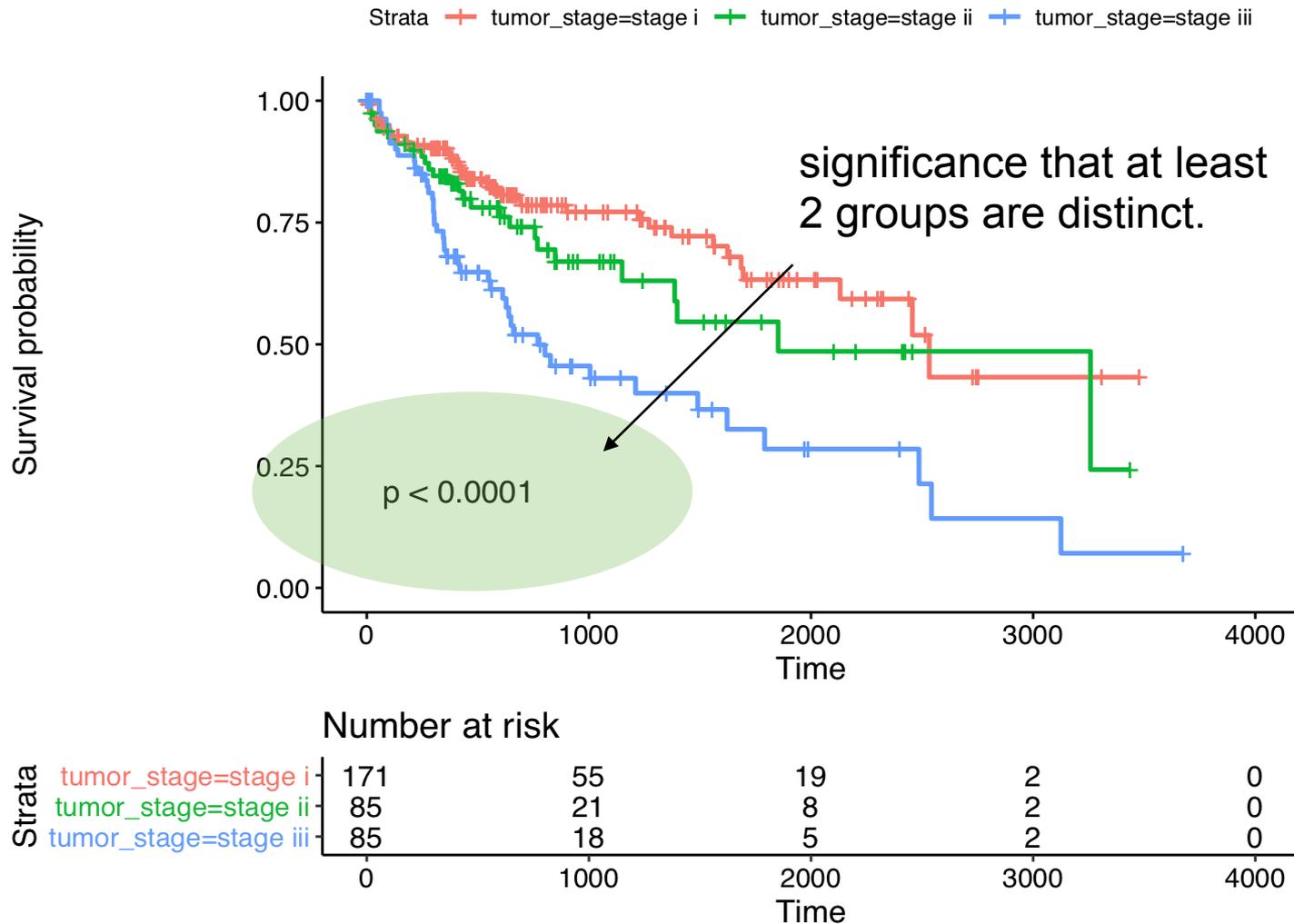
Kaplan-Meier plot



Kaplan-Meier / Log-Rank Test

KM and LRT can compare several groups at a time.

Survival vs Tumour stage at diagnosis



Survival Analysis and Biological Markers

How to perform survival analysis on biological markers?

1. Given their continuous nature of gene expression, Cox hazards test is recommended.
2. An alternative is to group patients by expression of a gene (low/high expression) and use Kaplan-Meier plots (seen in practical).

Important: if you test several markers you need to correct for multiple testing!!!



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