

Computational Human genomics

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Chapter 1

Introduction

1.1 Basic principles

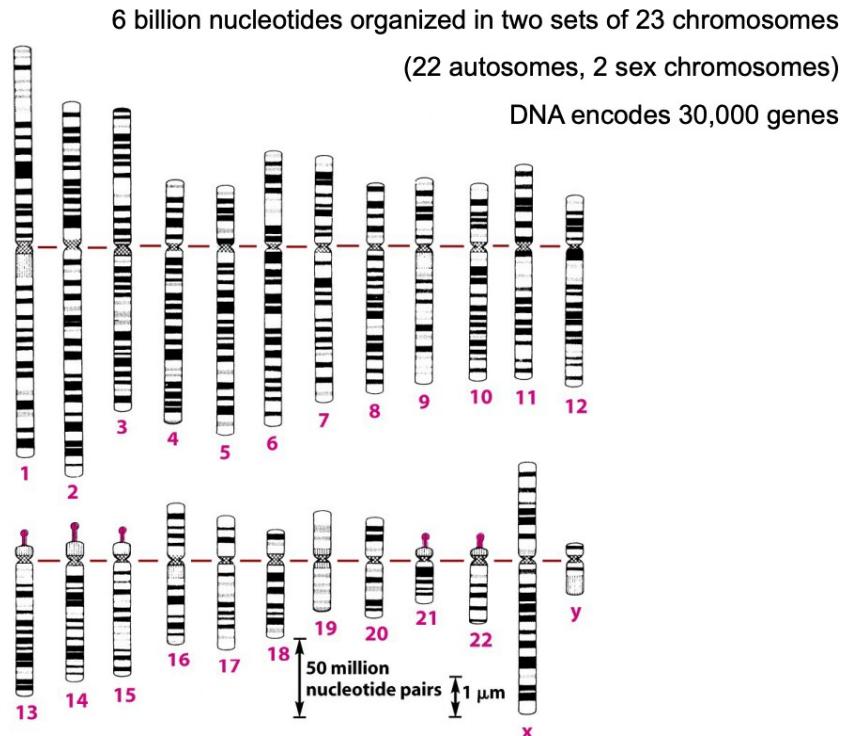


Figure 1.1

The words variations, aberrations and lesions are often interchanged. Aberrations and lesions are mainly used for acquired lesions, instead variations are mainly used for the inherited ones.

1.1. BASIC PRINCIPLES

1.1.1 Genetic Make-Up

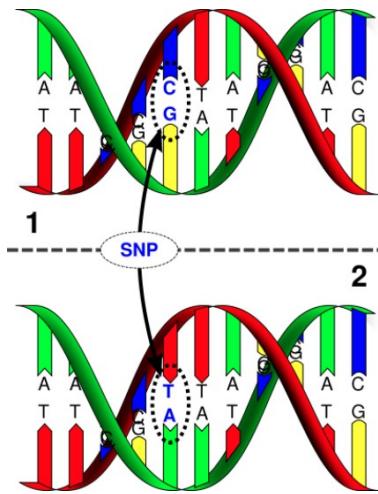


Figure 1.2

Single Nucleotide Polymorphism (SNP) is a sequence variation affecting single bases (point mutations) (figure 1.2).

The genomes of two unrelated individuals have about 1% of different bases → that percentage corresponds to the SNPs.

But looking at the **Copy Number Variants (CNV)**, that difference will be way higher than only 1% DNA not present in only two copies, but in multiple, single or even zero copies (hemizygous loss, homozygous loss). They are less known as inherited type of variants because they are harder to detect and identify, but they provide a lot of uniqueness in each of us.

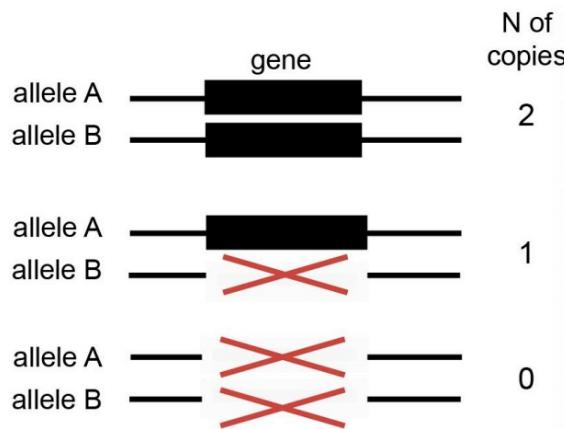


Figure 1.3

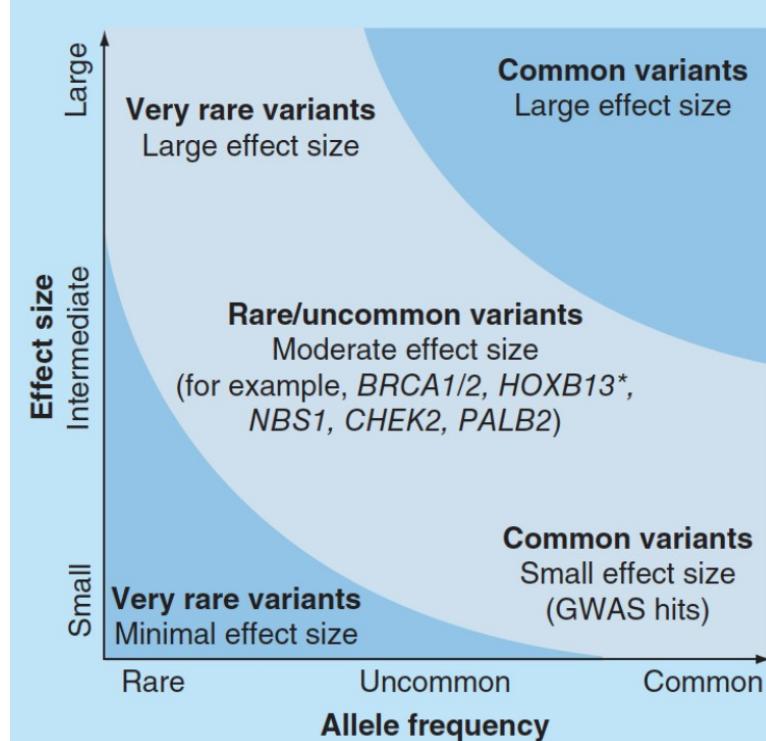
Why are SNPs and CNVs so important?

1.1. BASIC PRINCIPLES

They are responsible of human diversity → genetic changes
Hundreds of CNVs per individual and 20% of them potentially affect protein-coding genes

Differences in Genetic Make-up:

Very common variants are variants that are distributed in the population as the common allele, so that 1/2 of the population has a heterozygous genotype at that position, 1/4 has a homozygous genotype for one allele and 1/4 has a homozygous genotype for the other allele.



The **penetrance** is the proportion of individuals carrying an allele (or a genotype) that also expresses the trait (phenotype) associated with it. Obviously, penetrance is directly associated with the size of the effect produced by the variant.

The **allele frequency** is calculated by dividing the number of times the allele of interest is observed in a population by the total number of copies of all the alleles at that particular genetic locus in the population.

The allele frequency is low with very rare variants

Well known variants: BRCA1/2, HOXB13, NBS1, PALB2, CHEK2 → they have moderate size effects, meaning that all the people who have the variants, have the disease

1.1.1.1 Differences in genetic Make-Up, example

Absorption, distribution, metabolism and elimination (ADME) genetic variants determine pharmacokinetic variability of certain compounds, influencing the patients' treatment response. Both common and rare variants are involved.

1.1. BASIC PRINCIPLES

Table 1. Comparison between pharmacogenomics approaches.

PGx Approach	GWAS	SNPs Panel	Candidate SNP
Sample size	Tailored for large populations	Tailored for small populations	Tailored for small populations
Number of investigated markers	Larger numbers	1–2 thousand	Smaller number
Hypothesis	Hypothesis-free and hypothesis generating	Hypothesis-free and hypothesis generating/PK and PD coverage	Selected on a priori knowledge
Study Design	Exploratory	Confirmatory/Exploratory	Confirmatory
Limitations	False Negative/control for multiple testing	Coverage of limited genes	False positive/non-replication of results/low genetic coverage

PGx: pharmacogenomics; GWAS: genome-wide association study; SNP: single nucleotide polymorphism.

Three main ways to study genetic variants:

- GWAS (genome-wide association study)
- SNPs panel
- Candidate SNP

For example, in terms of hypothesis, if I study all the variants in the human genome and I query them in a large population, I generate data without specifying SNP to search. Instead, if I have a very specific hypothesis, for example I want to query if a SNPs in the CYP gene relates to the conversion of androgens to estrogens, I don't need to run an GWAS or a wide SNPs panel. I can query those SNPs because I have an *a priori* hypothesis and I want to test them.

This type of differential design for an experiment it is not only true for inherited variants and ADME genes, but also to predisposition to diseases and to study human tumors.

Precision medicine → treatment (or dosage) of a patient based on their individual traits: takes into consideration genetic and genomic of the individual and tumor/disease cells

- Drink beer and turn red → ADME gene
- Athletes with a deletion of a gene, the steroids were not found in the anti-doping tests

1.1.2 Acquired DNA aberrations

Somatic variants are the variants NOT inherited from parents and not transmitted to offspring. They are:

- **Single Nucleotide Variants (SNV)** are somatic changes of single nucleotides present in only certain cells, instead of SNPs that are present in all cells of our body.
- **Indels** are changes that involve few nucleotides by INsertion and DEletion

1.1. BASIC PRINCIPLES

- **Rearrangements** are mutations that can involve events like translocations, inversion, chromothripsy,... usually these events are caused by breakage in the DNA double helices at two different locations, followed by a rejoining of the broken ends to produce a new chromosomal arrangement of genes, different from the beginning
- **Somatic copy number aberrations (SCNA)** are somatic changes similar to CNVs. They can be every change related to the number of copies like loss of a portion of a genome, loss of both alleles, extra copies...

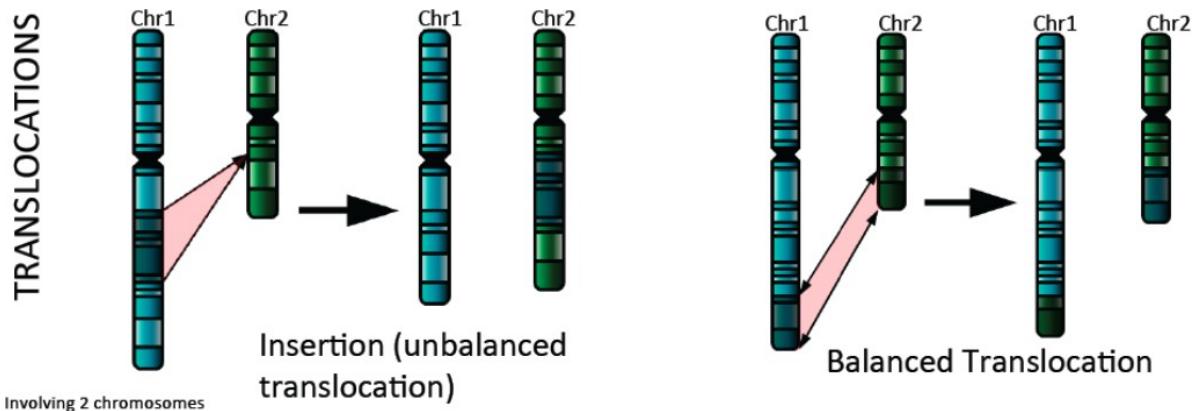


Figure 1.4: translocations

Rearrangements include:

- **Balanced translocation** (figure 1.4): you conserve the quantity of DNA, there isn't any loss or gain.
- **Unbalanced translocation:** A genomic portion is translocated from a chromosome to another, there is not vice versa.
- **Inversions** in only ONE chromosome: everything is normal instead in the break points.

1.1. BASIC PRINCIPLES

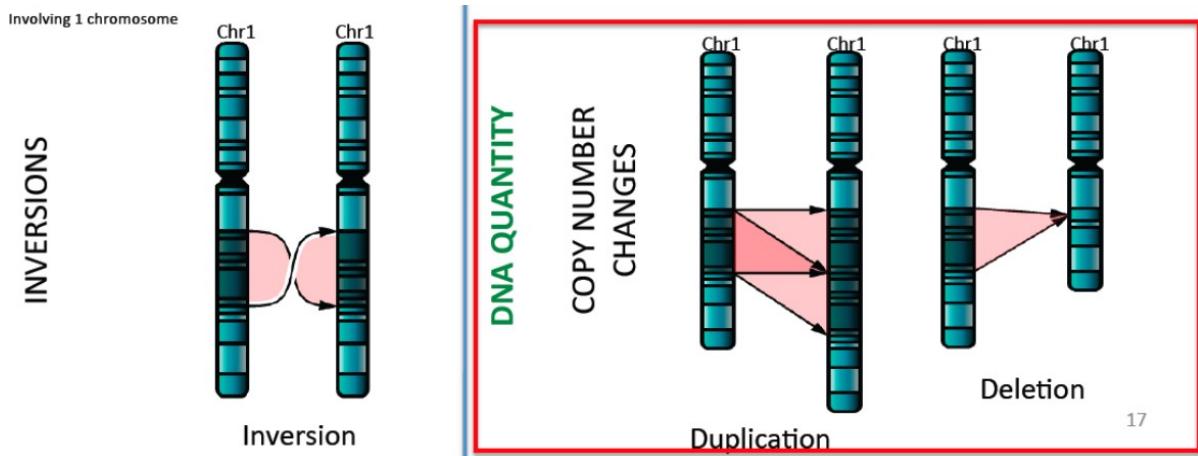


Figure 1.5: Duplications inversions and deletions

- **Copy number changes:** duplication or deletion. It could happen in the same chromosome but also in different chromosomes.

Other important modifications:

- **Chromoplexy:** a class of complex somatic DNA rearrangements whereby abundant DNA deletions and intra- and inter-chromosomal translocations that have originated in an interdependent way occur within a single cell cycle.
- **Chromothripsis:** a clustered chromosomal rearrangement in confined genomic regions that results from a single catastrophic event, usually limited to one chromosome.
- **Kataegis:** a phenomenon that is characterized by large cluster of mutations (hypermutation) in the genome of cancer cells. An APOBEC family enzyme might be responsible for the kataegis process.

1.2. EXPERIMENTAL APPROACHES

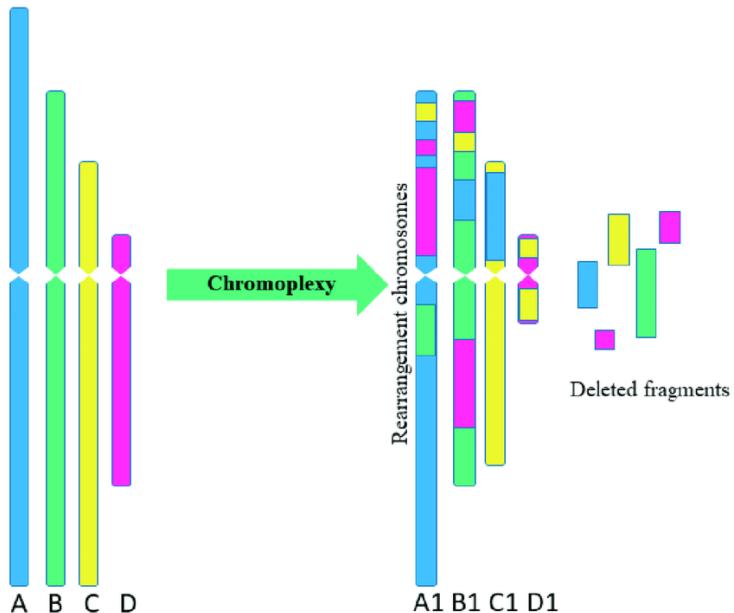


Figure 1.6: Chromoplexy

When an aberration (clonal) occurs, all the cells will harbour the aberration and at some point another aberration (subclonal of the other) could appear in just one cell line. The **clonal** aberration is present in all the cells, the **subclonal** aberration is inherited in just one cell line. Clonality is an important information that allow us to study evolution.

1.2 Experimental approaches

Experimental techniques to detect variants/aberrations **prior to NGS**: a failure because it was very hard to determine the starting points of the aberrations.

1.2. EXPERIMENTAL APPROACHES

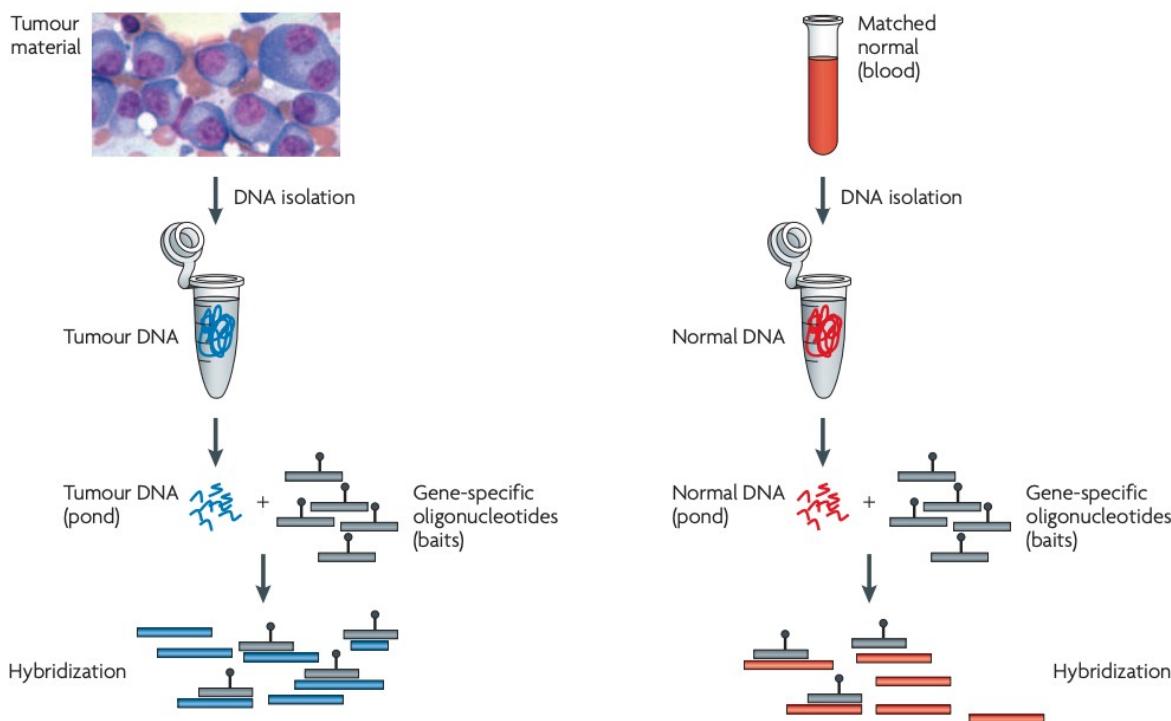


Figure 1.7: Meyerson et al. 2010, “Advances in Understanding Cancer Genomes through Second-Generation Sequencing.”, Nature Reviews Genetics, <https://doi.org/10.1038/nrg2841>

Bulk of tumor tissue/cells from the blood procedure (figure 1.7):

- 1) DNA isolation.
- 2) Gene-specific oligonucleotides (**baits**) that get hybridized onto the tumor DNA → the baits have a tag that allows them to be isolated.
- 3) The DNA does get fragmented.
- 4) The captured DNA is eluted and prepared into sequencing libraries.
- 5) Sequencing.
- 6) Aligned to the bait sequences.

We repeat the procedure for healthy cells of the same individual in order to **detect somatic mutations**.

1.2. EXPERIMENTAL APPROACHES

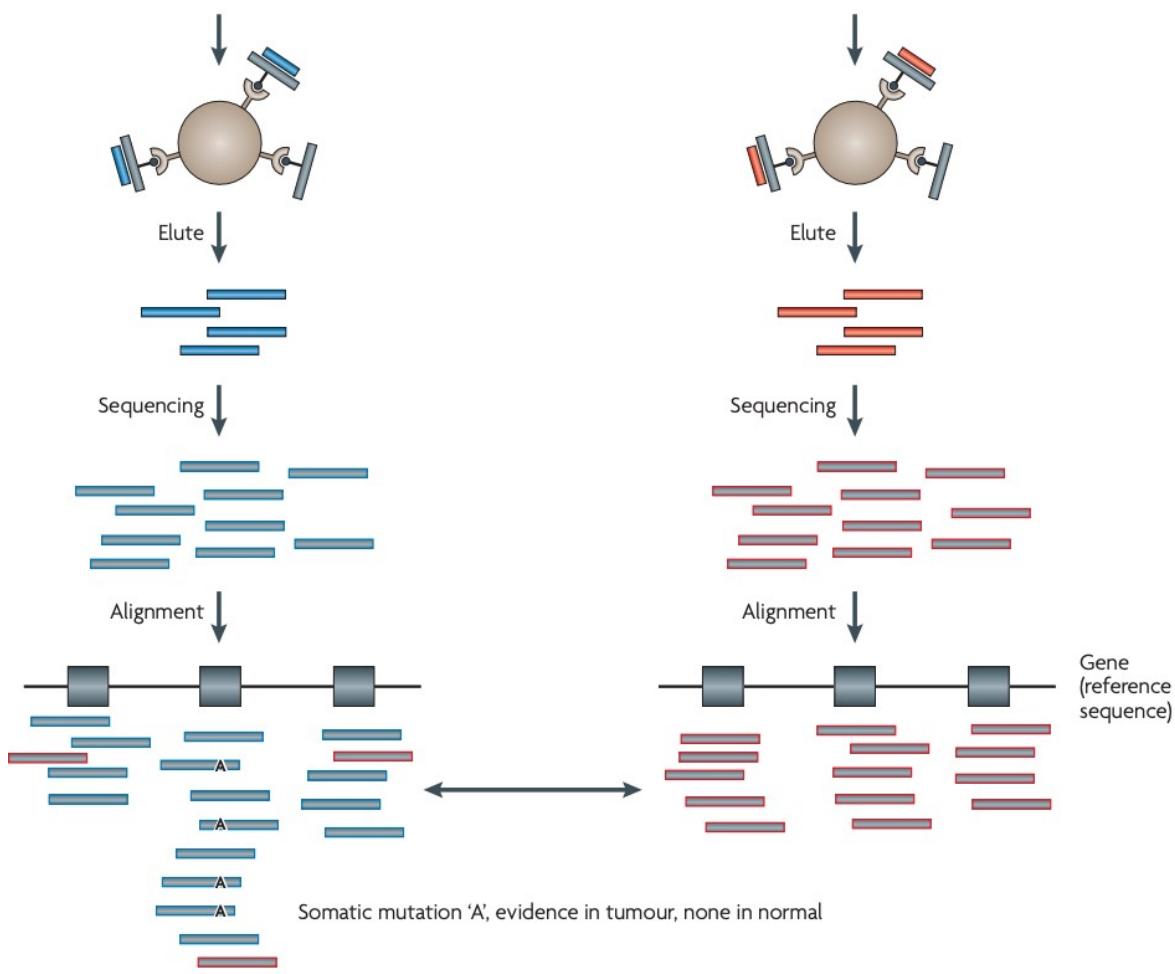
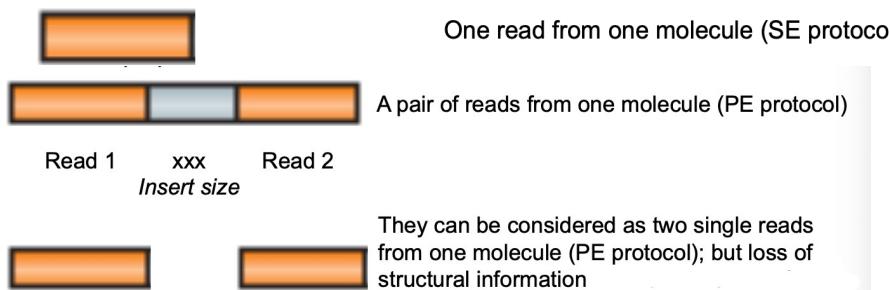


Figure 1.8: Beads capture

We sequence baits because is way cheaper (exons of 50 bases instead the whole genome)

After fragmentation procedure, before adding the adapters, we can choose between two different sequencing approaches 1.2:



1.2. EXPERIMENTAL APPROACHES

- **Paired End (PE) sequencing**

You will sequence only one part of a molecule (length of 150 bp → based on the power of the sequencing machine we are using). You will know exactly 150 bp for every molecule you sequence, but you lose information (the second end of the pair).

- **Single End (SE) sequencing**

You information about the length of the DNA portion between the ends. It's more expensive, but:

- it gives information about the localization of the molecule
- you can treat each end as single read

1.2.1 Information after reads mapping over reference genome

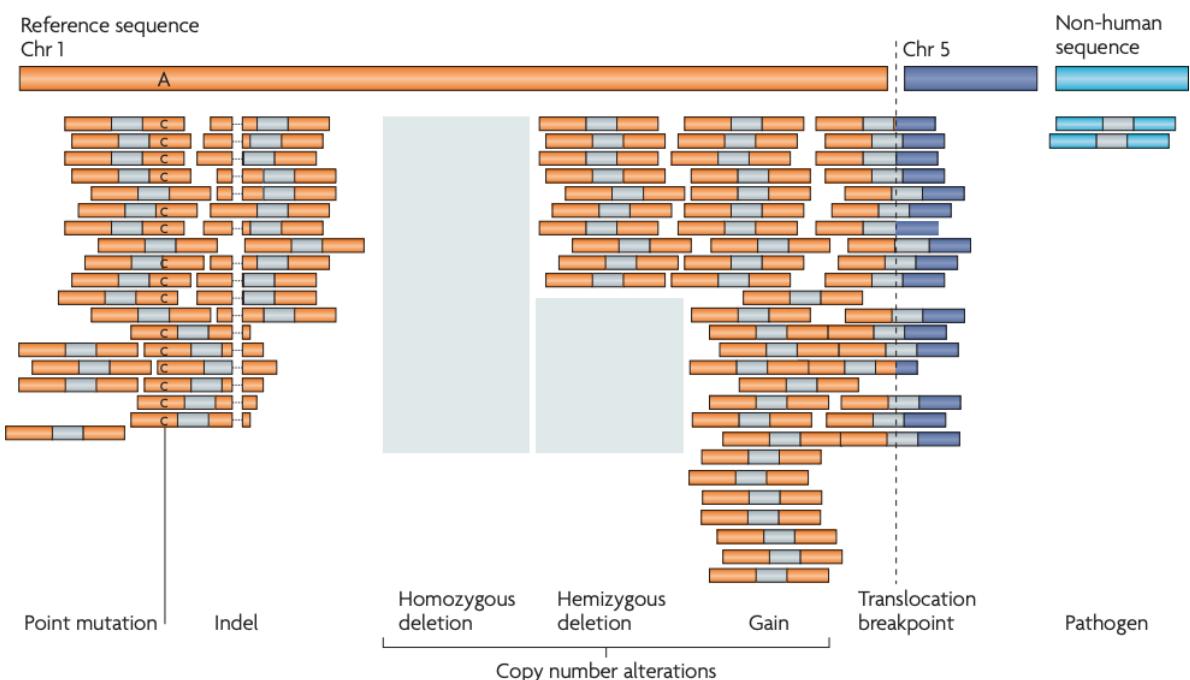


Figure 1.9: In the following picture: a view of reads that are mapped against reference genome and what we would look if we have any of the variations that we mentioned

Following the mapping of the reads over the reference genome, different types of genomic alteration/information can be detected:

- You can clearly identify **point mutations**. If a point mutation is present in the molecule that you sequenced and present on both alleles of the genome, it can be seen in all the reads very clearly.
- You might see **indels** (shown here by a dashed line). You will see a little space because the reference genome has more nucleotides than the sequenced molecule.

1.2. EXPERIMENTAL APPROACHES

- If you have **homozygous deletion**, you don't see anything mapped in that portion: there's no DNA. Doesn't matter if SE or PE.
- If you have **hemizygous deletion**, you see the read mapped to that portion where the hemizygous deletion is sitting, that is more or less proportional to half of the reads that you have in regions where you don't have a copy number change. Doesn't matter if SE or PE.
- If you have **gain**, what you get is higher number of reads aligned against that part of DNA, underlying the fact that the molecule you sequenced has extra DNA for that portion of reference genome. Doesn't matter if SE or PE.
- **Translocation breakpoint** are very important!! You will have one end mapping the chr1 and the other end mapping the chr5. Those two ends come from the same molecule of the *target cell* (the cell we sequenced), it means the cell has a translocation between chr1 and chr5. Without the PE protocol you cannot have this result.

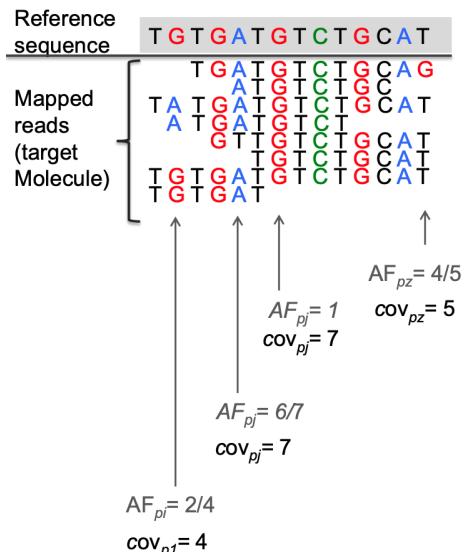


Figure 1.10: View of sequence alignment

The **local coverage** (cov) as shown in figure 1.10 at position i is the number of reads that span p_i .

The **allelic fraction** (AF) as shown in figure 1.10 at position i is the proportion of reads that supports the reference base in p_i (= the reference or the alternative allele).

1.2.2 Whole Genome Sequencing Coverage

$$cov = \frac{LN}{G} \quad (1.1)$$

where:

1.2. EXPERIMENTAL APPROACHES

- **L** is the read length.
- **N** is the number of mapped reads.
- **G** is the haploid human genome length.

This is super important because it saves us time and money when we design an experiment. When you design an NGS experiment, you should know before what is the type of coverage you need to answer the question you wanna ask with your experiment. For example, if you want to look at the genotype of SNPs (inherited polymorphisms at single side), you don't really need a coverage which is above 10 or 15. So you can design your experiment in order to have an average coverage equal to 10 or 15. To do that, you reverse the equation and count how many reads you need to generate to achieve that goal.

N.B.: The number of mapped reads will be always lower of the number of reads generated by the machine (than the expected). There might be duplicates that you might not be able to use because there might be reads that have a quality below the threshold you intend to use.

1.2.2.1 Difference between sequence coverage and physical coverage

A graphic view of how **SE (Single End Sequencing)** or **PE (Paired End Sequencing)** can be used:

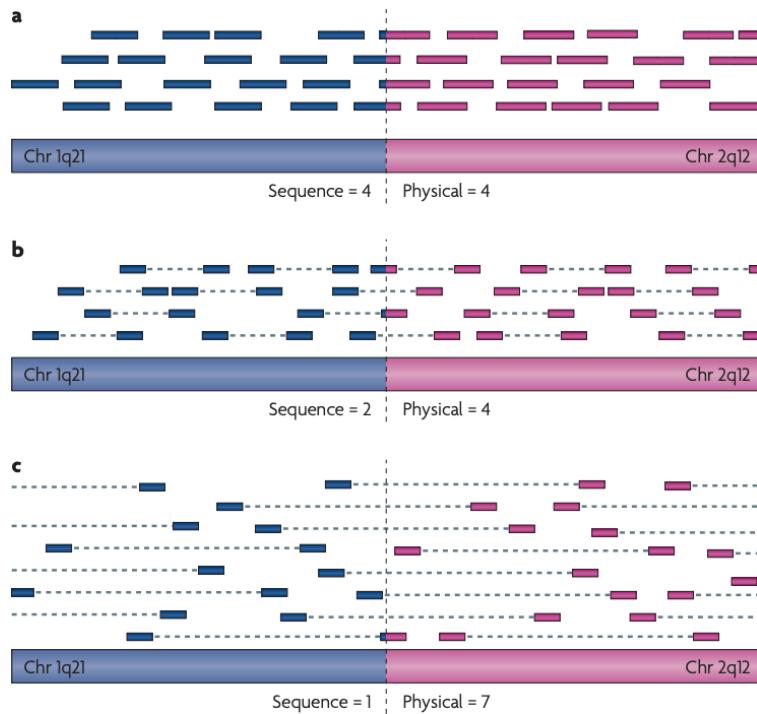


Figure 1.11: Panel A - SE protocol; Panel B - PE protocol; Panel C - PE protocol

1.2. EXPERIMENTAL APPROACHES

Three different scenario are depicted that vary in the length of the DNA fragments that are sequenced. **Sequence coverage** represents the number of sequenced reads that cover the site; this affects the ability to detect point mutations. **Physical coverage** measures the number of fragments that span the site; this affects the ability to detect the rearrangements, based on paired reads that map to different chromosomes. It is a way informative type of coverage: for instance for translocations, deletions ...

In Paired End sequencing protocols, the physical coverage is always higher than the sequence coverage. Choosing the method illustrated in panel 3 (figure 1.11).

Making estimation of intended coverage and observed coverage is very important. Below I will report an example:

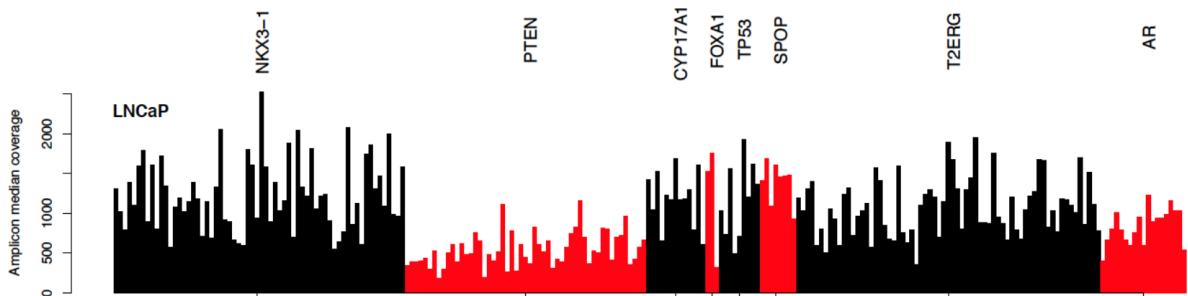
Example coverage observation:

In these panels were designed to sequence a set of 10 genes that the researchers were interested in for prostate cancer. They designed this panel, sequenced cell lines on this panel and observed the following points

- On *x*-axis: the genomic location
- On *y*-axis: the local coverage (amplicon median coverage = each bar represents the local coverage of about 30 bp)

The different colors represent the different genes

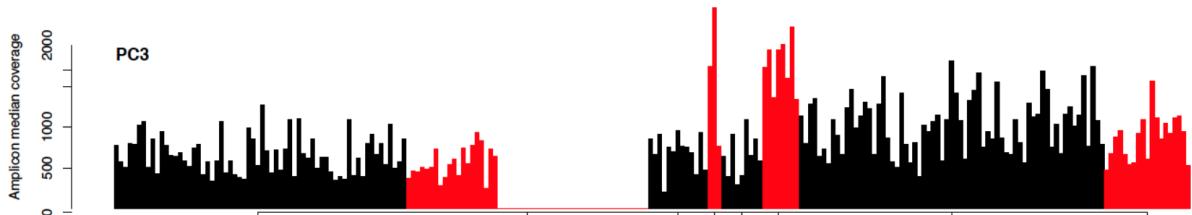
- 1st **panel:** Local coverage (pile-up) of selected areas (targeted sequencing assay): 7 genes
 - + 1 multi-gene region (T2ERG). Alternate colors indicate targeted areas The barplot show a single sample (LnCaP cell line; cancer cell line) data.
 - Apparent **deletion** of PTEN (monoallelic deletion) because the local coverage of PTEN is significantly lower than the one from other genes.



- 2nd **panel:** Local coverage (pile-up) of selected areas (targeted sequencing assay): 7 genes
 - + 1 multi-gene region.
 - Monoallelic deletion and partial biallelic deletion of PTEN because one portion is deleted and one not. PTEN has a **partial homozygous deletion**.
 - The PC3 cell line shows a little bit of gain in the gene SPOP and FOXA1.

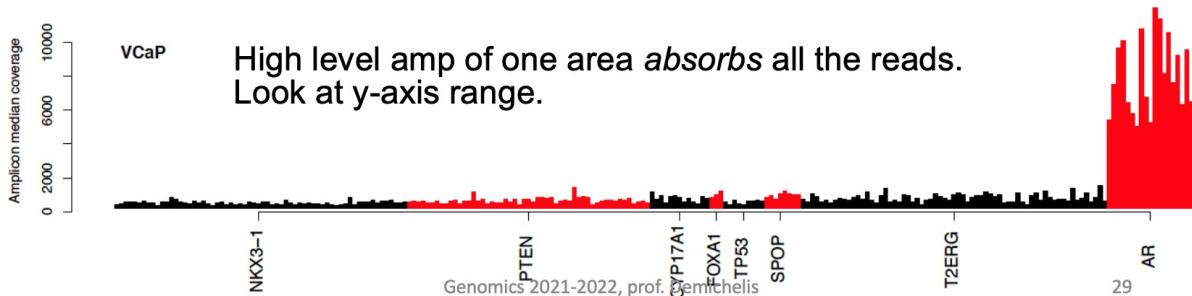
1.3. THE REFERENCE SEQUENCE OF THE HUMAN GENOME

- The average coverage for the PC3 cells is approximately the same as the previous sample.



- **3rd panel:**

- There's no homozygous deletion but has a high level **amplification** of one area *absorbs* all the reads. Massive amplification of the Androgen Receptor (AR) → error: because it inhibits the sensitivity of detecting copy number changes in any other gene, the signal is excessively intense.
- When designing a panel we must pay attention and make sure that we don't have potential aberration that basically will draw all the attention of your experiment and leave you without information or **sensitivity** in all other regions.
- It's easy to increase the experimental coverage (i.e. the sequence depth) at later point. Provided your original sample/library is still available, you can perform another run of sequencing and then combine the output from different runs.



1.2.2.2 Note that this isn't possible with array-based technologies.

What are the limiting factors of NGS DNA-seq experiment, in any?

Repeated regions due to **short reads**

What is the problem of short sequencing on long genome?

- Complexity regions
- GC content

1.3 The reference sequence of the human genome

Many years ago, some people claimed that the entire human genome was sequenced but it wasn't true at all. There were still unknown or missing regions. In 2022 we finally have the complete human reference genome sequence.

1.3. THE REFERENCE SEQUENCE OF THE HUMAN GENOME

But we need to consider the polymorphisms, there is no **unique** genome. How to integrate them into a single reference genome? There is a consortium that deals with these problems. They assemble a reference genome that reflects the most common (in the whole population) sequences at each position of the human genome, but also tracks information of everything that is polymorphic. So that we can use the latest release of what they built as reference genome and then use databases to learn about all the polymorphic sites and all the features of every polymorphic variants.

Genome Reference Consortium: ([Genome Reference Consortium link](#)) where you can find different versions of human reference genome

UCSC Genome Browser on Human: ([UCSC link](#))

where you can upload different versions of the reference

1.3.1 Interpreting pair orientation

Using IGV (Integrative Genomics Viewer) (see chapter 3 for more details)



The main characteristic of IGV is that it is a main view viewer: all the information are in one window.

On the x-axis there's the genome coordinates at the top, the reference genome at the bottom (we can select the reference genome we prefer).

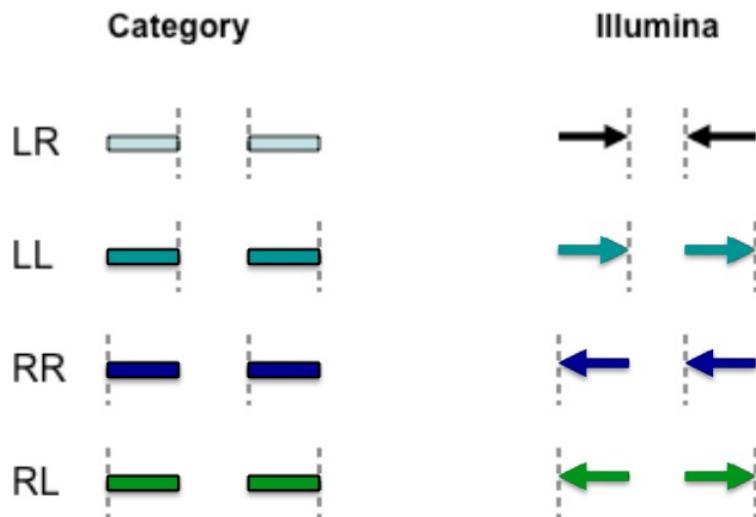
Along with the data tracks there is the local coverage of the kb shown in the window (of the sample we are looking at).

1.3. THE REFERENCE SEQUENCE OF THE HUMAN GENOME

You can get any information you want of any single read that you are uploading, very useful to see difference from the reference genome because every aberration or whatsoever is highlighted by a different color in the local coverage of a nucleotide base. Moreover, it gives information about the quality of the read and the bases, if you have a PE protocol, it tells you also information about the PE for each of them.

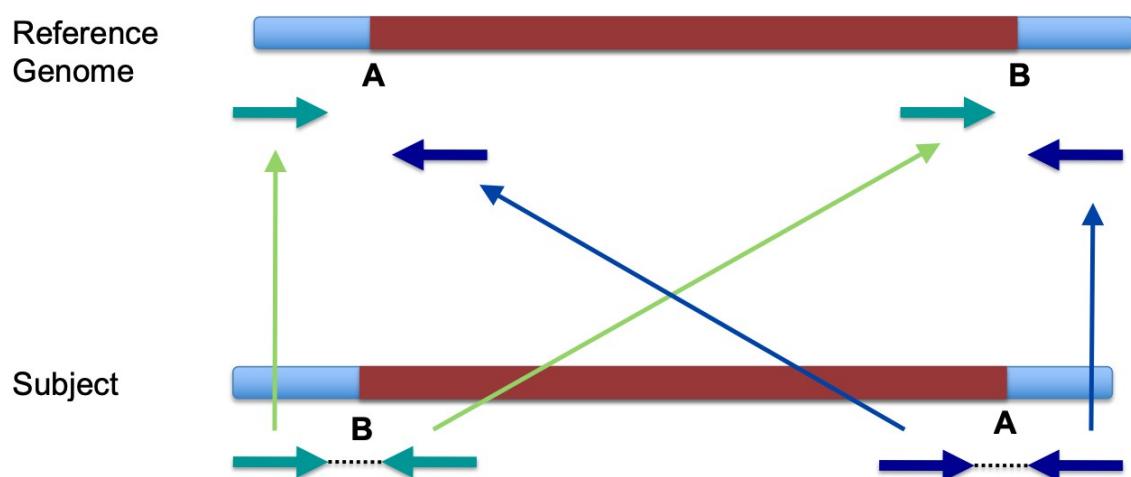
The **orientation** of paired end can be used to detect structural events, including:

- Inversions
- Duplications
- Translocations



1.3.2 Inversion

A segment of DNA is inverted



1.3. THE REFERENCE SEQUENCE OF THE HUMAN GENOME

The most important pairs are the ones that stand between junctions because they are the most informative ones.

Here one end mapped where it was on the reference genome while the other end reversed its orientation.

In IGV:



Information that help us:

- The **insert size** from the target molecule (= the subject) is way longer. For all the pairs that are at the breakpoint, the insert size is different from the expected.
- The **orientation** is different.
- If you look at the local coverage, you can see a **drop** in two points: at the breakpoints. The reads that are mapping the junctions cannot map the reference genome because the breakpoint sequence is altered in the reference genome. So, if we have an inversion in only one of the two alleles, then the reads coming from the allele with the inversion will not contribute to the local coverage at the breakpoint. The contribution to the local coverage will come only from one allele.

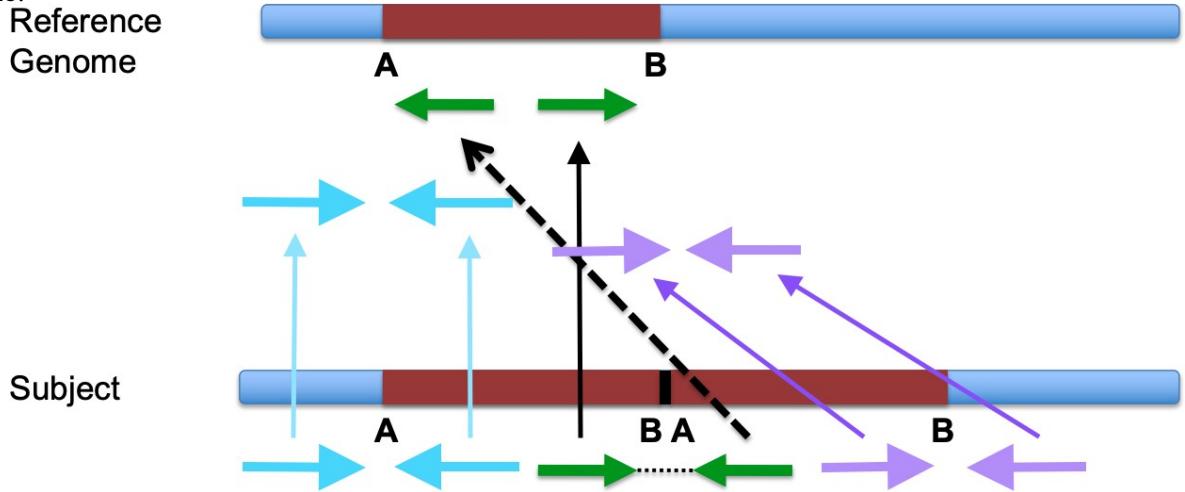
Moreover, we can notice that the coverage on the middle part does not change significantly from the coverage on the sides.

1.3. THE REFERENCE SEQUENCE OF THE HUMAN GENOME

When you align reads against a genome, you can allow for a certain mismatches or partial alignment. So, if you impose certain thresholds to your aligner, you can also say that if there are reads that align for 80% and have 20% of sequences misaligned, you align them in any case. So you will have reads that are correct up to the breakpoints and the browser will shows the mismatches beyond the breakpoint. So, you can have a partial drop of coverage because you allow mismatches in your alignment.

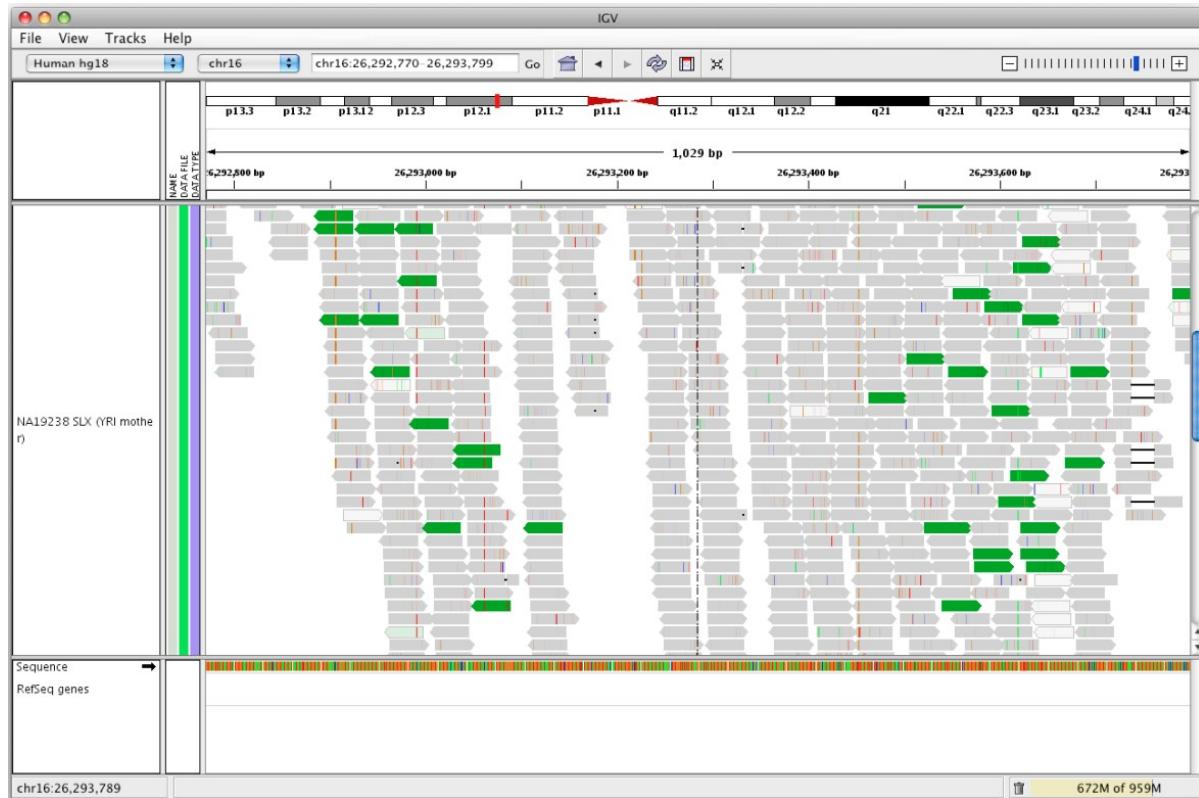
1.3.3 Tandem duplication

A segment of DNA is duplicated and inserted in the target molecule adjacent to the original one.



So, as result, the orientation instead of going inward goes outward.

1.3. THE REFERENCE SEQUENCE OF THE HUMAN GENOME



What do you expect to see from coverage? We will have a gain in coverage that is proportional to the extra copy. We need to pay attention to the double because it is a double contribution of that allele, but if a tandem duplication happens only in one allele and the other allele has his own one copy, then the local coverage corresponding to the tandem duplication will be 3/2 of the expected coverage.

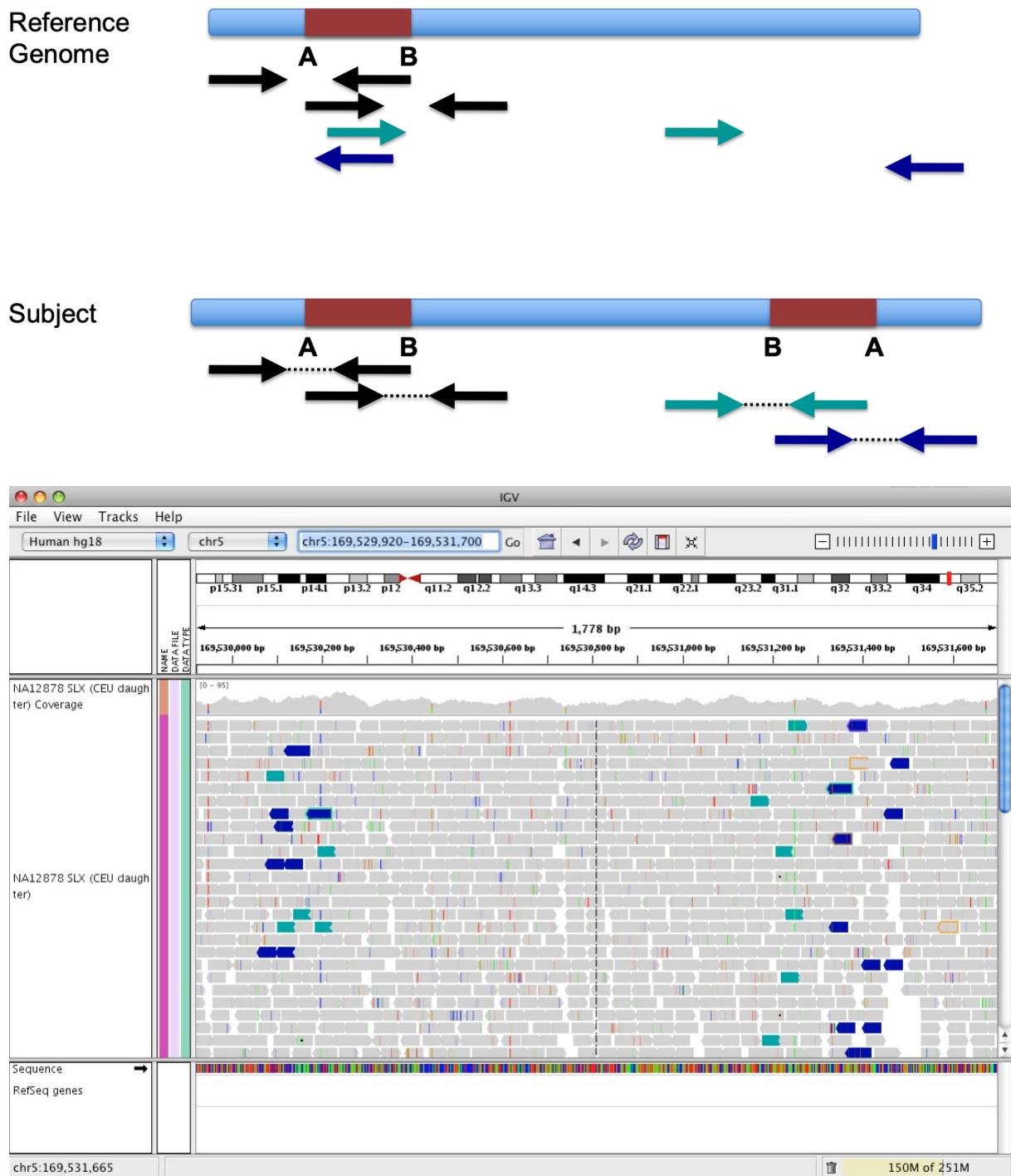
If you have a read that maps BA, do you expect to see it in the mapped reads? Partial mapping. As we said before, if you allow your mapper to have some mismatches of a certain percentage of bases from your reads, you can still see some coverage contributed on one end of the segment and mismatches on the other side.

For what concern the junctions, you shouldn't see any difference of coverage because that sequence exists only once in the target molecule. The local coverage increases only in correspondence of the segment AB.

1.3.4 Inverted duplication

The duplication is inverted but it's not located near the original fragment, but somewhere else.

1.3. THE REFERENCE SEQUENCE OF THE HUMAN GENOME



There is a gain of coverage in the duplicated region and a tiny drop in the break points where the sequence exists in only one allele.

1.3.5 Deletion

Deletion of a segment of DNA:

- If the deletion is larger than the size of the reads, we should see half of the coverage in the deleted regions.
- If the deletion is shorter than the size of the reads, we should see a tiny little space corresponding to the missing nucleotides.

Chapter 2

Genetic Fingerprinting

Genetic fingerprinting is a technique used to identify some characteristics of a genome (a pattern of variable elements), like SNPs or minisatellites, in order to **uniquely characterize a genome**. Genetic fingerprinting can be used to compare a genome with a reference sample or to compare different genomes between each other, in order to determine their diversity or analogy.

DNA fingerprinting is applied in different fields:

- In **Forensic**, for identification purposes;
- In **lineage related tests**, for cells or humans. Eg. paternity test, hereditary tests.
- For the certification of the **origin of cells used in the laboratory**, to make sure that the cells are the right ones and that there are no major genetic drifts. Needed when using certain cell lines, for publishing purposes.

Variants used for genetic testing

There are different variants that can be used for genetic fingerprinting, such as Single Nucleotide Polymorphisms (SNPs) or Inherited Copy Number Variations (CNVs) (see chapter 1.1). Basically everything that is inherited and that is a polymorphism can be used in genetic testing, however some variants are more amenable than others.

SNPs are the most amenable ones since they are simple, abundant in the genome and easy to detect in sequencing data at any coverage depth. For these reasons, in this lesson we will focus on the development of **SNP-based genetic tests**.

2.1 SNPs features

2.1.1 Hardy-Weinberg equilibrium and Minor Allele frequency

One property of SNPs which has to be taken into account when using SNPs for genetic testing is the **Hardy-Weinberg equilibrium**. In population genetics, the Hardy-Weinberg equilibrium states that allele and genotype frequencies in a population will remain constant from generation to generation under neutral selection, so in the absence of other

2.1. SNPs FEATURES

evolutionary influences, like genetic drift, mate choice, sexual selection, mutations, population structures. Also, it requires randomness in sexual matings, equal proportions of males and females, infinite size.

In the simple cases following the Hardy-Weinberg equilibrium, a single locus with two alleles denoted A and a with frequencies $f(A) = p$ and $f(a) = q$, respectively, the expected genotype frequencies under random mating are $f(AA) = p^2$ for the AA homozygotes, $f(aa) = q^2$ for the aa homozygotes, and $f(Aa) = 2pq$ for the heterozygotes. In the absence of selection, allele frequencies p and q are constant between generations, so equilibrium is reached.

SNPs that respect this equilibrium are also the most studied, thus more informative.

2.1.2 Minor Allele Frequency

Also, when performing genetic fingerprinting, the aim is to maximize the probability to have different genotypes in unrelated individuals. For this reason, the more advantageous SNPs will be the ones in which the allelic frequency of the variants is the higher possible. Highest variability in the population allows to distinguish better more individuals.

Number-wise, a frequency of $\frac{1}{3}$ for each SNP would maximize the variability, but those SNPs wouldn't be in HW equilibrium and we might have missed calls. Therefore, the optimal SNPs to detect individuals' differences and similarities are those with genotype frequencies: $P_{AA} = 0.25$, $P_{BB} = 0.25$, $P_{AB} = 0.5$. 50% of individuals for that SNP will have a heterozygous genotype, 25% a homozygous genotype for the reference allele, 25% for the alternative allele.

This is equivalent to say that best SNPs will be the ones with **MAF** = 0.5. Minor allele frequency (MAF) is the frequency at which the second most common allele occurs in a given population.

Some useful projects:

- **dbSNPs**: is a database of small scale nucleotide variants. The database includes both common and rare singlebase nucleotide variation (SNV), short ($=; 50\text{bp}$) deletion/insertion polymorphisms, and other classes of small genetic variations. <https://www.ncbi.nlm.nih.gov/snp/>.
- **HapMap3**: is the third phase of the HapMap project whose aim is to develop a haplotype map of the human genome to describe the common patterns of human genetic variation in order to allow researchers to find genes and genetic variations that affect health, disease and individual responses to medications and environmental factors. The HapMap is a catalog of common genetic variants that occur in human beings. It describes what these variants are, where they occur in our DNA, and how they are distributed among people within populations and among populations in different parts of the world. <https://www.sanger.ac.uk/resources/downloads/human/hapmap3.html>

2.1.3 Haplotype Blocks

Another important feature to consider for SNPs selection are **Haplotype blocks**. Haplotype blocks are blocks along the genome that tend to be inherited as segments. In these

2.1. SNPs FEATURES

sizable regions there is little evidence for historical recombination and only a few common haplotypes are observed.

So for example, if there are 10 SNPs in a block of 1 MB, the genotype of one specific SNP in that block gives an indication the genotype of the other SNPs in the same block, since they are inherited together. Hence, if there is a haplotype block, there is no point in sequencing all SNPs in that block, it is sufficient to select some specific SNPs. Also, when running a fingerprint assay, there is no point in using all SNPs in a haplotype block since they won't bring additional information independently.

SNPs in the same HB are said to be in **Linkage Disequilibrium** (LD). Linkage disequilibrium measures the non-random associations between alleles or polymorphisms at different loci. A higher LD indicates a SNPs with a stronger tendency to co-segregate. Haplotype Blocks are therefore commonly represented with *linkage disequilibrium plots* 2.1. In these plots, SNPs are represented in a way that does not respect the genomic distance, but the order along the genome (position of each SNP relative the others).

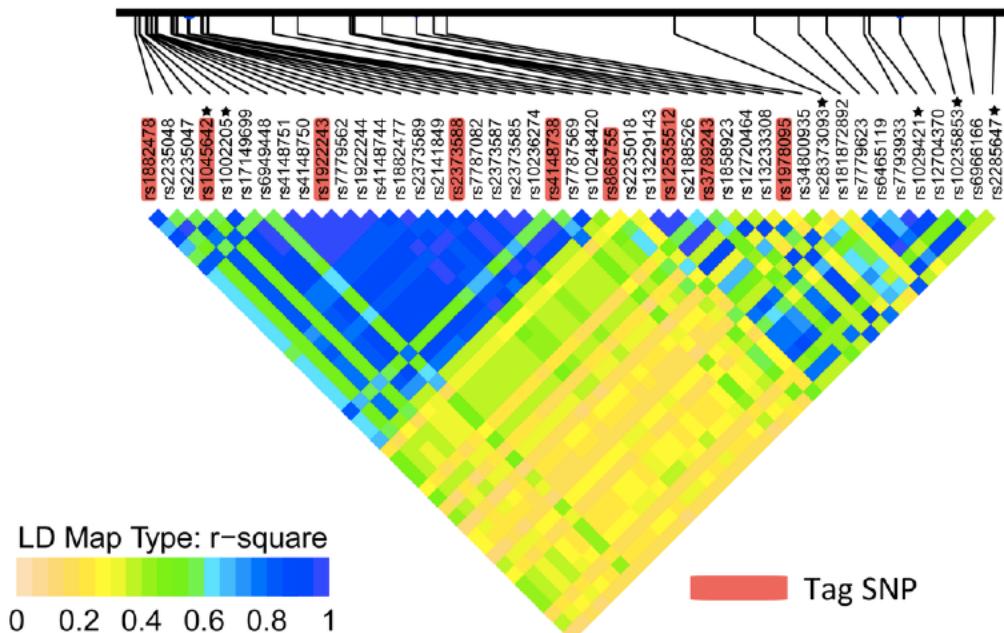


Figure 2.1: Linkage disequilibrium plot.

In figure 2.1, the colors indicate the strength of pairwise linkage disequilibrium (LD) according to r^2 metrics. Tag SNPs are shadowed in pink. A **Tag SNP** is representative of a region with high linkage disequilibrium and represents a group of SNPs (called haplotype).

2.1.4 Other SNPs features

- Choose SNPs that are in areas that are not likely to undergo somatic aberrations. So exclude chromosomal locations which undergo frequent somatic aberrations. Eg. ar-

2.1. SNPs FEATURES

eas commonly deleted in tumor will produce LOH but probably also no calls, since there is no DNA.

- Choose SNPs equally represented/spread all around the genome (not in specific chromosome regions).
- Select autosomal only SNPs.
- Select SNPs in exons. If we were to run a targeted assay, this would cover more exons instead of intrones. It will also be more probable to have signal from a non-DNA assay, for example if calling a genotype from RNA sequencing data (even though it is not always done).
- Exclude/include disease or drug response associated loci.
- Include/exclude loci with significantly different MAF in different ethnicity. If we include them we can also have a lineage type of tests in the same assay.

2.1.5 Number of SNPs to select

If we want to build a test to run genetic fingerprinting using SNPs, **how many polymorphic loci (SNPs) should be tested?** We want to make sure that the measure of the test will be able to differentiate unrelated individuals. But we must also remember that many variables must be taken into account, possible mismatches in particular. Those can be due to the sequencing process itself (experimental mismatches) but also to changes due to somatic events (biological mismatches). All these events can be used in the test with a different weight, based on how likely they are.

2.1.5.1 Experimental mismatches : Genotype call error rate

During sequencing, each machine will produce some errors, resulting in some loci for which no data will be available. If those loci include some SNPs of interest, then no call will be associated to that SNP. Experimental mismatches are related to the error rate of the technology used, they are platform dependent.

2.1.5.1.1 Some examples: In each example in figure 2.2 there are two samples with the same number of potential SNPs: 24. To determine the difference/similarity (concordance) of the two samples we can look at the genotype for each position and count mismatches.

Legend: 'A' stands for 'AA' (e.g. homozygous genotype for the reference allele); often referred to as Aa. 'B' stands for 'BB' (e.g. homozygous genotype for the alternative allele); often referred to as Bb. 'AB' stands for heterozygous.

- **First Example:** over the 24 loci, there is only one mismatch. This translates to a level of concordance of 95.8%. Those 2 individuals are highly related or DNA comes from the same samples.
- **Second example:** there is only one mismatch but there are some 'na', indicating that for some positions we don't have a call (not available data). Therefore, in this case the concordance is measured out of 22 SNPs and is equal 95.4%.

2.1. SNPs FEATURES

Legend: 'A' stands for AA; 'B' stands for BB. Often referred to as Aa and Bb.

PROBLEM STATEMENT 1

How many polymorphic loci to test?

<i>i</i>	1 2 3 4 5 6 7 24
S1	A A B AB AB B B A A A AB AB A A B B AB B AB AB A A B	
S2	A A B AB A B B A A A AB AB A A B B AB B AB AB A A B	
	1 mismatch (concordance 95.8%)	out of 24 SNPs
S1	A A B AB AB B B A A A AB AB A A B B AB B AB na AB A A B	
S2	A A B AB A B B A A A AB AB A A B B AB B AB AB A A na	
	1 mismatch (concordance 95.4%)	out of 22 SNPs
S1	A A B AB AB B B A A A AB AB A na na na na na na na na na	
S2	A A B AB na B B A A A AB AB A na na na na na na na na na	
	no mismatches (concordance 100%)	out of 12

11

Figure 2.2: 'A' stands for 'AA' (e.g. homozygous genotype for the reference allele); often referred to as Aa. 'B' stands for 'BB' (e.g. homozygous genotype for the alternative allele); often referred to as Bb. 'AB' stands for heterozygous.

- **Third example:** here a lot of 'na's are present, leading to have only 12 SNPs available. This brings to a concordance of 100%.

Different examples produced different levels of concordance. What do we trust the most?

The first set of SNPs is the one that we trust the most, because it has the higher number of available SNPs. Wider number of SNPs provides the most reliable information.

2.1.5.2 Biological mismatches

In the context of disease samples and tumors, many somatic events can happen, like deletions, gains of copies, homozygous deletions, etc. Some common ones are:

- **Loss Of Heterozygosity (LOH):** event that results in loss one parental copy of a region which results in the genome having just one copy of that region. If that region contained a heterozygous locus (e.g. SNP), there will be loss of Heterozygosity. $AB \rightarrow A$.
- **Gain Of Heterozygosity (GOH):** due to a mutation in a site often polymorphic through inheritance. These are pretty rare. $A \rightarrow AB$.
- **Double Mutation (DM):** very rare.

Biological mismatches can be properly modeled in our assay. We can, in a data driven way, assess the error rate for the genotyping for some specific SNPs or run tests. We can also think in terms of SNP-specific or tissue-specific probabilities.

2.2. GENETIC DISTANCE

$$\text{Loss Of Heterozygosity (LOH): } (AB,A) \quad P(AB,A) = P(AB) * P(A|AB)$$

$$\text{Gain Of Heterozygosity (GOH): } (A,AB) \quad P(A,AB) = P(A) * P(AB|A)$$

$$\text{Double Mutation (DM): } (A,B) \quad P(A,B) = P(A) * P(B|A)$$

Figure 2.3

The main point is that all mismatches must be taken into consideration. For this, all implemented tests use *more than the minimal number of SNPs* that allow to identify individuals.

2.2 Genetic Distance

Having defined the number of SNPs to use, with maximum MAF and other amenable characteristics, the genetic test should provide a measure of some sort, which will be the output metric, associated with a probability of the measure to be correct.

As a simple measure, we can count the number of loci where two samples show different genotype and normalize on the total number of queried loci, defining a certain level of discordance (or concordance). The output value will be the '*genetic distance*' between the two samples given the selected loci. The distance is proportional to the number of discordant calls.

In figure 2.4 we can see an example of a typical graph used to measure the genetic distance using SNP-based genetic testing. We have 4 samples with a set of 5 SNPs for each one. The distance is measured among all possible pairs, whose indexes are reported on the x-axis.

- s1 and s2 have 3 A in common, one locus has no call and another one produces a mismatch. 1 mismatch out of 4 produce a distance of 0.25.
- samples s1 and s3 have 5 mismatches out of 5, so a distance (or discordance) of 1.

If we put that into an equation in 2.4 will have that: for each position i (SNP) between sample 1 and 2 we can have 1 if the genotype is different, 0 if they are identical. Then we determine the distance D by summing up the different scores obtained for each SNP. We can associate different weights w_i to different mismatches (depending on Gain Of Heterozygosity, Loss Of Heterozygosity, Double Mutation) or we can put all equal to one. Then we devide by the total number of SNPs for which we have available calls vNSNPs for both the sequences in comparison, which will be lower or equal to the total number of SNPs, NSNPs.

This other example at figure 2.5 shows the distance, measured by genetic fingerprinting, of a collection of 160 samples of cell lines.

The number of possible pairs corresponds to: $\frac{160 \cdot 159}{2}$ (number found in the x-axis).

By applying this measure to a larger collection of samples like this one, with many SNPs, we expect to find an **average distance** among all possible pairs that very unlikely will be close to 1.

2.2. GENETIC DISTANCE

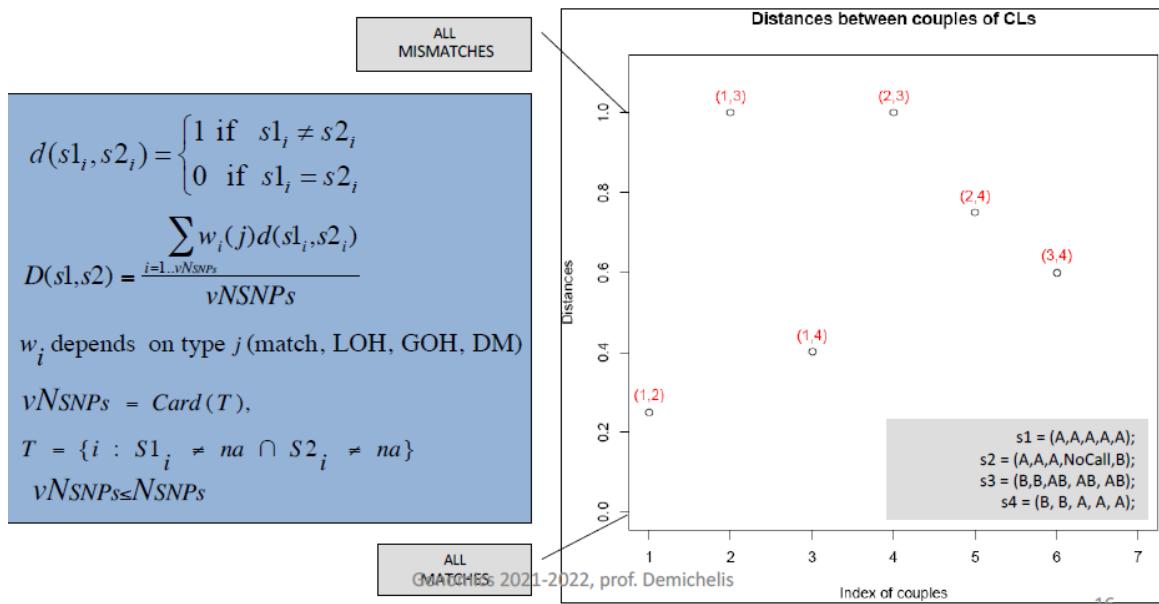


Figure 2.4: Genetic Distance graph with 4 samples

The MAF of the SNPs is 0.5 but it will never happen that, with a high number of SNPs, the discordance will be 1, meaning that the SNPs will be all different. We will have an average distance that in this case around 0.5, since by chance we all share some genotypes on a large number of SNPs.

Here they found certain pairs with a very low distance, sometimes almost equal to zero (dots at the bottom). This was a surprising result because it shows that those pairs, which were suppose to be different cell lines, were actually not different cell lines (only less than 70% of SNPs have available calls).

In this last example at figure 2.6 genetic fingerprint was performed on a collection of 160 tumor samples, with a larger SNP array (more than 100.000 SNPs).

From the analysis, two samples with very low distance were observed. One of the two samples came from a Rapid Autopsy Program and the other one from a xenograft model.

RAP are programs for which patients at the end of their life agree to donate their tumor tissues which can be used for research. In these very complex but highly valuable programs, the material must be taken within two hours after death. Those samples are usually highly characterized but after a while the track of the patient's identity is lost. Here, what happened is that one man who donated tissue to this program was sequenced and for some of those metastasis models were generated and implanted into a mouse and a xenograft model was derived. Thanks to fingerprinting it was possible to determine the same origin between xenograft and patient.

The power of this technique is very high, it allows also to identify and remove things that we don't want in our study. Eg. if running a study (like a GWAS study) on a certain interesting geographic area, we will want to remove the members of the same family because that would skew the results. Genetic fingerprint can be used for this purpose.

2.2. GENETIC DISTANCE

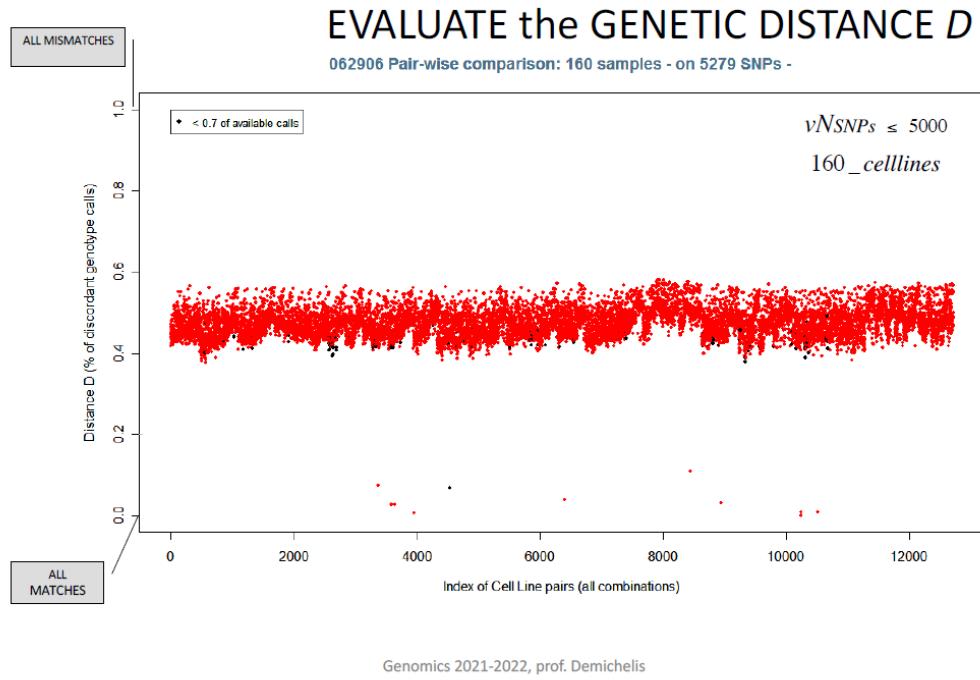


Figure 2.5: Genetic Distance graph with 160 samples

2.2.1 Some questions

Q1: Would the average of unrelated samples distance increase or decrease after selection of ideal SNPs?

If we use SNPs likely to be different among individuals and we use them to determine the measure of the ditance, then the average value of unrelated individuals will increase.

Q2: Is it likely to obtain a genotype distance $D = 1$?

We get distance 1 only if we are looking at too few viable SNPs. Whereas with a well selected pool of SNPs, and a high enough number of SNPs, it is very unlikely that the distance is equal to 1.

2.2.2 Further considerations

How does the genetic distance among different samples change when varying the number of selected SNPs used to perform the test?

The genetic distance among many samples, with an array of 5.3K SNPs, was measured, using a decreasing number of SNPs (from the initial total number of SNPs to decreasing numbers of highly selected SNPs) 2.7.

2.3. BUILDING A SNP-BASED GENETIC TEST

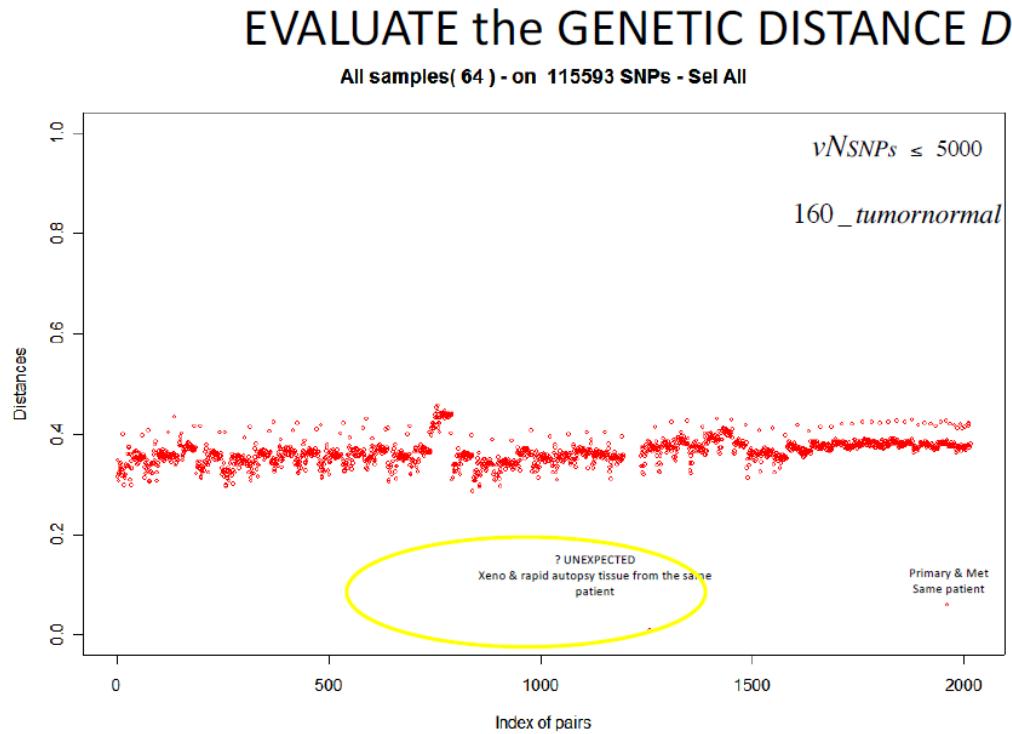


Figure 2.6: Genetic Distance graph with tumor samples

It is noticeable that, in the second plot where 80 SNPs matching the required characteristics were selected, the average Distance across all pairs is higher than in the previous example, in which all available SNPs were used (~ 0.45 vs. ~ 0.65). Also, the standard deviation of greater. Decreasing the number of SNPs to 60, then to 40 and 20 leads to have the same average distance between pairs, which settles around 0.66, but higher standard deviation.

In reality we always need enough SNPs, enough information, in order to prevent unexpected issues and to be sure that for any pairs of sample we have enough information to trust our measure.

2.3 Building a SNP-based genetic test

Building an identity test base on SNPs is a MULTI-STEP process, consisting in:

1. Definition of a genotype/genetic distance to compare samples;
2. Definition of SNPs requirements, based on the intention of the assay.

2.3. BUILDING A SNP-BASED GENETIC TEST

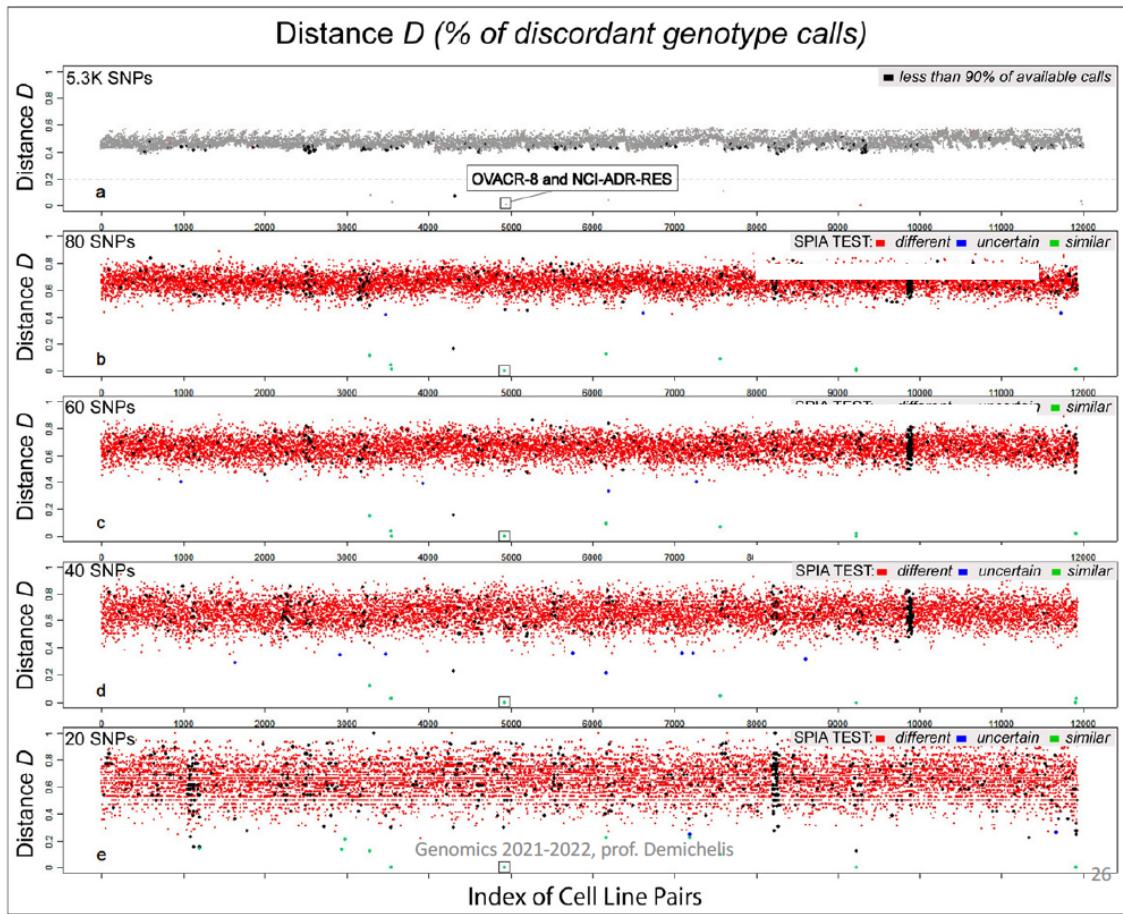


Figure 2.7: Genetic Distance graph at deacreasing number of selected SNPs

3. Selection of SNPs:

- This can be done in a data-driven manner, through an iterative procedure of training and test on known sample set;
- Or, performing the selection based upon MAF and Hardy-Weinberg equilibrium. For example, using HapMap data.

4. Implementation of a probabilistic test (different, uncertain or similar)

5. In silico validation on independent/multiple dataset.

6. Validation on cell lines genotyped on independent platform.

We have already seen some of the steps needed (1, 2, 3), we now pass to the following ones.

2.3.1 Implementation of a probabilistic test

Other important questions which we have to answer to when designing a genetic test are:

2.3. BUILDING A SNP-BASED GENETIC TEST

- What is the *threshold* on the genotype distance to call two samples 'identical' ('similar') or 'different'?
- How *confident* would the call be?
- What is the *minimum number* of loci needed for a robust test?

It could be useful to have a probabilistic test to determine if the measure of the test is correct at which level of confidence. We can use a probabilistic approach to compare observations with expectations (gold standard).

Under the assumption that SNP calls at different loci are independent, we can think in terms of Binomial distribution. Each SNP can be considered as a trial,

- n = number of SNPs in the assay,
- k = number of matches,
- p is the probability of match and $(1 - p)$ of mismatch.

Then the probability of having k matches (successes) out of N SNPs (trials) follows the binomial distribution. With n , np and $np(1 - p)$ large enough, we can use the **Gaussian approximation** of the Binomial distribution with $K_{\text{mean}} = np$ and $sd = \sqrt{np(1 - p)}$.

With something that simple we can add a probabilistic test in our assay, defining an area of confidence given by $K_{\text{mean}} \pm m \cdot sd$ where m is the number of standard deviations used to define the thresholds which will lead to have a smaller or wider confidence area.

So for example: given two unrelated samples, we reason in terms of '*what is the probability of having a certain number k of matches over a total number of n SNPs, therefore a certain value of D^2* '.

The probability mass function for unrelated individuals is shown in figure 2.9 with a blue dotted line and indicates that there is a low probability of having both a very low and a very high number of matches (it is assumed that the comparisons of samples follow a binomial distribution, where higher and lower values, compared to the mean, have less likely).

We can also think in the opposite term: given two related samples, what is the probability of having matches? As represented by the red dotted line, in this case there will be a high probability of having many matches.

Using these probabilities we can set two thresholds which will define 3 regions:

- A '**not pair**' region for which the two samples will be considered as 'different'.
- a '**pair**' region for which the two samples will be considered as 'similar'.
- and an '**uncertain**' region, a grey zone, for which no certain result can be produced.

Then we can move the grey area based on what we want to be certain of and on how many SNPs we have.

By decreasing the number of SNPs, the grey zone will become more tiny, making the result more difficult to interpret. For example, a difference of only 2 matches could lead to opposite conclusions.

By contrast, with more SNPs the area will be wider and easier to interpret. Hence using a number of SNPs greater than the minimum number is better, otherwise there will be many uncertain calls.

2.4. FURTHER CONSIDERATIONS AND EXAMPLES

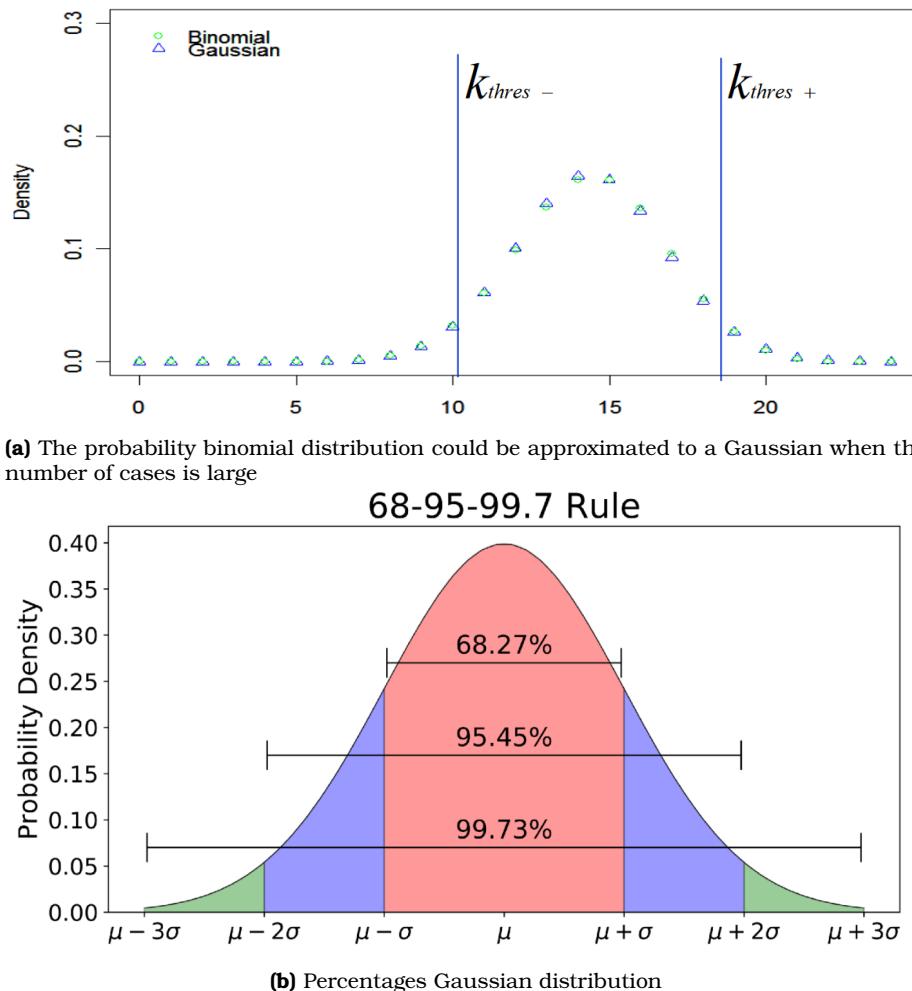


Figure 2.8: Approximation of a binomial distribution to a Gaussian and percentages related.

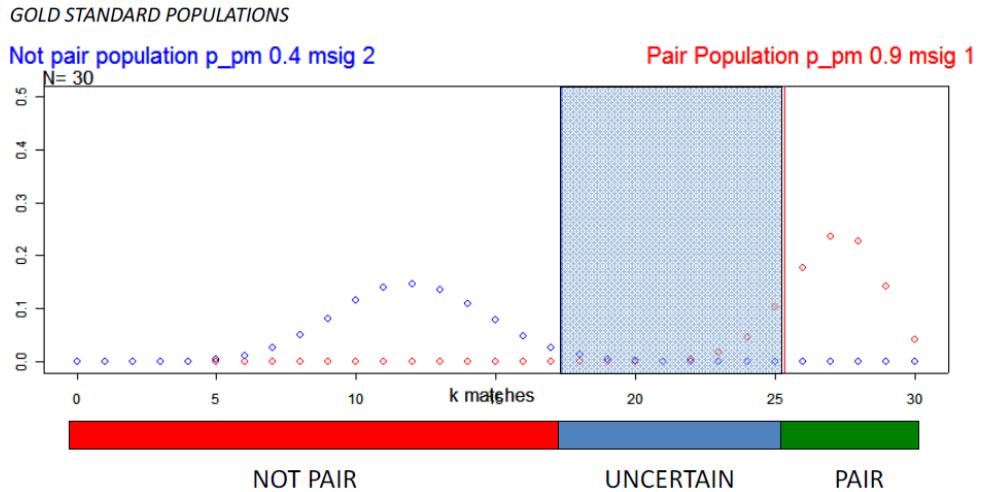
2.4 Further considerations and examples

In the past, before sequencing area and SNPs array area, short tandem repeats were commonly used for genetic fingerprint. They were used on gels to distinguish related and unrelated individuals, eg, for the initial paternity test. **Di-nucleotide markers** are the most informative class of microsatellites. Generally they are more informative than SNPs, though selected SNPs perform quite well.

Inherited copy number variants can be used too for a fingerprinting test, but not all of them. The more amenable for this test are the loss type of CNV. In the population there will either a copy number of 2 or 1 or 0. If both parents have heterozygous pair of CNVs it will be possible that I have a homozygous deletion. If both parents have 2 copies at a site that is polymorphic in the population, we will have a genotype equal to 2 copies.

2.4. FURTHER CONSIDERATIONS AND EXAMPLES

PROBABILISTIC APPROACH DOUBLE TEST



Varying the m_{PAIR} and $m_{NON-PAIR}$ we set how CONSERVATIVE the test is.

Figure 2.9

If we think about **gain** of CNV then it becomes messy, because when combining multiple copies and have a add up we cannot distinguish what comes from what pair, so we cannot use them to identify an individual.

2.4.1 Example 1: Cell line passages

A mass use of these genetic tests is done to assess **genetic changes in in-vitro cultivation**. Cell lines go through multiple passages in which they are used and stored. Genetic fingerprinting can be used to assess if among different passages the cells have remained the same, if they were mislabeled or if major genetic drifts happened. Genetic fingerprinting is also used in studies of tumor evolution, lineage plasticity, heterogeneity across metastasis across individuals or a single tumor.

Lineage plasticity, the ability of cells to transition from one committed developmental pathway to another, has been proposed as a source of intratumoural heterogeneity and of tumour adaptation to an adverse tumour microenvironment including exposure to targeted anticancer treatments.

In this example (figure 2.10), two types of prostate cell lines which underwent multiple passages were used: **N15C6** (passages from 48 to 63) and **N33B2** (passages from 21 to 39). The cell lines were profiled with a SNPs array and the assay was run. All passages of each cell lines were compared with all other passages. We expect all passages to have the same genetic fingerprinting in the same cell line.

2.4. FURTHER CONSIDERATIONS AND EXAMPLES

However the results obtained using the **full array of SNPs (50k)** (subfigure 2.10a), showed that some pairs which should be exactly identical (distance equal to zero) are actually a bit different (points at the bottom-left). By contrast, by using a set on only 54 SNPs (subfigure 2.10b), this diversity is not detectable, indicating that using the restricted number of SNPs could make us loose some information.

In order to understand this increase of distance, they looked at each chromosome to see if there were problems that justified increase the increased distance expected to be equal to zero in that cell line. All chromosome were tried. If we focus only on the SNPs spread across Chromosome 11, we observe that there is a major difference for certain passages with respect to the initial ones, only for one cell line (N15C6). This was due to the way the cells were immortalized (insertion in chromosome 11), look to figure 2.11.

2.4.2 Individual's Relatedness (genotype-distance)

The HapMap consortium sequenced hundreds of individuals for different ethnicities and also used trios. **Trio sequencing** is a technique which involves the sequencing of the genome of mother, father and son/daughter. Trios provide major information for haplotype blocks, for identifying regions related to inheritance, ecc.

By looking at the data based on *SPIA Assay* (subsection 2.4.5) (a genotype base assay which measures distance) at figure 2.12 we see that self-self pairs have distance zero, as expected; samples within each ethnicity have a certain average distance, which is lower than the distance observed among different ethnicities. Differences in distance among mixed samples are due to the fact that the SNPs used had on average higher MAF in some populations than in others.

We also notice that in trios the distance is not 0 and is not equal to the median distance of unrelated individuals. This can be used for paternity tests or even in forensic science (figure 2.13).

2.4.3 Example 3: Cancer susceptibility test

The data showed refers to a study where they were looking for polymorphisms that increase the **likelihood of prostate cancer**. In these studies, if relatives are present in the cohort, only one of them is taken to avoid skewing the results. When looking for signs of cancer susceptibility by performing genetic fingerprinting, the division based on the degree of relatedness was determined 'for free' and could be used to remove unwanted samples from the cohort.

2.4.4 Genetic structure of the human population

Understanding the genetic structure of human populations is of fundamental interest to medical, forensic and anthropological sciences.

The goal of association studies is to **identify DNA variants** that affect disease risk or other traits of interest. However, association studies can be confounded by differences in ancestry.

Misleading results could arise if individuals selected as disease cases have different ancestry, on average, than healthy controls. The differences in the markers wouldn't be

2.4. FURTHER CONSIDERATIONS AND EXAMPLES

due to the disease itself at this point, instead it would be caused by the different origin. If in a study all controls are of the same ethnicity and the test is done on an individual of a different ethnicity than the test is biased. If we run a GWAS study using two ethnicities and we want to use the same markers of susceptibility worldwide, it won't work.

Especially in medicine and in the study of human evolution it is important to **track the genetic background of individuals** that are involved in studies in order to understand if the individuals are from a homogeneous population or from genetically distant ones. More and more, clinical studies must have declarations of the checks and interpretation of the data of the genetic background of the individuals present in the study. It is very important to come to results for which we know exactly what is the applicability. To avoid spurious results, association studies often restrict their focus to a single continental group.

Advances in high-throughput genotyping technology have improved the understanding of global patterns of human genetic variation and suggest the potential to use large sample sets to **uncover variation among closely spaced populations**. One important piece of information to consider when developing methods to understand the genetic structure of a population, is to think in terms of variance, which is also relevant for human diseases. Many SNPs have different MAFs in different populations. If we use those, and are able to have all of them in a simple computational way, we could be able to infer what is one individual's genetic background in terms of origins (e.g. Chinese origins).

The easiest mathematical approach to assess how well SNPs can distinguish ethnicity is by using **Principal Component Analysis (PCA)**. By running a very simple PCA on a set of SNPs including SNPs with different MAF in different populations we can, in a space, distinguish different ethnical groups. And we could also start thinking at individuals' origins.

How accurately can one predict an individual's geographic-ethnic background based upon his/her genetic barcode?

2.4.4.1 Example paper: 'Genes mirror geography within Europe'

In the study seen during lectures they used a 500.000 (500k) single nucleotide polymorphism array. Information about the country of origin of grandparents, parents and other relatives was used to determine the geographical location that best represents each individual ancestry. They ran a combined study where they used a supervised search to find the best SNPs to make inference and then they tested it on another set of individuals. By using high confidence data (individuals with high confidence origin data) and by using the genotypes of highly informative SNPs for specific region-related inheritance, they were able to **rebuild the map of some of the countries in Europe** 2.14.

It is true that by using properly selected variants it is possible to distinguish **individuals coming from different countries**. The way those SNPs are selected is very similar to the process seen for genetic fingerprinting, but pushing for the selection of variants that are different in terms of MAF in different populations.

Clusters that are a bit more dense and distant from the others (like the Spain/Portugal cluster) could be due to the fact that many SNPs selected are typical of that area and are therefore able to maximize the difference between that area and the others (so it is a

2.4. FURTHER CONSIDERATIONS AND EXAMPLES

data-related 'issue').

Focusing on Switzerland, they could even make inference on the linguistic canton 2.15. It is possibly true that in country where some regions have very different cultures (e.g. marriage within the same area) might be different between each other.

2.4.4.2 Summary and notes

Low-frequency alleles tend to be the result of a recent mutation and are expected to geographically cluster around the location at which the mutation first arose. Hence, they can be highly informative about the **fine-scale** population structure.

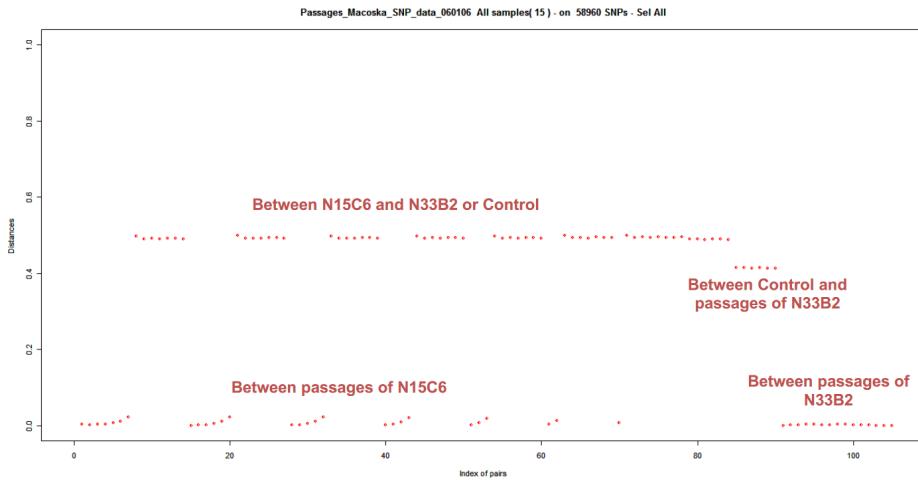
Despite low average levels of genetic differentiation among Europeans, close correspondence between genetic and geographic distances was found. When mapping the genetic basis of a disease phenotype, spurious associations can arise if genetic structure is not properly accounted for.

2.4.5 SPIA Assay

In a hand on lesson we performed ourselves a SNP-based genetic distance test using the R package 'SPIAssay'. You can find an R Markdown of that lesson in the folder 'Additional material'.

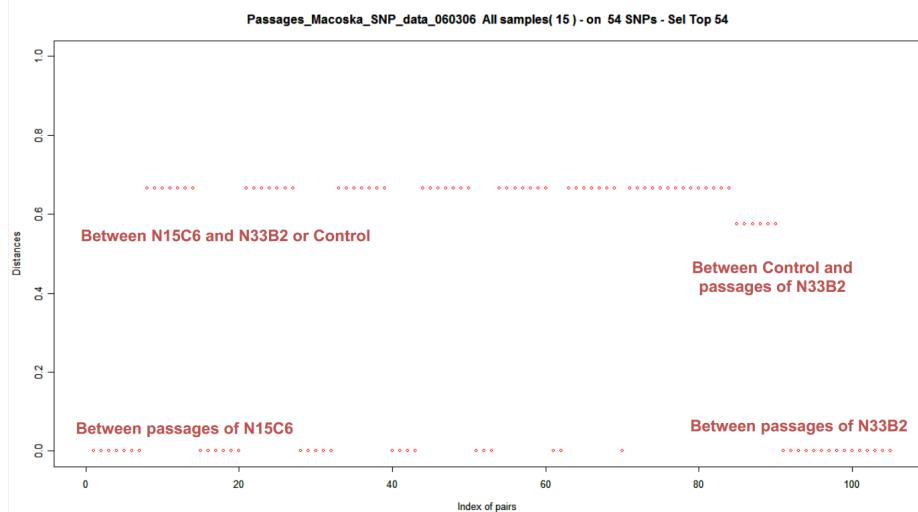
2.4. FURTHER CONSIDERATIONS AND EXAMPLES

50K chip – 58960 SNPs



(a) Comparing passages with the total amount of available SNPs

Highly selected 54 SNPs



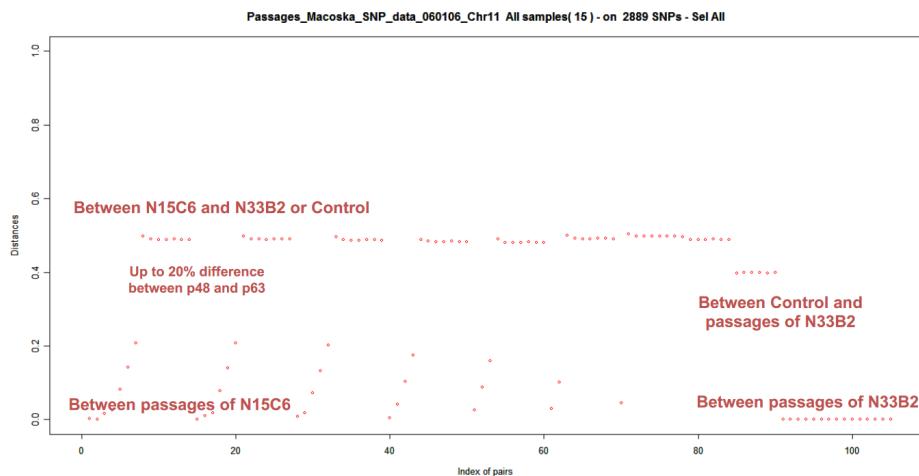
(b) Comparing passages with 54 SNPs

Figure 2.10

2.4. FURTHER CONSIDERATIONS AND EXAMPLES

Figure 2.11: Comparison passages regarding chr 11

Chromosome 11 -2889 SNPs



2.4. FURTHER CONSIDERATIONS AND EXAMPLES

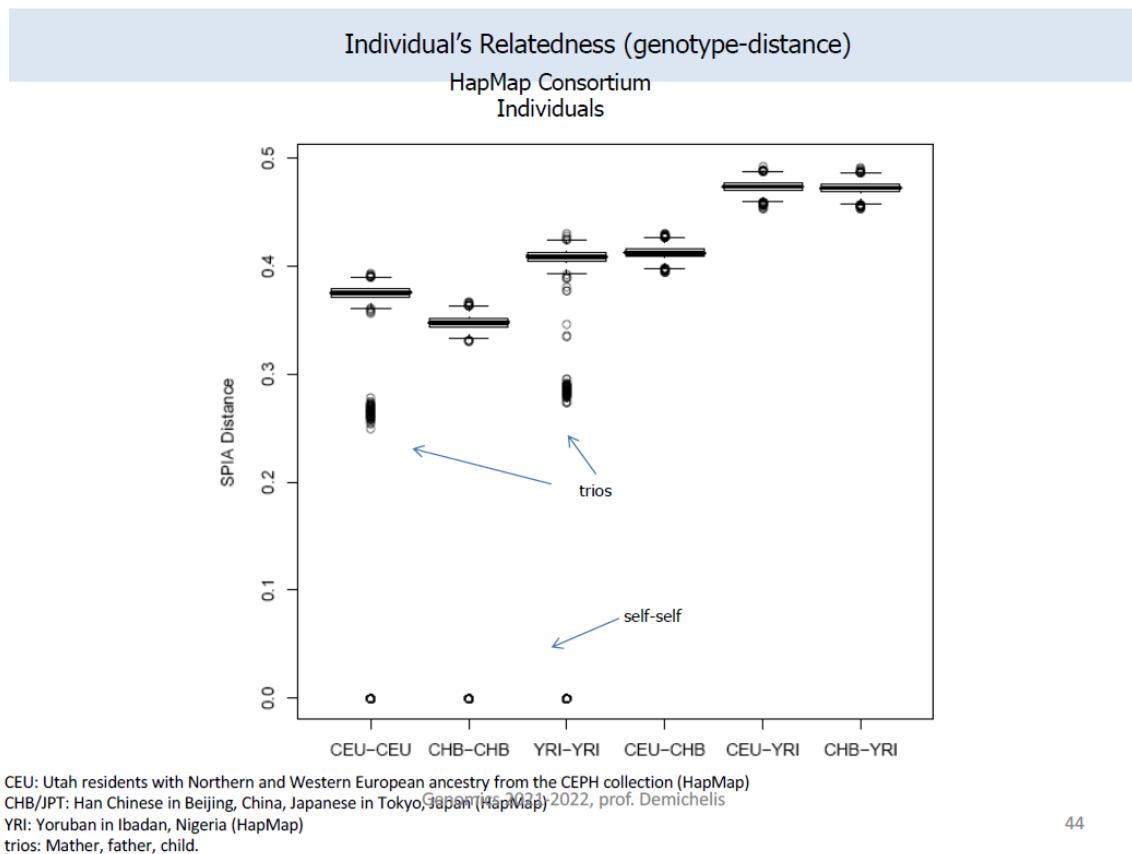
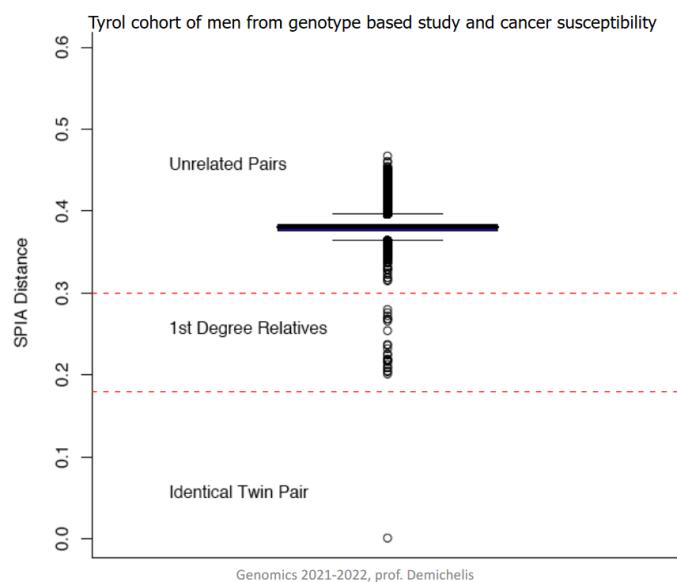


Figure 2.12

2.4. FURTHER CONSIDERATIONS AND EXAMPLES

Figure 2.13: Relatedness between individuals can be evaluated through SNPs



2.4. FURTHER CONSIDERATIONS AND EXAMPLES

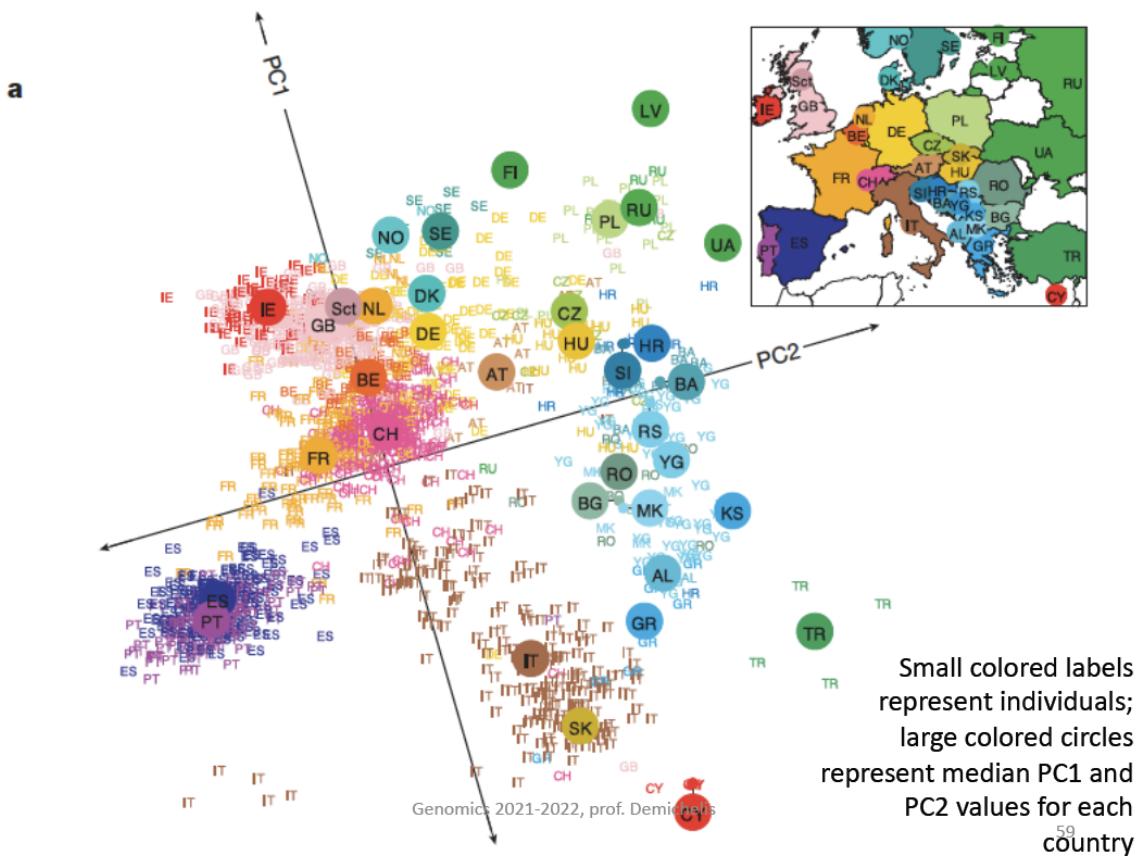
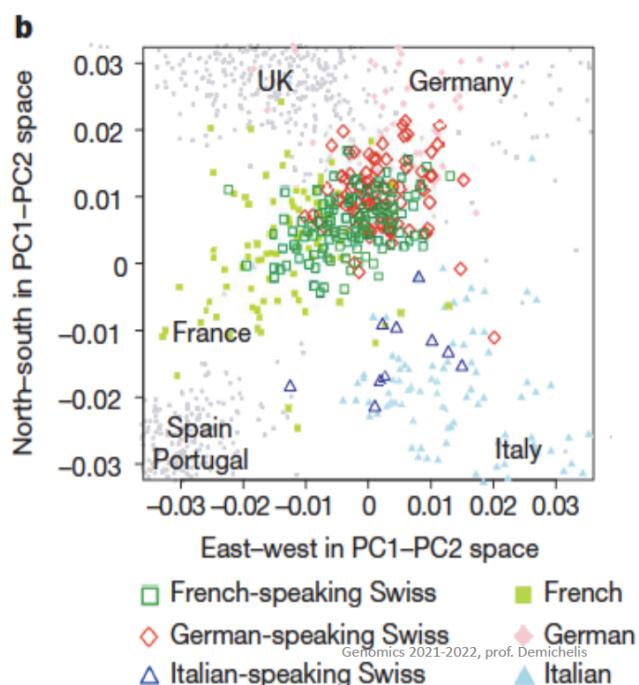


Figure 2.14: Distinction of European regions thanks to SNPs.



Switzerland: differentiation by language

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Figure 2.15

Chapter 3

IGV (Integrative Genomics Viewer)

3.1 Main characteristics

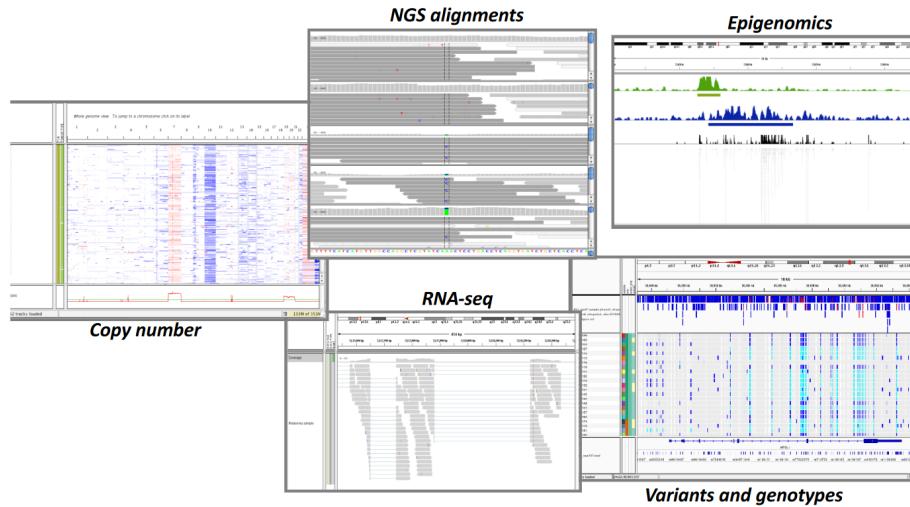
The human genome nowadays is being explored extensively thanks to exons and whole-genome sequencing, epigenetic surveys, expression profiling of coding and noncoding RNAs, single nucleotide polymorphism (SNP) and copy number profiling, and functional assays. Those findings are essential to pave the way for the future **precision medicine**, which is an approach for disease treatment and prevention that takes into account individual variability in genes, living environment, and lifestyle for each person. The scope is to administer the right drug, at the right time and at the right dose for each individual.

Below, some of the main utilizations of IGV, also represented in figure 3.1.

- **NGS alignment**
- **Epigenomics studies**
- **Copy number evaluations**
- **RNA-sequencings**
- **Identification of variants and genotypes**

3.1. MAIN CHARACTERISTICS

Figure 3.1: All the important usages of IGV



The IGV software is an **high-performance lightweight visualization tool** for interactive exploration of large, integrated genomic datasets. It supports a wide variety of data types, including next-generation sequence data, and genomic annotations. Data sets can be loaded from local or remote sources, including cloud-based resources.

It allows to move, zoom in and out quickly over different genomic scales (subfigure 3.2a), and also to jump in precise positions of the sequence. It is possible to search for genomic coordinates or gene names. For each resolution scale ("zoom level"), the aggregated data is divided into tiles (subfigure 3.2b) that correspond to a region viewable on a typical user display. Each tile is subdivided into bins, with the width of a bin chosen to correspond to the width represented by a pixel at that resolution scale. The corresponding data tiles for each zoom level are stored in the binary Tiled Data Format, or TDF, which has been optimized for fast tile retrieval.

A *tiled data file (TDF) file (.tdf)* is a binary file that contains data that has been preprocessed for faster display in IGV. TDF files are generated by using the *igvtools* package (*toTDF* command).

3.1. MAIN CHARACTERISTICS

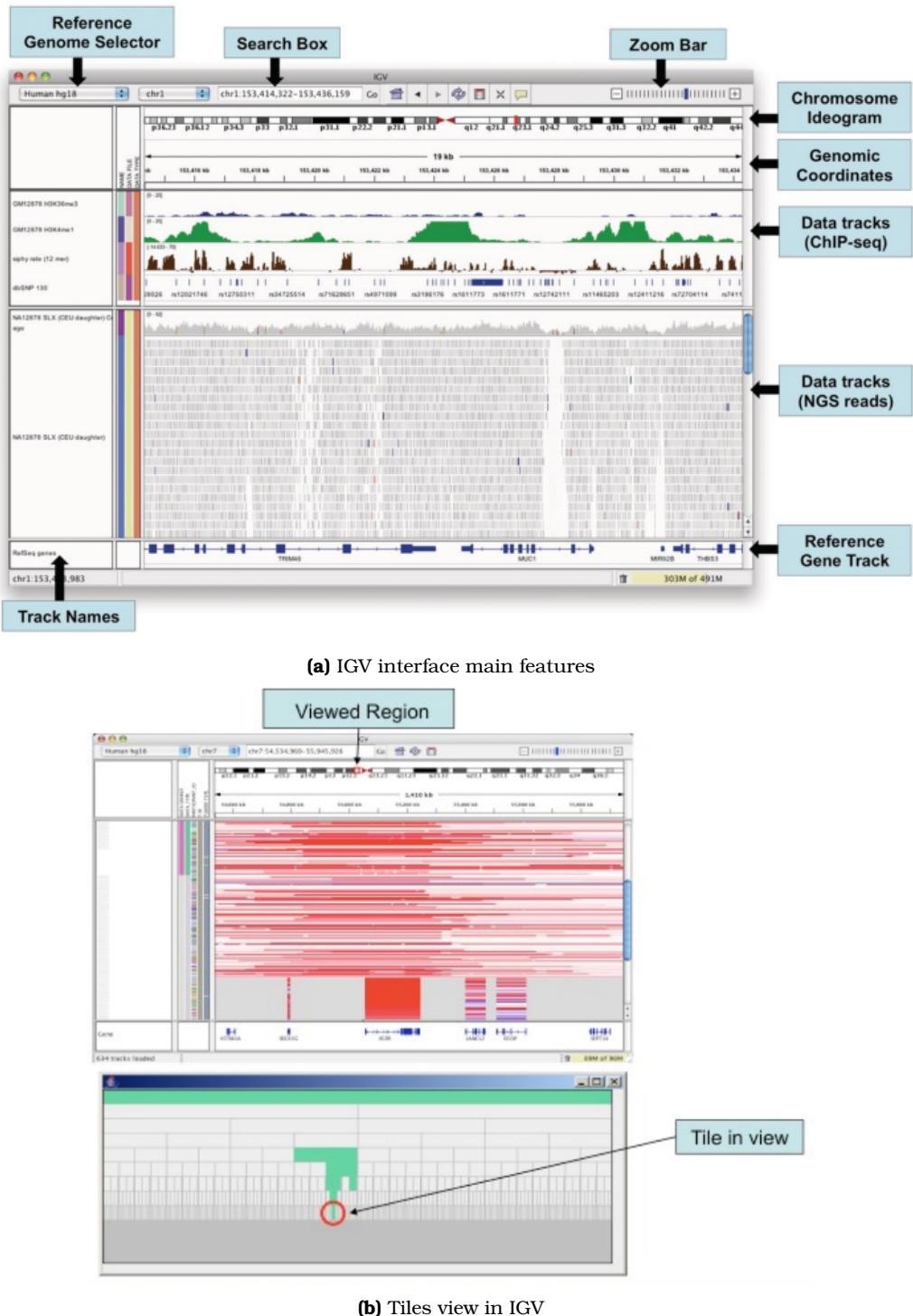


Figure 3.2: IGV navigation

3.1. MAIN CHARACTERISTICS

Importantly, **tile** sizes for each zoom level are constant and small, and also, a single tile at the lowest resolution (spanning the entire genome) has the same memory footprint as a tile at the very high zoom levels (might span only a few kilobases).

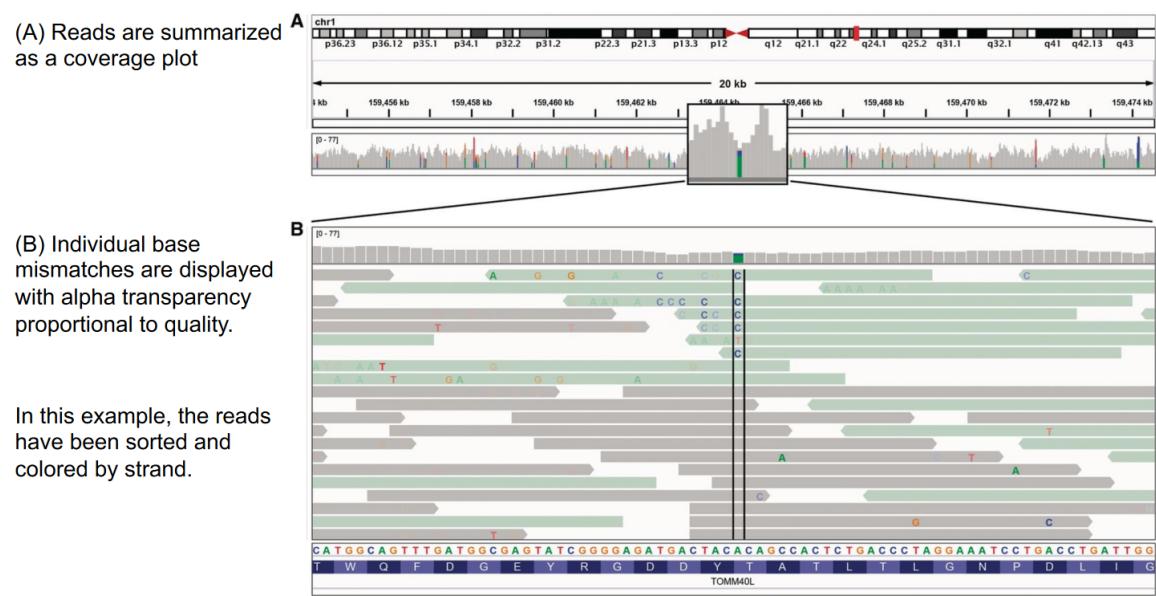
Tiles no longer in view are discarded as needed to free memory. Navigation through a data set is similar to that of *Google Maps*, allowing the user to zoom and pan seamlessly across the genome at any level of detail from whole genome to base pair.

Pixel resolution errors, occurring when data density exceeds the constraint given by the number of pixels available for display, could be solved through data aggregation. As the user zooms below the 50 kb range, individual aligned reads become visible. It is possible then to zoom further, and see the bases at each position.

Annotations for specific genomes could be found consulting the UCSC Table Browser (UCSC table).

Other information regarding IGV are present in the Supplementary information - Integrative Genomics Viewer pdf file.

Figure 3.3



3.1.1 Igvtools

Igvtools comprises a set of utilities to prepare large files for efficient display.

3.2. SOME OF THE MAIN UTILIZATIONS

Figure 3.4: igvtools possible operations, the "count" function allows to generate coverage data, and it takes in input a BAM file. The obtained file could be then loaded with the "Load pre-computed coverage data" commandq

count	- Computes alignment coverage from BAM files - Produces TDF or WIG files
toTDF	- Converts sorted data file to binary tiled data (TDF) - Supported file formats: WIG, bedGraph
sort	- Sorts file by genomic start position. - Supported file formats: BED, GFF, GTF, PSL, SAM, BAM, VCF
index	- Creates index for large genomic annotation files and alignments. - Supported file formats: BED, GFF, GTF, PSL, SAM, BAM, VCF

3.1.2 Session Files

Sessions are an integral part of IGV, allowing users to share their data and views with other users simply and accurately. Session files describe the session in **XML**.

Figure 3.5: Structure of the XML file

Required - These elements are required in a session file. All session files must follow XML standards.

- <Global>: Contains information about the general state of IGV when the session was saved
 - genome= The genome id
 - locus= The genomic range selected when the session was saved
 - version= The session version (this must equal '3')
- <Resources>: An enclosing element for all Resource elements
- <Resource>: Contains the location and other important information for your data files; for instance, a Resource could be a DAS server, BED file, or sequence alignment
 - name= The name of the track for single track files
 - path= The path IGV uses to access the resource
 - url= The URL path to the resource / UCSC Track Line Url

Optional - These elements are optional in a session file and are added by IGV to help determine the placement of the data and visual style choices.

- <Panel>: Contains information about the placement of Tracks in the visual panels
 - name= The display name for the Panel
 - height= The default height for the Panel
 - width= The default width for the Panel
- <Track>: Details information about every track in a session
 - color= The default color for the data in the track
 - expand= Whether the track is expanded or not
 - height= The default height of the track
 - id= The id assigned by IGV to this track: 2021, Demichelis
 - name= The display name for the track

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3.2 Some of the main utilizations

(I will not write down all the passages needed to obtain the figures represented below, as they are included in the exercise file delivered by the professor)

3.2. SOME OF THE MAIN UTILIZATIONS

3.2.1 RNA-seq alignments

Figure 3.6: the height depends on the quantity of reads connecting the different exons.

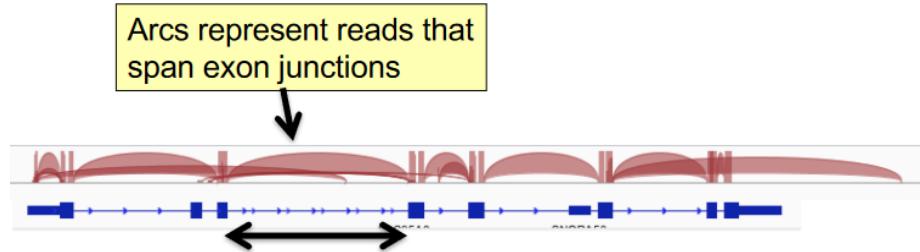
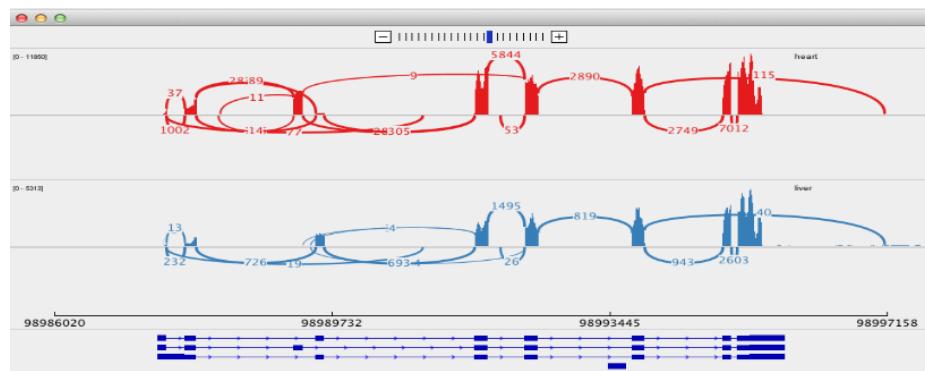
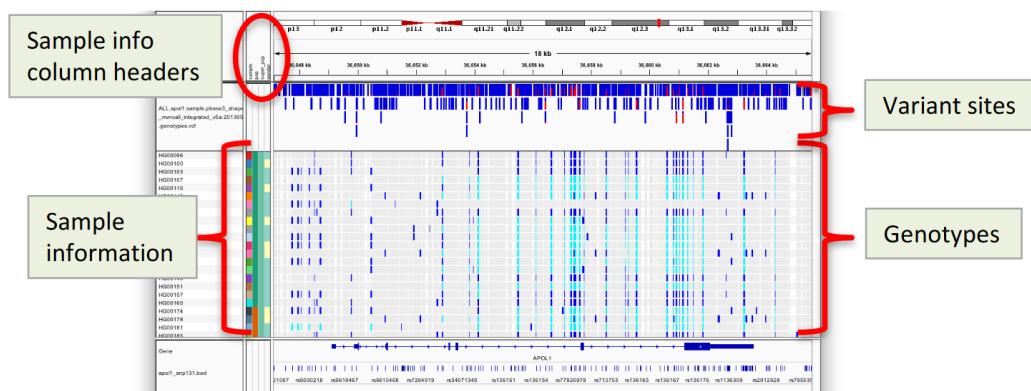


Figure 3.7: Sashimi plots: The number of reads connecting exons are represented here on the curved lines. The peaks represent coverage within exons.



3.2.2 Study of variants

It is possible to study variants from different samples.



It is also possible to sort the samples in different ways and to group them considering

3.3. EXERCISE

different characteristics.

3.3 Exercise

The goal was to read pairs/end order/coverage/insert sizes at following coordinates (hg19). Interpret, if possible, as inversion, inverted duplication, tandem duplication, or deletion.

Figure 3.8: Tasks performed

Task A (1:5)

Refer to IGV_Exercises.pdf and finalize exercises (typo CAP9->CAPN9)

Task B

Upload from folder

NA12878.mapped.ILLUMINA.bwa.CEU.high_coverage_pcr_free.RR.bam

Inspect read pairs/end order/coverage/insert sizes at following coordinates (hg19). Interpret, if possible, as inversion, inverted duplication, tandem duplication, or deletion. (Color by pair orientation)

Region 1 chr1:11,043,245-11,061,901

Region 2 chr5:9,410,315-9,413,699

Region 3 chr7:31,576,117-31,599,940 (tricky)

Region 4 chr12:12,540,452-12,550,470

Region 5 chr5:79,041,411-79,054,952

Task C

Save a session. Inspect xml file using a text editor.

[igv]

1. load BAM file

2. go to first genomic region

3. save session (*.xlm)

4. open *.xlm with text editor

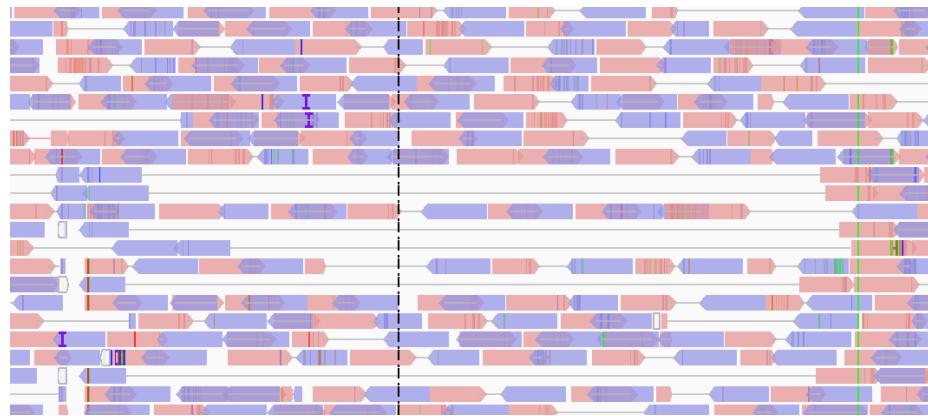
Genomics 2020-2021, Demichelis

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3.3. EXERCISE

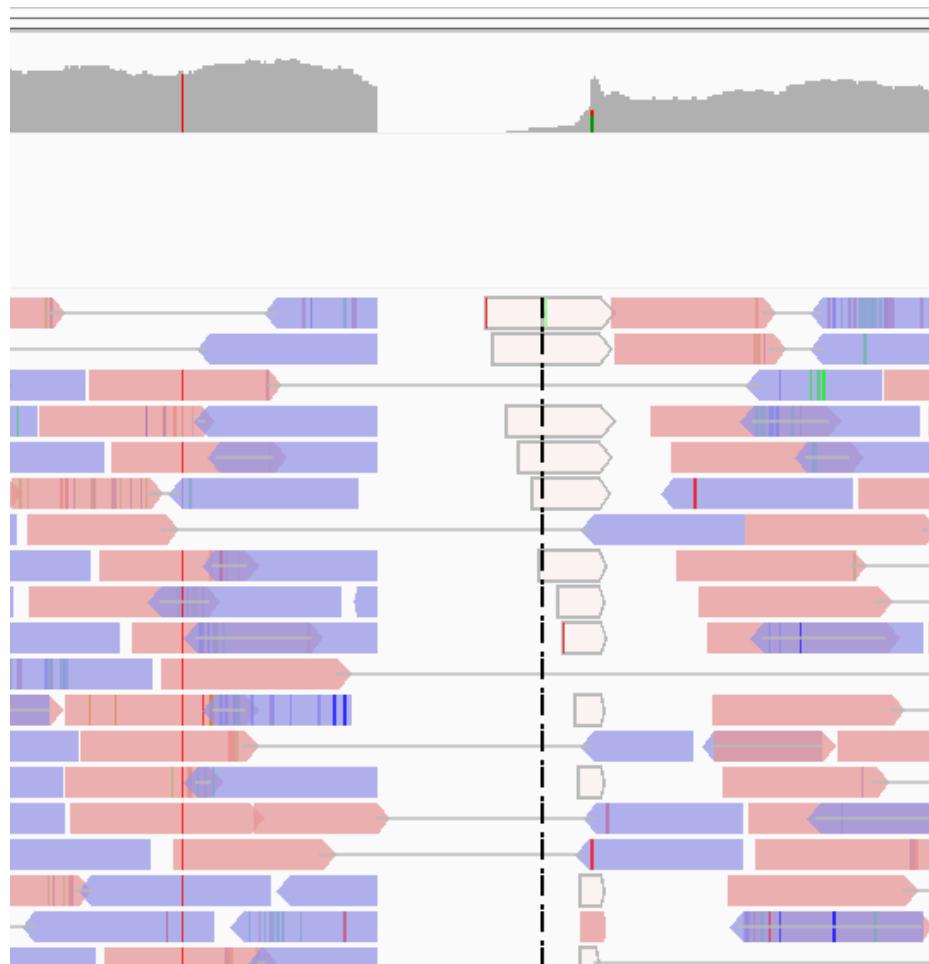
3.3.1 Task B

Figure 3.9: chr1:11,050,009-11,055,137: It could be a tandem duplication on one of the two alleles and a deletion on the other allele. The reason why I would suggest the presence of a deletion is due to the fact that the coverage remains quite constant, despite of the duplication.



3.3. EXERCISE

Figure 3.10: chr5:9,410,315-9,413,699: it is quite clear that both the alleles were deleted in that region, because of the decrease in coverage



3.3. EXERCISE

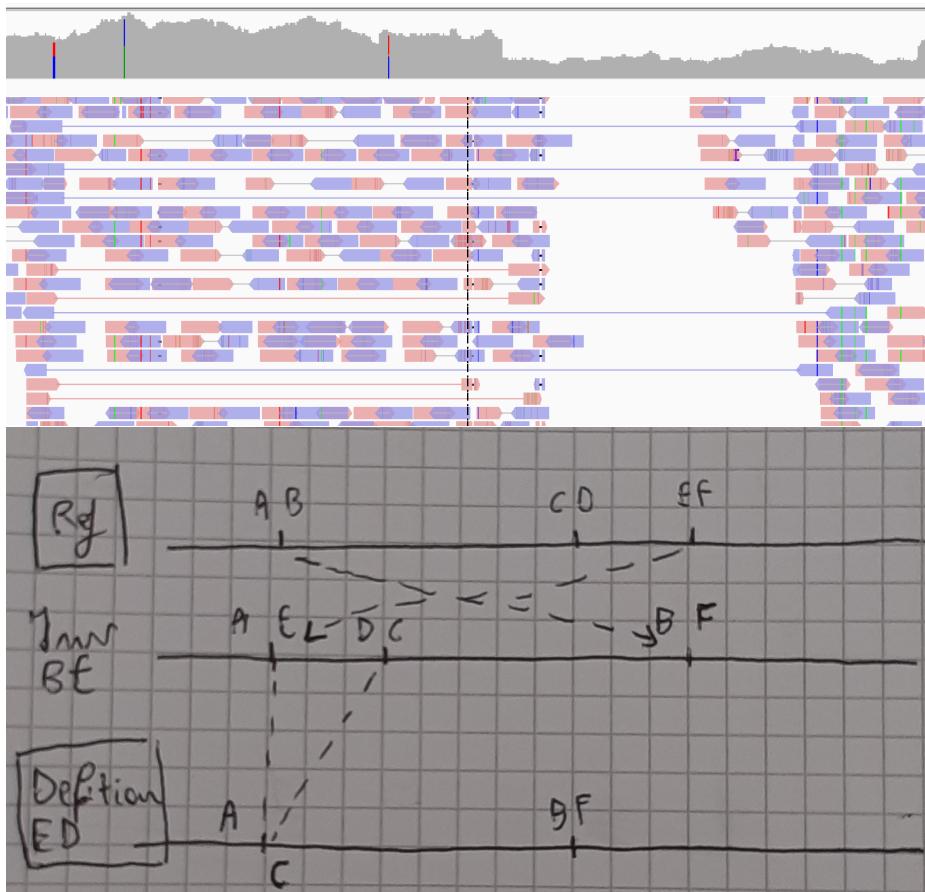


Figure 3.11: chr7:31,576,117-31,599,940: Basically you have an inversion between B and F, and after the deletion of the ED portion, the other allele remains normal.

Chapter 4

Tumor evolution studies via NGS data

4.1 Why studying tumor evolution?

Understanding tumor evolution is useful for:

- **Academical purpose;** mainly for research
- **Clinical purpose;** the order of somatic events during tumor evolution can be relevant when considering the management of a patient, e.g. it can affect the treatment decided by the tumor board. A **tumor board** is an organism present mostly in research oriented hospitals (but also non research oriented ones and its popularity is increasing), and it is composed by different specialists (oncologist, genetist, radiologist, computational biologist, pathologist and others) which manage the patients jointly. Aside from clinical purposes, this organism is also useful for training new experts.

4.2 Tumor heterogeneity

A tumor can arise due to:

- A single (or few) strong driver mutation (in oncogenes or oncosoppressor genes)
- Several mutations that gradually change the cell phenotype without leading to cell death

In both cases the mutations are somatic events due to stochastic processes, mainly due to carcinogenic substances that damage DNA therefore causing mutations (but also physical phenomena such as radiations and others). These mutations are mostly associated to cell growth; this is why tumor cells often undergo clonal expansion and create a mass. The speed at which the mass grows and mutates is dictated by the mutations that occurred.

A tumor mass can be either homogeneous or heterogeneous; in general, the more aggressive and old the tumor is, the higher the degree of heterogeneity. Higher heterogeneity

4.2. TUMOR HETEROGENEITY

usually correlates to drug resistance (since some of the clonal populations might be able to better resist the drug compared to others).

New Generation Sequencing allows to study all the somatic mutations that occurred in a cell, both the cancer related ones and the benign ones. By sequencing with an appropriate depth, you can infer in which fraction of the cell population a certain mutation is present; this allows to reconstruct the clonality of the tumor and the mutation history. Notice that since very deep sequencing can give errors, you usually need to check different loci in order to consolidate your result.

Tumor heterogeneity can be subdivided into:

- **Inter-individual heterogeneity:** tumor from patient A is different from that of patient B
- **Intra-individual heterogeneity:** tumors from the same patient might differ
 - **Spatial heterogeneity:** synchronous tumor masses in the same patient might differ
 - **Temporal heterogeneity:** a tumor might change over time, due to spontaneous or drug induced selection
 - **Intra-lesion heterogeneity:** an individual tumor mass might present different lesions (which display different clones with different mutations, therefore different phenotypes, treatment resistances and so on)

Almost always, many of these heterogeneities are present simultaneously.

Notice that genetic heterogeneity does not necessarily reflect morphological heterogeneity (e.g. different prostate lesions might look the same when stained but then display different markers using *in situ* immunochemistry). Moreover tumor mass size does not necessarily correlate with aggressiveness (hence imaging is not enough to study tumors). Heterogeneity might cause problems in the interpretation of the spectrum obtained via Sanger sequencing, since the sample might contain different sequences for the same locus, hence leading to an overlap in the peaks. In case of different lesions, we can define tumor burden and features of each of them via individual sequencing.

Tumor evolution can happen in two ways:

- **Linear evolution:** genetic instability leads to new tumor clones and if those display some advantage compared to the previous ones, the older ones get replaced by the new ones (otherwise the new clone dies down). In this case you generally have low heterogeneity.
- **Branched evolution:** genetic instability leads to the formation, from an ancestral clone, of different clonal populations which can coexist in the same or different tumor masses. In this case you generally have high heterogeneity.

A metastasis can either have:

- **Monoclonal origin:** meaning that it originates from tumor cells coming from a single lesion. In this case you have similar features as the starting mass and overall low heterogeneity within the metastasis.

4.3. ALGORITHMS TO STUDY TUMOR EVOLUTION

- **Polyclonal origin:** meaning that it originates from multiple tumor cells coming from different lesions. This phenomenon is called **multiclonal seeding** and it leads to high lesion heterogeneity. Moreover, the fact that it displays some of the features from each of the parental lesions makes the analysis more complex.

As previously mentioned, tumor heterogeneity plays a big role in defining treatment resistance. We talk about two types of drug resistance:

- **Primary resistance:** the pre-treatment tumor mass already contains cells that are resistant to the treatment; the treatment kills the non-resistant cells, hence the resistant clone expands.
- **Acquired resistance:** the pre-treatment tumor mass does not already contain cells completely resistant to the treatment; the clones that can survive the treatment the best could then mutate in order to acquire a treatment immunity mechanism.

In case of primary resistance, the tumor might already display some biomarkers pointing to some treatment resistance; this is useful for the tumor boards in order to avoid needless harmful treatments. However, no biomarkers for each treatment are known, plus the tumor can always evolve unpredictably and acquire a new resistance.

4.3 Algorithms to study tumor evolution

You can study tumor evolution using information from:

- Samples from the **same subject**, from different time points or lesions; this way you can reconstruct mutation order and metastatic processes within the individual (base on shared or not mutations).
- Samples from **different subjects** affected by the same pathology (e.g. prostate cancer); you use recurring patterns across individuals, this way you can reconstruct more generic features of the pathogenesis, for instance:
 - Very common mutations in the pathology (those shared across many individuals)
 - Mutations that tend to happen in a specific order (take for instance two mutations A and B; if in the majority of tumors which present both lesions, B is almost always subclonal to A, then probably A tends to happen prior to B).

For more in depth reading (clickable links):

- *The evolutionary history of lethal metastatic prostate cancer, Gundem et al, Nature 2015*
- *Punctuated evolution of prostate cancer genomes, Baca et al, Cell 2013*

NOTE: I did not add some pictures even though they were commented in class because I think that the relevant part was understanding the points listed above.

In general, when you have some tumor data, you try to see which of your models best fits the progression.

There are several aspects that must be taken into account during this type of analysis; most of them are useful in comprehending the pathology and its mechanisms, but at the same time they make analyzing the NGS data more difficult. Some of these aspects are:

4.3. ALGORITHMS TO STUDY TUMOR EVOLUTION

- **Heterogeneity** (inter-patient, intra-patient, intra-tumor)
- **Time dependence** (tumor changes overtime)
- **Treatment status** (was the tumor treated, if yes how?)
- **Admixture DNA** (presence of non-tumoral DNA, *explained more in depth below*)

In a tumor biopsy you could have (and this is generally the case) other cells that are not tumoral (healthy tissue cells, stromal cells, leukocytes...). It is then defined the concept of **admixture**, which is *the fraction of non-tumoral DNA within the sample*. Admixture is then used to define **tumor purity**, which is

$$\text{tumor purity} = 1 - \text{admixture}$$

To sum up, a fully tumoral sample would have admixture equal to zero and purity equal to one. The opposite holds for healthy tissue (purity equal to zero, admixture equal to one). Deconvoluting the sequences derived from admixed DNA complicates NGS data analysis, but also provides useful information:

- Aggressiveness of a lesion; in general, the lower the purity, the better the outcome
- Defining whether a mutation is actually part of a subclonal tumor population or it is just admixed DNA

The most useful feature from NGS for characterizing tumor evolution (clonality, purity and so on) are:

- Copy number mutations
- Point mutations
- Single cell sequencing
- Polymorphic information (which SNPs does the tumor have)

The algorithms used to study tumor evolution use **informative SNPs**, meaning:

- SNPs for which the individual is heterozygous (hence they vary from individual to individual)
- SNPs for which the allelic fraction is easily measurable

Making parsimonious assumptions (mainly that all clones have the same growth rate), these algorithms allow to study any form of genetic aberration.

REMINDER: Do not confuse the following concepts:

- **Minor allele frequency:** frequency of the alternative allele for a locus in the **entire population**
- **Allelic fraction:** frequency of the alternative allele for a locus in a **single individual**. It is a local property of the individual. In terms of NGS this becomes:

$$\text{Allelic fraction} = \frac{\text{locus reads with minor allele}}{\text{total locus reads}}$$

4.3. ALGORITHMS TO STUDY TUMOR EVOLUTION

Other important concepts to consider are:

- **Neutral reads:** reads equally representing parental chromosomes
- **Beta fraction:** percentage of neutral reads. Beta goes from 0 to 1; the closer the value to 1, the closer the reads are to a 50/50 split among parental sequences, the closer the value to 0, the closer the reads are to a 100/0 split in favour of either parental sequence.

NOTE: The way to compute beta values is in the slides but skipped during the lecture
The **allelic fraction** for an informative SNP can be:

- 0 if the alternative allele was deleted
- 1 if the reference allele was deleted (the non-alternative one)
- Around 1/2 if both alleles are present in equal proportion
- Some other value in the range (0,1), that could be due to duplication, heterogeneity (admixture and/or subclonality), errors and so on. In this case some further information might be required (for instance the coverage)

The **beta fraction** can be:

- 0 if either allele was deleted (hence you have only one)
- 1 if both alleles are equally-represented (normal condition)
- Any other value in the range (0,1), and this is also due to heterogeneity and other factors.

Notice that allelic fraction and beta fraction give similar information, but the beta fraction is not allele specific, hence it is better suited to study genetic abnormalities.

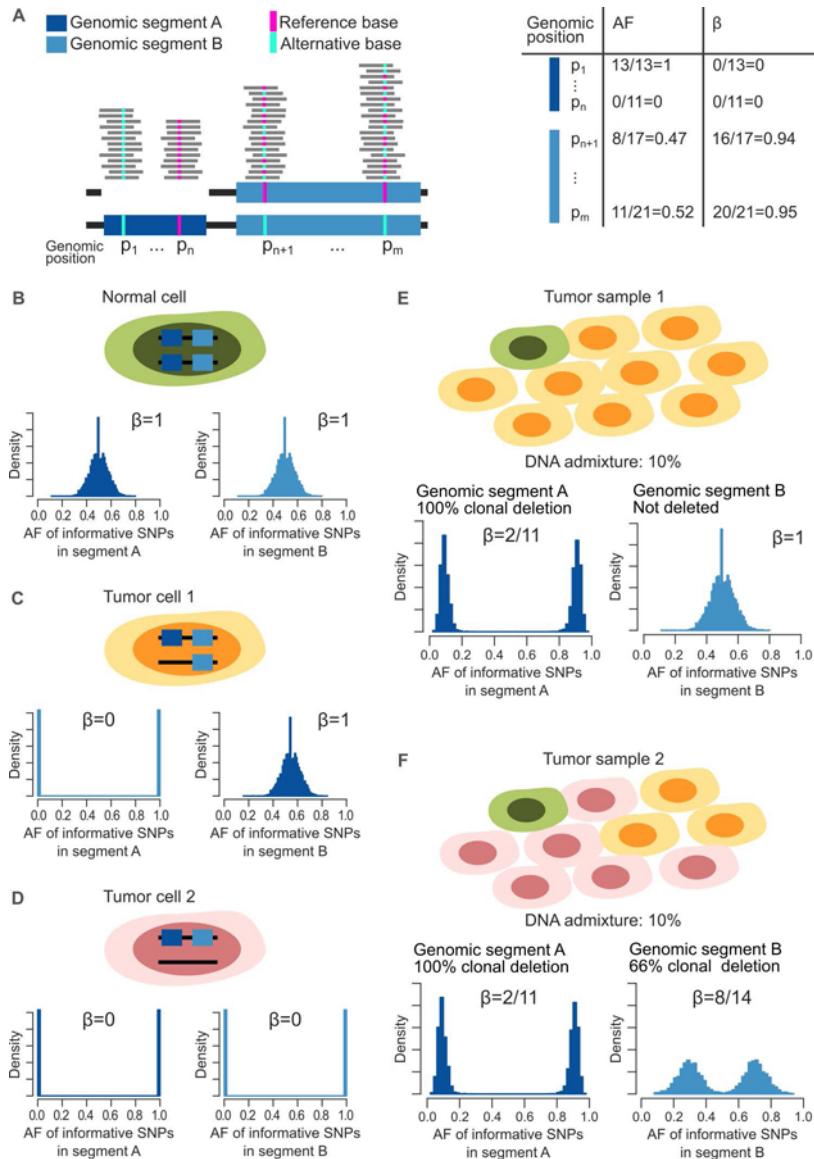
Using allele frequency and beta fraction, informative SNPs can be used to reconstruct the genealogy of the mutations. If there is a deletion of a region then you have a loss of heterozygosity for all SNPs in that region (since you chose informative SNPs, hence heterozygous ones); then based on the mutations present or absent in the different clones of the lesion (since you do not have a perfectly homogeneous mass) you can reconstruct their order.

When designing a test you need multiple informative SNPs for each genomic fragment of interest. Moreover you have to choose alleles that have high MAF (hence the minor allele frequency is still rather high), since those are more likely to give you information.

For more in depth reading (clickable links):

- *Ploidy- and Purity-Adjusted Allele-Specific DNA Analysis Using CLONETv2, Davide Prandi, Francesca Demichelis, 2019*

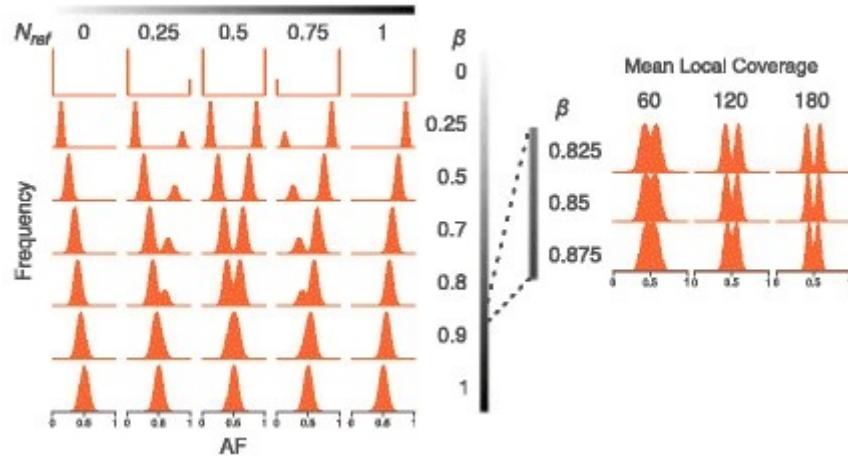
4.4. ESTIMATING ADMIXTURE AND CLONALITY



4.4 Estimating admixture and clonality

Notice that the beta fraction correlates with the shape of the distribution of the allelic fractions of the informatics SNPs in the read; with beta = 1, you have a normal distribution with mean 0.5, with beta = 0 you have two sharp peaks at 0 and 1, with any intermediate value you have to peaks which can be partially overlapping for values close to 1. Notice that increasing the coverage does increase the resolution of the peaks. For this reason increasing the coverage (with beta constant) does increase the ability to distinguish clonality, especially of populations that are only some degree of difference from each other.

4.4. ESTIMATING ADMIXTURE AND CLONALITY



To estimate the admixture/clonality of a cell population:

- Measure the allelic fraction and beta fraction of each informative SNP of a genomic region
- For each region try which of the models fits your data the best (basically map the distribution of the allelic fractions of the region against prefitted reference distributions)
- You can then compute the local and the global admixture:
 - **Local admixture** is a measure of the fraction of cells displaying a certain lesion with respect to another; for this reason local admixture is used as an estimate for **clonality**
 - **Global admixture** is a measure of how many cells, on average, have a lesion; this can be used to estimate the **DNA admixture** (purity) of the sample

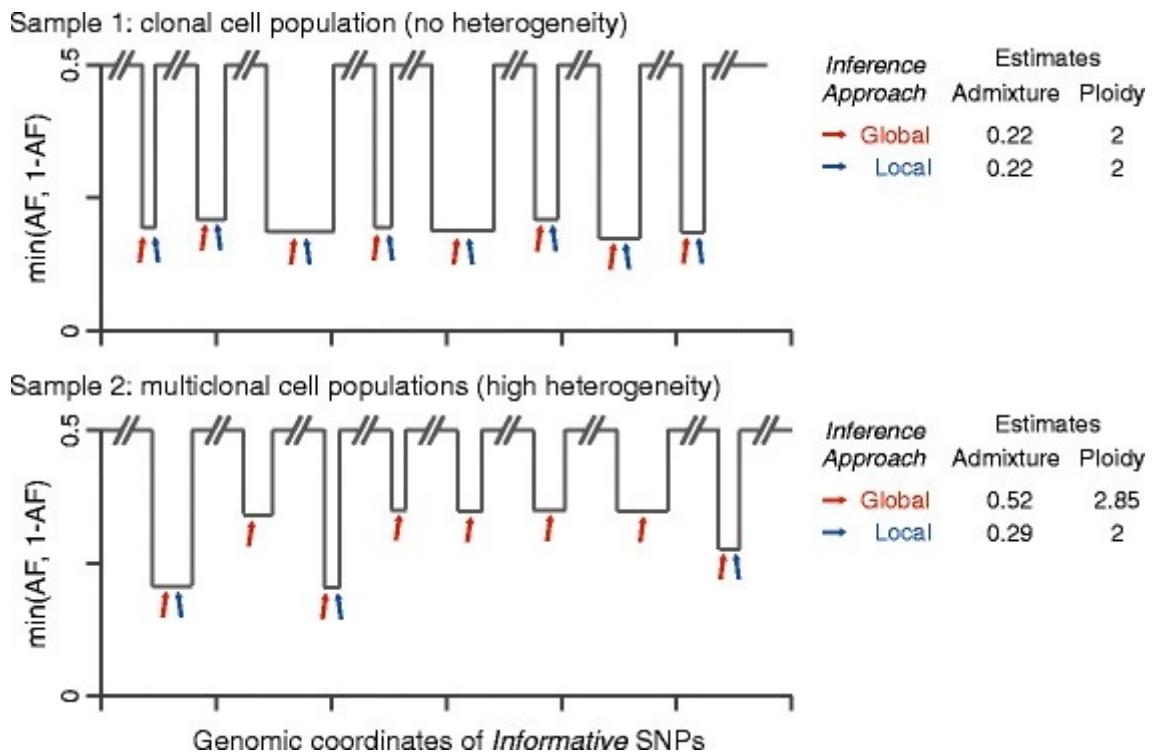
Hence this technique allows you to distinguish purity and subclonality.

Graphically you obtain a plot with:

- On the x axis, the chromosomal coordinates indexed by informative SNPs. The longer the horizontal segment, the bigger the considered region.
- On the y axis, the MAF values for the informative SNPs. MAF values are mirrored on 0.5, since you do not care about distinguishing the alleles. Any drop below the 0.5 value means that the region does not have a 50/50 split. The deeper the drop the deeper the difference in the representation of the alleles.

In the example picture, the top subplot shows drops which have very similar depth, hence global and local admixture are similar and there is very low heterogeneity. In the bottom subplot you have differences in local and global admixture, hence we can infer the presence of different clonal populations.

4.4. ESTIMATING ADMIXTURE AND CLONALITY



For this type of analysis is always useful to have the **match normal DNA** (the non-tumor DNA of the subject): match normal DNA is usually obtained from leukocytes in the blood, otherwise one could somehow deconvolute the signal of the admixed cells.

Another graphical representation in bidimensional space is the following plot:

- On the x axis the **log2 ratio**, meaning

$$\text{log2 ratio} = \log_2 \frac{\text{local tumor coverage}}{\text{local normal coverage}}$$

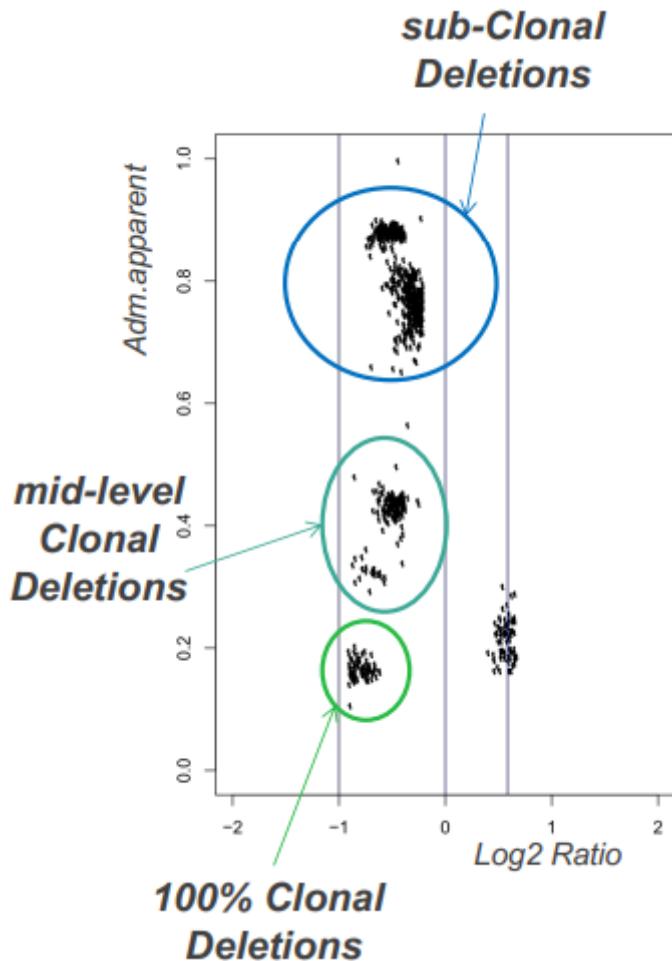
This indicates how abundant cancer DNA is with respect to healthy DNA (gain of DNA if above zero, loss of DNA if below zero).

- On the y axis the **apparent admixture**, which is defined as

$$\text{Adm. apparent} = \frac{\beta}{2 - \beta}$$

Notice that this measure refers to each individual deletion/abnormality.

- The dots which represent the individual genomic segments. The dots tend to create multiple clusters and the closer two points are, the more probable the events they represent are close to each other in time.



You can compute clonality using the formula:

$$\text{clonality} = \frac{1 - \text{Adm. apparent}}{1 - \text{Adm. global}}$$

An example of how heterogeneity can lead to difficult to interpret results can be found in the following paper: *Unraveling the clonal hierarchy of somatic genomic aberrations*
 NOTE: The case study was rushly explained during the lecture, but in my opinion it did not provide any further information; this is one of the papers uploaded on moodle.

Chapter 5

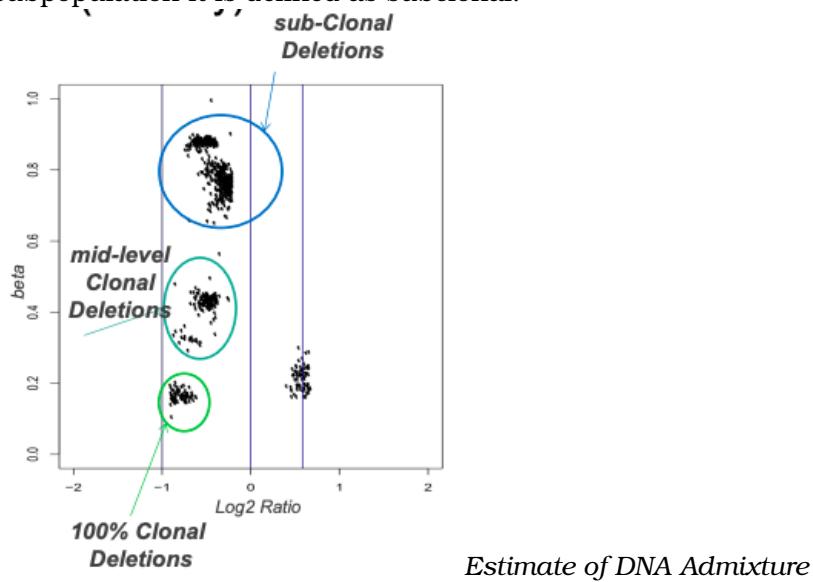
Tumor evolution studies (continued)

5.1 Recalls from the previous lecture

At the basis of tumor evolution is the concept of how to use informative SNPs: SNPs for which a specific individual has heterozygous calls so that set of SNPs is unique for every individual.

This property is connected to the fact that when we have the loss of an allele, the allelic fraction of the informative SNPs within that lesion will be informative of the lesion and its depth (clonality = what's the fraction of tumor cells that very likely harbor that lesion).

We can also have different population of cells, when a set of lesions is present in every population it is said to be clonal whereas when a specific set of lesion is harbored only by a subpopulation it is defined as subclonal.



Log2 Ratio is the log₂ of the ratio of the tumor over the normal that applies to array

5.1. RECALLS FROM THE PREVIOUS LECTURE

data signals (intensity of the signals) but also to the local coverage of a tumor BAM file over a normal BAM file.

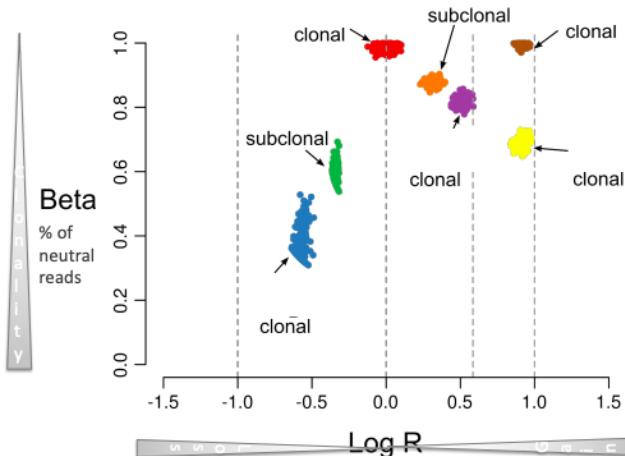
In the figure each dot is a genomic segment or a gene that clusterize in the space and when dots are in a same cluster it means that they very likely share the same copy number status and also the same level of clonality.

Beta is a variable that goes from 0 to 1 and provides information of the number of reads that equally represent the two alleles; when beta is equal to 1 the concept of admixture (1-purity) is equal to 1 meaning that purity is equal to 0 if we are at the top of the y scale it means that there's no signal related to tumor content, while the lower we go, so the closer we get to 0, the higher the tumor content and the level of clonality is.

If we use this equation we can assess the level of clonality of a cluster.

So the graph in the figure puts in relation the copy number status (\log_2 ratio) and the purity/clonality of the sample (Beta); the more we go towards the left the fewer number of copies, the lower on the y axis the higher the clonality.

The best proxy of the quantity of tumor content present in a sample is done using the lowest cluster.



We have losses and gain of DNA copies,

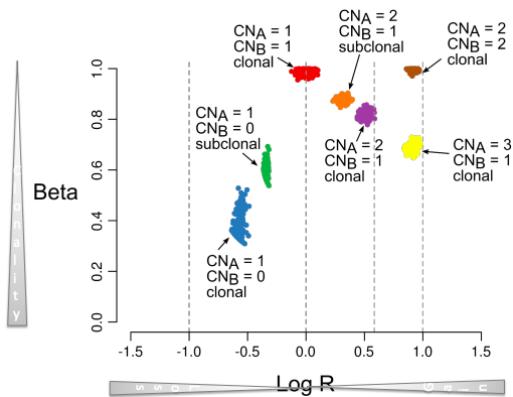
moving on the x axis.

The beta is related to the clonality so the lower we go the more clonal the signal is.

The only difference from the previous figure is the presence of extra clusters:

- The blue cluster with deletions is the most clonal one
- Both blue and green clusters had deletions, since they have a negative \log_2 ratio, but the green ones are less clonal than the blue ones
- In $\log_2 R = 0$ and $\beta = 1$, where there's the red cluster, we have a status of no copy number changes (wild-type status in terms of copy numbers). This basically represents a total number of alleles which is the same in both the tumor and normal sample.
- All the other clusters with a positive \log_2 ratio had a gain of DNA

5.1. RECALLS FROM THE PREVIOUS LECTURE



In this figure the number of copies that correspond to all the clusters in the space is also reported.

- Blue one: one copy of DNA, so we have a deletion
- Green one: also one copy of DNA but with subclonality

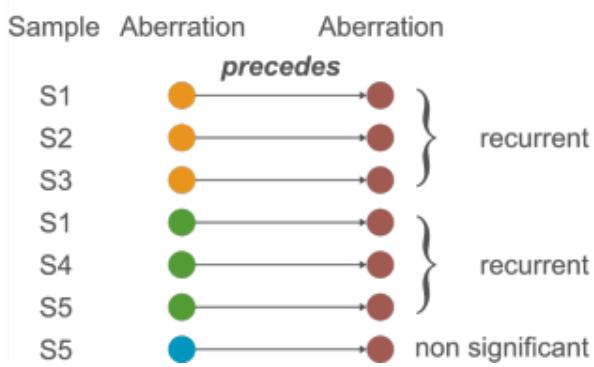
This is how we can map in the space the status of clonality and the number of copies for a specific segment in the genome.

So again, the lower we go the more clonal the clusters are, the more left the deeper they are in terms of loss of DNA.

We can use these information to build *evolution maps*.

The first thing to do is to look, within each individual, at concomitant deletion where one is subclonal to the other one.

Ordered aberrations



In the figure:

- In sample 1 the brown lesion is subclonal to the orange one, and that same lesion is also subclonal to the green one.
- In sample 2 we have again the support of the relation between the brown and orange lesion with the same level of subclonality (brown subclonal to orange).
- In sample 3 is the same as in sample 1 and 2.

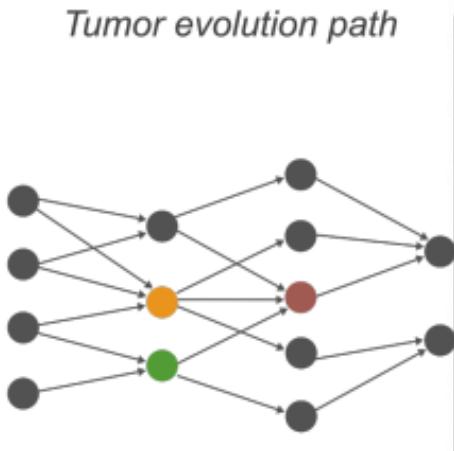
5.1. RECALLS FROM THE PREVIOUS LECTURE

- Samples 4 and 5 have the same concomitant green and brown lesions again with the same level of subclonality.
- In sample 5 only we also have another concomitant lesion (blue subclonal to brown).

So we perform this analysis for all the concomitant lesions in our sample and we start drawing the arrows to keep track of what is subclonal to what. We compile this list across all individuals and look for how many times we see support for the same relationship in the same direction.

In our case we can say that the relationship going from orange to brown is supported by 3 out of 5 individuals; the same can be said for the green going to brown. The blue one is instead not significant since it's supported by only one individual.

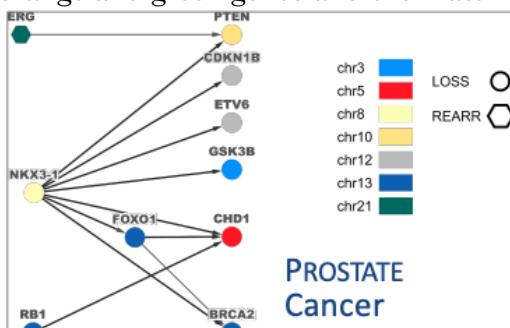
So having multiple observation supporting that aberration x precedes aberration y (i.e. aberration y is subclonal to aberration x) we can build an evolution chart.



The orange and the green which have no relationship between them, are at the same level on the x axis in the path and they both go into brown.

So one can assume that the more clonal a lesion is the more likely it is that it occurred earlier during the evolution (time is on the x axis of the path), and we can look for recurrent relationships among lesions.

In principle we can say that the grey ones at the beginning happened at the same time point and then at a second time point, the tumors in our set of samples, underwent loss of orange and green genes and then later they both underwent loss of the brown gene.



If we do that in large datasets (lung cancer melanoma, prostate cancer ...) we can come up with all the dependencies that were observed and

5.1. RECALLS FROM THE PREVIOUS LECTURE

that were supported by more than one individual (e.g. in prostate cancer we can say that a loss in NKX3-1 precedes the deletion of PTEN).

Even if we have hundreds of BAM files on whole exon sequencing data from large collections all that we can build are evolution maps with at most three layers (pretty disappointing).

This has multiple reasons, one of them is that:

- To build a relationship which is statistically significant between two genes we need to have multiple instances of that relationship (in many samples) which means that we need to have co-occurrence of the two lesions and subclonality of the second lesion with respect to the first in a significant number of individuals compared to the total number of individuals that have co-occurrence. So if co-occurrence occurs in N individuals and subclonality of the second lesion to the first one occurs in a fraction of those, only if this fraction is significant with a proportion test out of the total number, then we can build the path.

Therefore we are tremendously limited by co-occurrence of lesions.

To boost the reconstruction of these paths gene families or pathways have been exploited.

E.g. if we are dealing with PTEN which is a tumor-suppressive gene relevant in a specific pathway (PF3K), then it doesn't matter if we have deletion or inactivation of the same genes in the same pathway, what matters for the tumor evolution is that that specific pathway is altered and so what we can do is start aggregating signals from genes that belong to the same pathway.

So if individual 1 has a relationship between gene A and some gene in a specific pathway (PF3K) and individual 2 has a relationship between gene A and a second gene in that same pathway, then we can assume that maybe they have the same effect and so we can aggregate the information on the landing gene.

So instead of going from gene 1 to gene 2 we go from pathway 1 to pathway 2, and in terms of numbers what we gain is that the co-occurrences are counted including all the gene lesions with the same function in pathway 1 and all the gene lesions with the same function in pathway 2 (if we consider the inactivation of the gene then we have to consider all the lesions that inactivate the gene and not others).

We can then run a simple test to build our path.

With this method we start having some more data to look for major changes during the evolution of the tumor pathway.

E.g. in prostate cancer we'd identify a set of pathways that are more or less at some level altered in earlier staged disease and that then trigger or are precedent to our pathways. Doing so we can learn more in terms of the biology of the disease evolution.

We can also decide to go for a mix model or a mix approach, where for certain genes we go at the pathway level while for other we treat them separately.

There are also more complicated ways to make inference of tumor evolution. Some try to avoid the hypothesis that the more clonal a lesion is the more likely it is to happen early, because we know it's not always the case; it might be in untreated samples but not in treated samples. In a treatment regimen, because of drug pressure selection, specific resistant clones harboring a specific lesion can take over due to their higher rate of proliferation, so in this case if we see a lesion that appears to be more clonal it doesn't really mean that it happened earlier, it may be that it had a higher proliferation and so it's

5.2. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

taking over (and we see it as apparently clonal but it's in fact a late event) -> important concept in precision medicine.

So simplistic approaches like the one discussed are proper for untreated (in terms of drugs) primary diseases.

Evolution charts can also be boosted via the combination of multiple molecular layers.

5.2 Ploidy and purity correction on $\log_2(\frac{T}{N})$ data

How can we use measure of the tumor purity and the effect of the tumor ploidy?

How can we compare two different samples for which we quantify completely different levels of tumor content?

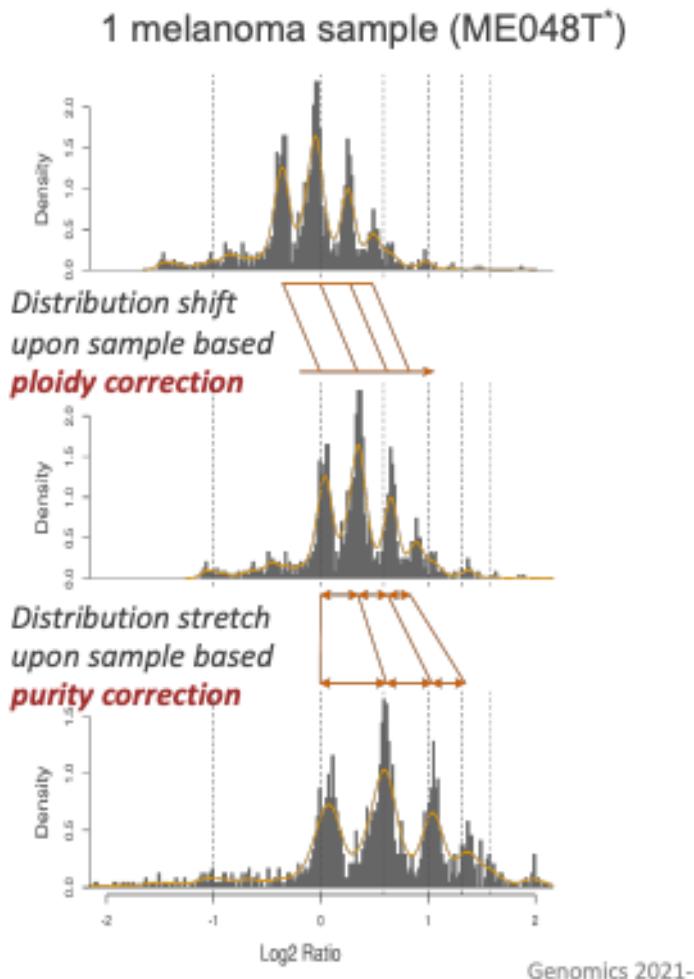
E.g.: we have a sample a 100% pure and with 50% of clonality (a lesion present in 50% of the cells) and a second sample with a tumor purity of 10% and a clonality of 100% (a lesion present in 100% of the cells), we need a way that allows us to compare numbers without having to convert everytime for every lesion the depth of the lesion based on the tumor content, so we need an equation that we can apply to every individual data that puts everything on the same level

(same concept as gene expression normalization).

The coverage makes data coming from different samples comparable because we normalize everything to the total coverage, but when we deal with diseased cells we can have contamination from the admixture, so we need an extra step.

The step, once we know how to assess the tumor purity and ploidy, is quite simple: we need to adjust the data for tumor purity and ploidy.

5.2. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA



* raw data from Berger et al. Nature. 2012 May 9;485(7399)

Schematically

In the figure we are looking at one tumor sample: a whole genome sequencing of one melanoma sample.

We see multiple peaks which correspond to different copy number states.

Let's suppose we have a genome with a backbone of three copies but we sequence a bulk and we don't have 100% purity but 80% (so 20% is contamination).

Ploidy correction

Computationally we assess the ploidy through the copy number space and then correct the data.

From the tumor and the normal we obtain something like the first graph, and we could wrongly assume that the main peak is always in 0 (wild-type state of the genome), but it shouldn't.

In fact, if we assess the ploidy and overall we see a backbone state of three copies for our genome, then the main peak should be shifted toward three.

So, the *ploidy correction shifts the distribution* towards the right (second graph).

Purity correction

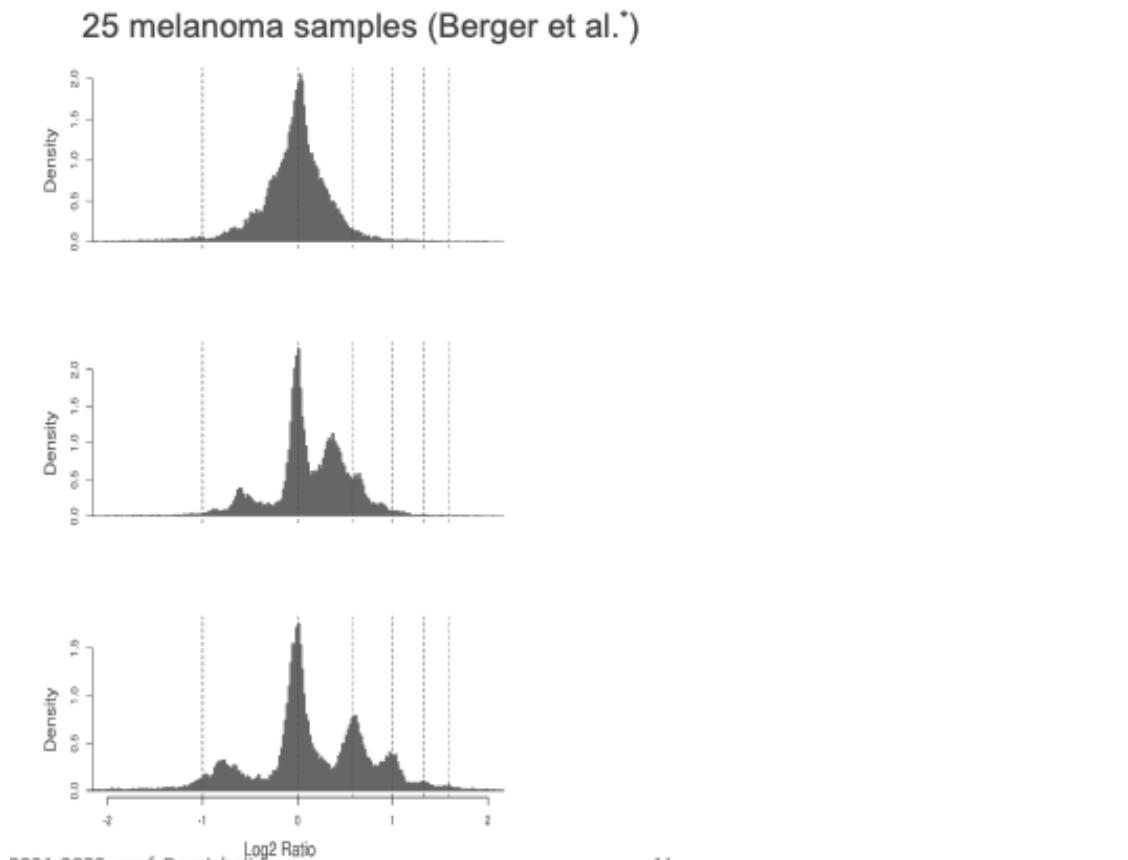
5.2. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

We correct our data and the *purity correction causes a stretch between the peaks*, since tumor admixture dilutes the signal. So, the effect of purity correction is a wider spread between the peaks (third graph).

+ add the example graph

- If we have one extra copy in our tumor, the log2 ratio will be around 0.58 and so we would expect that the signal will peak around that value; for two extra copies we'd expect a peak around 1 and so on.
- We'll have the peak of the normal state around 0 and then if we have an underrepresented allele in our tumor we'd get another peak around -1 for the hemizygous deletion and then the homozygous deletion.
- If our signal is not 100% pure tumor (so diluted by normal cells), the peak at -1 and 0.5 would be closer to the 0 peak for uncorrected data.

When we correct for tumor purity we stretch the distribution to go to the correct positions.
E.g.: 25 whole genome sequencing of melanoma samples



- 1st graph: The distribution of the log2 data of uncorrected signal, every melanoma sample is highly aberrant with a ploidy that is different between different individuals and a purity that is also different between different individuals. But we do have the tumor ploidy and purity so we can correct the data.

5.2. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

- 2nd graph: we correct for ploidy
- 3rd graph: we correct for purity too

If we don't correct our data we'll see much noise (as in the first graph). From the corrected data we learn that:

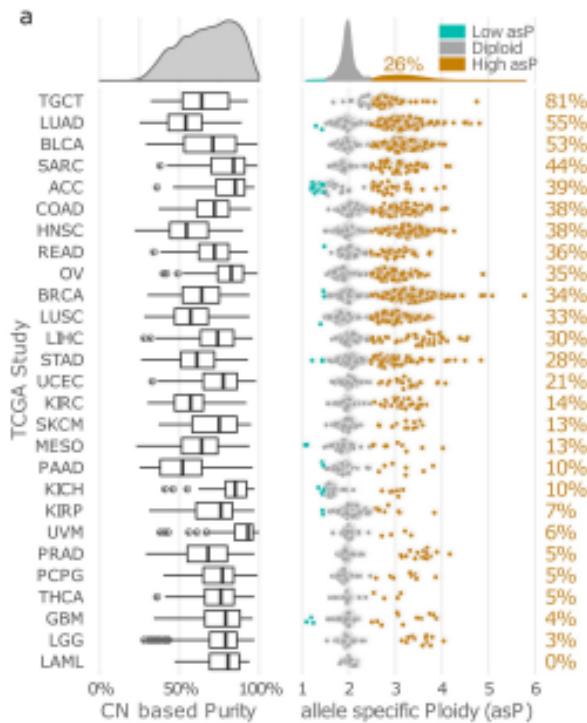
- A lot of tumors have a backbone ploidy of two
- There are some hemizygous deletion not perfectly centered in one but closer to one in the 3rd graph if compared to the 1st
- Some signal is compatible with homozygous deletion
- We have a reasonable amount of signal for three copies which could come from a three-ploid status of some tumors.

These corrections are part of standard preprocessing.

Tumor Ploidy and Purity adjustment, corrected TCGA data

How commonly does suboptimal tumor purity affect proper copy number data analysis?

How common is it that purity is not equal to 100% and ploidy is not equal to 2 in any primary disease



In the figure we can see a list of tumor types, where every draw is a tumor type (lung carcinoma, bladder cancer, colon cancer, ovarian ecc.). On the x axis we have tumor purity (1-admixture) going from 0 to 100% and for each type we can see the distribution of the tumor purity analysis of all the samples from the TCGA dataset.

5.2. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

Every tumor type has a different number of sample profile

Looking at the GBM (glioblastoma multiforme), the middle vertical line is the median signal of the distribution, there are outliers shown and the black horizontal line represents the interquartile range.

Altogether across 27 tumor types they were able to assess the tumor cellularity, clonality and all in about five thousand of those, meaning that a great fraction of those had some optimal data (very strict criteria)

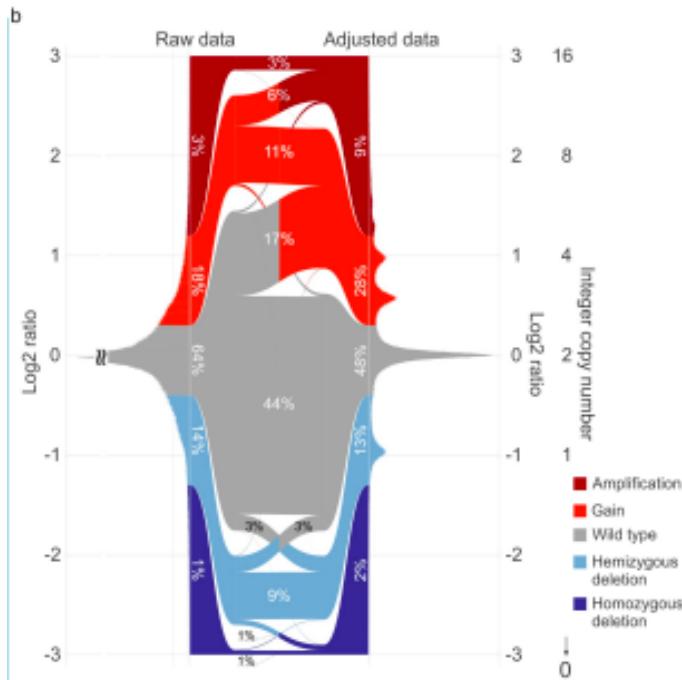
- The majority of the median distributions are above 50 %.
- The overall tumor cellularity was almost 70%.

If we look at ploidy: what is the fraction within each tumor type with a ploidy significantly above two?

In the graph they are sorted by decreasing percentage of tumors with a ploidy higher than two; for example, for the first and second tumor type, more than 50 % of the primary tumors have a ploidy status above two so either they underwent whole genome duplication (4 or more copies) or at least we have three.

Then we have some tumors with very low ploidy (blue dots) where at least one copy of the entire genome is completely lost -> low allele specific ploidy assessment.

The figure shows what happens to data when we correct for ploidy and purity



On the y axis we have the log2 ratio

- On the left side we have the raw data
- On the right side the adjusted data

5.2. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

We can see where correction for ploidy and purity takes the signal.

Focusing just on the first half we can see that

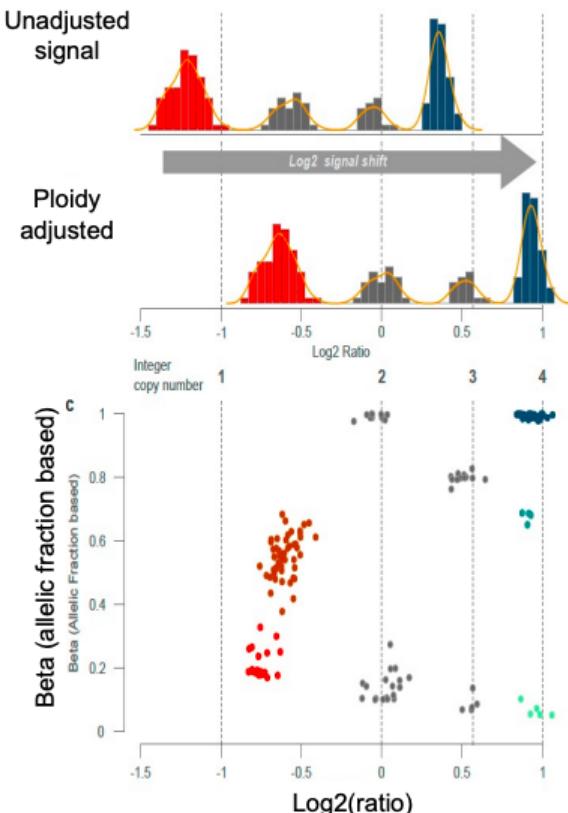
we have the same noise we've seen for the melanoma uncorrected data.

The correction of the data results in the reclassification of 30% of the totality of the segments (if we don't correct we have a wrong copy number classification in 30% of the cases)

Then there are certain copy numbers which are more or less affected by these corrections.

What's interesting is that the correction led to the doubling of the homozygous deletions that we were able to observe (these are very important because it means that the proteic product won't be there at all).

ALLELIC SPECIFIC ANALYSIS (CNA, CNB SPACE)



Thinking in terms of allele specific data:

1. We have unadjusted signal
2. We adjust
3. Then we can go to the beta-log2 ratio space where we can see that the data underneath the peaks are belonging to specific clusters

This suggests that by only looking at the log2 ratio we are unable to distinguish the presence of clusters with different clonalities.

The most interesting information is the lower cluster (on the x=0 axis):

5.2. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

- Even when the $T/N = 1$ (tumor/normal ratio) what we can have is a status of one copy and one copy or something that equally gives a log2 ratio equal to 0 but which still represents copy neutral loss of heterozygosity (CN-LOH), so two copies on one allele and zero copies on the other.

+ example figures (will be added soon, I have to draw them)

1st figure:

We have the loss of an allele on A so we'll have 2-1-2 copies

2nd figure:

We have the same situation on allele A but allele B is doubled so we'll have 3-2-3 copies

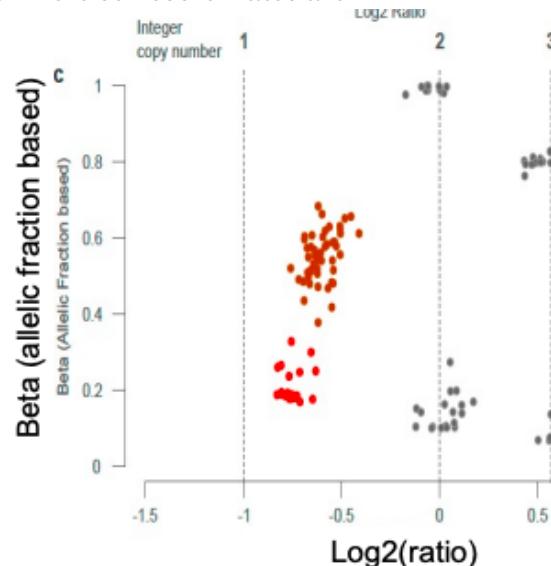
So, in this situation, the gene x will have two copies but both of them coming from the same allele (B).

Computing the log2 ratio in this situation we'll have the $\log_2(2/2)$ which will lead to the collocation on the 0 axis but on the lower part (due to the clonality).

The log2-beta statuses allows us to distinguish the copy-neutral LOH.

Also for the gain is the same (three copies from the same allele and zero from the other)

There are equations that allows us to go from here to a space where our coordinates are



the number of copies of allele A and number of copies of allele B.
four copies we can have different combinations:

- 2 copies of A + 2 copies of B,
- 3 copies of A + 1 copy of B
- 4 copies of A + 0 copies of B

The equations are not important, what's important is that once we have corrected the data then we can shift our analysis up to the level of number of copies of each allele for each gene.

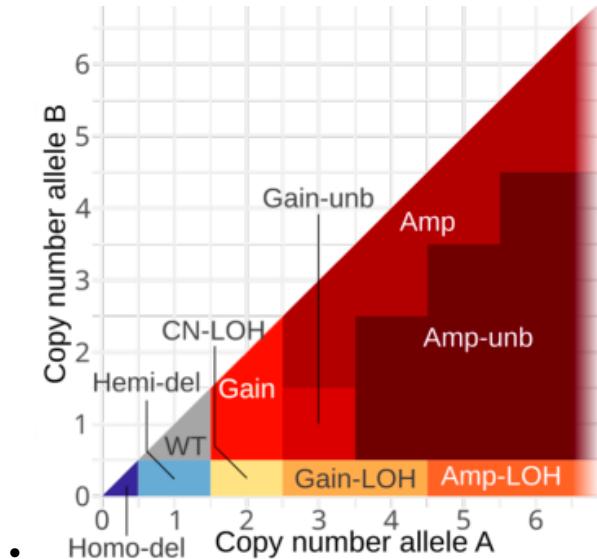
Why is this important?

E.g.: Let's imagine that for gene X we have one copy lost on allele A and a point mutation on the allele B which leads to unfunctional product so full loss of the protein.

5.2. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

If we instead are in the second case and the point mutation happened after the duplication then we'll still have an allele functioning, whereas if it happened before the duplication, we'd have again full loss of functional protein.

If we are able to distinguish the alleles we are able to also distinguish in which situation we are (which means we can distinguish between what's functional and what's not).



Extra graph with the same space allele a/ allele B where we can divide the space in terms of total number of copies and also what happens on both.

So, this whole computation allows us:

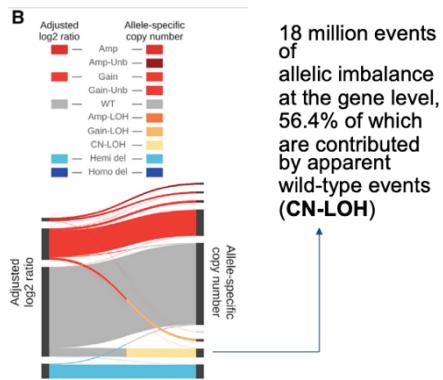
- To reclassify copy number status in the space by shifting and stretching
- To also assign a copy number A and B to every segment of the genome, which means to every gene

If we do that we can see that many of the segments that have a total number of copies equal to two are in fact 2+0 and not 1+1. This means that there is a significant fraction of the genome which is apparently wild-type but which actually underwent loss from one allele and a gain on the other. This event is called copy-neutral loss of heterozygosity (CN-LOH).

Copy-neutral because the number of copies doesn't change but there's been loss of heterozygosity.

From the TCGA data, they observed a relevant fraction of high copy number levels (4-5 copies) which all came from the same allele (one allele was lost and the other underwent multiple cycles of duplication).

5.2. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

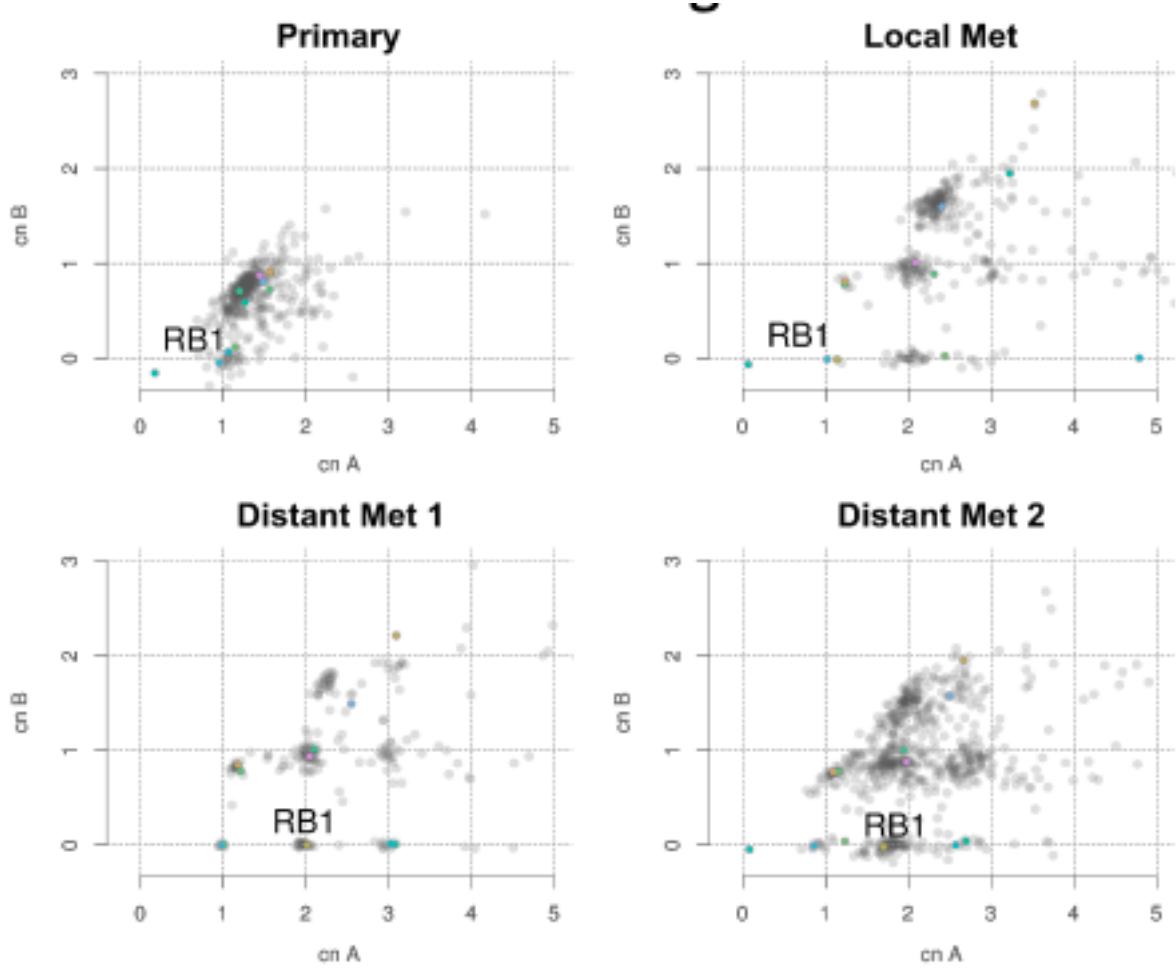


18 million events
of
allelic imbalance
at the gene level,
56.4% of which
are contributed
by apparent
wild-type events
(CN-LOH)

So, looking at the copy number only we'd say there's a gain (which is true) but we wouldn't have all the complete information (we also have to perform the allele analysis).

These information are relevant in precision medicine because there are ways to target genes exploiting loss of heterozygosity and up until now it was only used for deletions but now that's known, even if we have an apparent CN-LOH or we have a copy number gain LOH we can still consider to use the same approach.

5.2. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA



study – CNA, CNB real data example with multi-sample data from the same patient

We have one patient and we're looking at a primary sample, for which we plot the whole sequencing data in the copy number allele space and what we see (from the first plot) is that:

- There's a cloud of dots (every dot is a gene) which has a total number of copies around two
- There's a cluster that underwent hemizygous deletion so we only have one copy of all the genes in there
- There's one gene with a homozygous deletion (0,0).

Then we have three other metastatic sites for which they had biopsies so that they could run whole genome sequencing and perform the analysis of the data in the same space.

We have a local metastasis and two distant mets.

What we see:

5.2. PLOIDY AND PURITY CORRECTION ON $\log_2(\frac{T}{N})$ DATA

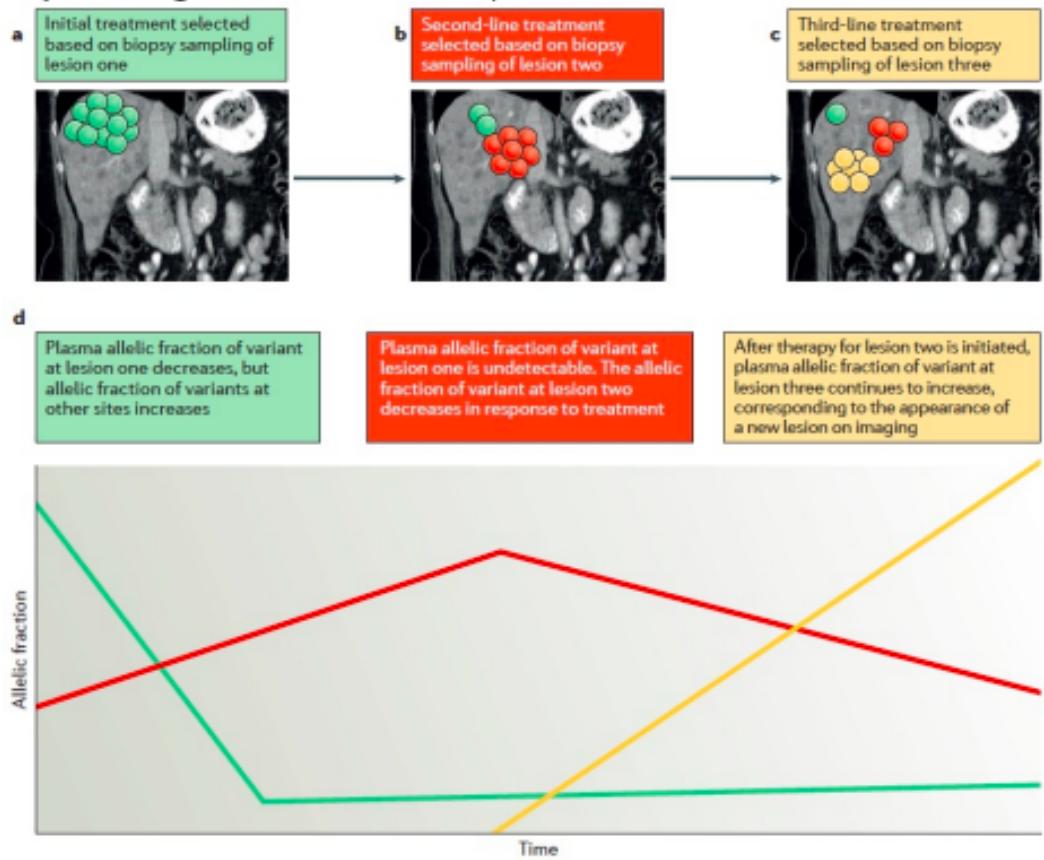
- In distant met 1 there's no homozygous deletion*
- In both the distant mets the gene RB1 gained an extra copy on allele A
- In all the mets there are extra gains of copies of all the genes (maybe there's been a whole genome duplication of some sort)
- In distant met 1 the data are as clean as to allow us to state that the data point in yellow/grey over the 1 is subclonal (if we have genes with 1+1 copy is equivalent to say it's a subclonal hemizygous loss, it means that all the cells have at least one copy and then some cells also have a second copy)
- In terms of evolution, very likely extra copies of the whole genome also in the local met after the loss of the second copy of the gene
- CN-LOH of many genes, including RB1
- Level of subclonality overall not high

*How's possible that there's a homozygous deletion in the primary tumor which is then absent in the distant mets? No DNA can be regained, it's impossible that the gene is reacquired, so probably the seeding of the distant mets happened before the loss of the gene.

Another way to track evolution is to have *serial time points*.

Application of longitudinal plasma profiling

Tumour heterogeneity and resistance to cancer therapies



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If we deal with biopsies over time we can track the evolution using the allelic fraction of a lesion.

E.g.: reasoning in terms of point mutations, let's say we have a point mutation at time point 0 in certain allelic fractions, which correspond to different subsets, we track the fractions over time.

Doing this we can make inference of which subsets appear during the treatment and are taking over (red one in the example figure).

Allelic fraction at any time point needs to be corrected for tumor content, otherwise we would not be able to compare multiple time points from the same patient.

Chapter 6

Tumor evolution studies via NGS data: SNVs-based methods

There is a large number of tumors where copy-number aberrations are minimal. Consequently, it is difficult to use copy number based approaches for these kinds of tumors. It is estimated that about 3% of primary tumors present flat genomes, meaning that they display very few copy number changes. These types of tumors are correlated to a better prognosis both in overall survival and progression-free interval, but relapses are still present so the assessment of these tumors is important.

In order to address this issue, some tools were developed to detect tumor purity via SNVs.

6.1 Rationale of somatic point mutation based assays

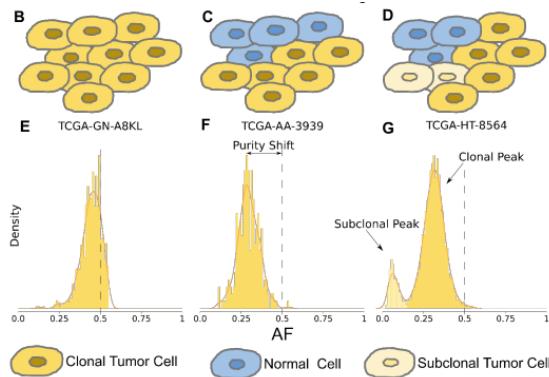


Figure 6.1: Peaks shift for a clonal tumor cell population and some mixed populations. Considering two genomic locations, healthy cells have genotype AA-AA, clonal tumor cells AB-AA and subclonal tumor cells AB-AB, where B is the alternative allele associated with a somatic point mutation

The distribution of allelic fractions of the clonal population only is symmetric, with the main peak around 0.5. A mixed population of clonal tumor and normal healthy cells

6.2. TPES (TUMOR PURITY ESTIMATION)

shows a shifted peak. The distance from 0.5 to the peak is proportional to the fraction of normal cells, because normal cells contribution moves the peak towards the side from the center (purity shift displayed in 6.1). A subclonal point mutation is identified with a second peak towards 0, because its allelic fraction is probably far distant from 0.5.

6.2 TPES (Tumor Purity Estimation)

Alessio Locallo (Demichelis' student, 2019)

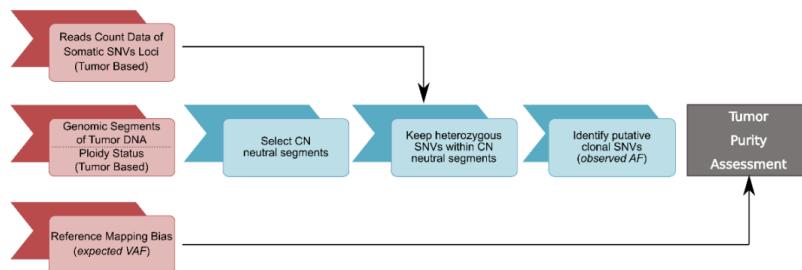


Figure 6.2: Workflow of the TPES algorithm

It is important to consider the **Reference Mapping Bias**: a polymorphic locus carrying a non-reference base is less likely to be mapped during the alignment process. With a perfect SNV (clonal, monoallelic, in highly pure tumor), allelic fraction will not be 0.5 because the aligner considers the variation as an error and sometimes discards the read containing it: some signal is lost.

TPES steps:

- **Selection of CN-neutral segments:** point mutations that are flat in terms of copy-number are perfect for flat genomes and easier to deal with. This is the first filter implemented by this tool: a threshold is set on the log2 of the tumor over the normal.
- When considering the **allelic fractions** of all the somatic mutations of whole genome, a major peak is expected around 0.5 (expected VAF). Other peaks can be originated from things that escaped the previous filter or from monoallelic mutations with copy-neutral LOH (loss of heterozygosity): in this case the allelic fraction results doubled. So another threshold on allelic fraction is needed (maxAF=0.55)
- Identification of **putative clonal SNVs**: the peak closer to 0.5 is the most useful to determine tumor purity. The others are related to subclonal events.

With enough point mutations and after peaks identification, purity is assessed with the following equation:

$$1 - \text{purity} = \text{admixture} = 1 - \frac{\text{observed VAF}}{\text{expected VAF}}$$

6.3. HOW MANY SNVS ARE NEEDED TO ASSESS TUMOR PURITY?

6.3 How many SNVs are needed to assess tumor purity?

The number of SNVs changes for each tumor type, so not all tumor types guarantee enough SNVs. The minimum number of SNVs needed to obtain reliable results can be assessed with a **comparative analysis**. The Spearman's correlations between the results of two different purity calling algorithms using decreasing number of SNVs are computed. The subsampling approach (which SNVs to consider?) is to subsample the SNVs as many times as possible to have higher confidence on the results. At each iteration, as many samples as possible are used, but the number decreases when the number of SNVs increases.

The computations determined 10 as the minimum number of SNVs needed to infer tumor purity. With this number, tumor was detected in 80% of samples by combining TPES and CLONET (CN-based). The 20% could be tumor-free or not detected samples. Since both SNVs and CN based methods failed, this 20% could be possibly detected with methylation.

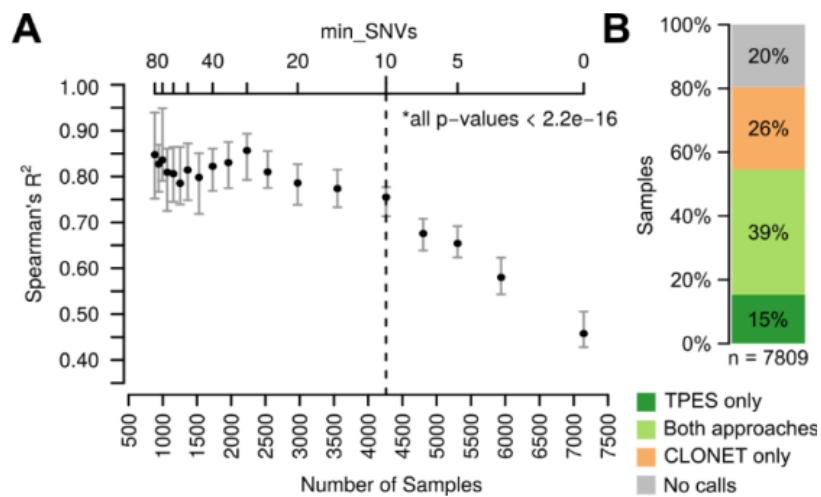


Figure 6.3: **A)** Correlation between two purity estimating algorithms (TPES and CLONET) with decreasing number of SNVs considered. **B)** Percentages of samples where tumor purity was assessed by the two tools (TPES considering with 10 SNVs)

6.4 Comparison between purity callers

TPES was compared to other tools that do the same thing but with a range of different methodologies: good correlation between the results was found, in particular with the CN-based algorithms. This shows that genomics is more reproducible in general to assess purity, while methods relying for example on image analysis give different results.

The best solution to assess tumor purity is to couple and CN-based and a SNV-based approach: some samples are only detected by one of the two so a combination gives the best results globally.

6.5. PROS AND CONS OF SNVS-BASED TUMOR PURITY ASSESSMENT

6.5 Pros and Cons of SNVs-based tumor purity assessment

Pros:

- Best-suited for CN neutral tumor genomes
- Applicable to a range of NGS techniques
- Fast and low demanding in terms of computational resources
- TPES is available as R package on CRAN

Limitations:

- Needs a reasonable number of putative clonal somatic heterozygous SNVs per sample
- Sensible to subclonal cell populations which could influence clonal peak detection

Chapter 7

Liquid biopsies in oncology

7.1 Liquid vs Tissue biopsies

Tissue Biopsy	Liquid Biopsy
Accurate and detailed view of one tissue only	Landscape overview, with resolution depending on tumor burden, releasing rates, metastases and tumor heterogeneity. It is possible to get an aggregated signal of different tumor cell populations
Single tumor	Possibility of getting signal from multiple tumor masses
Signal relative to a specific point in time	At a certain point in time, but multiple serial samples can be collected
Invasive and painful for the patient, not feasible for all the tissues	Minimally invasive (so it is possible to collect samples multiple times) and can be coupled with a routine blood draw
	It is possible to design specific assays to detect minimal quantities of tumor cells, for example the ones left behind after surgery. This is useful to detect minimal residual disease (MRD) and avoid tumor recurrence
	The collection of serial samples allows for example to track clonal evolution of the tumor over time, to catch treatment resistances early on and to monitor the patient's response to the treatment
	It can be used for early detection of cancer, many studies are trying to reach this objective

7.2. ISSUES IN THE INTERPRETATION OF CFDNA DATA

Material availability	
From needle biopsies, biopsies, surgical resections (if some material is left after the clinical protocol and the patient agrees to a research protocol)	From circulating tumor cells, extracellular vesicles, cell-free DNA (the most interesting). In healthy donors there is 4ng/ml cell-free DNA (below 10 anyway), in tumor patients 100s ng/ml (but the range is really wide). The numbers are higher if the tumor is metastatic and the treatment is also very influential on the quantity of cfDNA. Tumor patients under treatment have cfDNA quantities comparable to healthy people. Anyway, cfDNA quantity is influenced by a number of factors in addition to cancers so it is not a good diagnostic feature by itself
Tumor content	
Tumor content can be assessed with a microscope: the proportion of tumor cells compared to healthy cells is measured based on morphology with a simple staining of the tissue slide. So tumor content is assessed by counting cells and considering the magnification of the image. If subtyping is needed, a staining for markers is performed. Computational methods are also available	The fraction of circulating tumor DNA (ctDNA) is inferred with methods based on genomics (or possibly also methylation)
Tumor ploidy/aneuploidy	
Inferred with cytogenetics, FISH, or from NGS data	Inferred based on genomics but it is quite tricky

7.2 Issues in the interpretation of cfDNA data

7.2.1 Normalization on tumor content

When interpreting data from liquid biopsies, it is fundamental to contextualize a mutation after observing it. In order to associate a particular mutation to a particular diagnosis the signal has to be normalized based on tumor content. Without normalization, tumor content is the most influential variable on the patient's prognosis and this can be misleading. For example, one mutation could look like it is linked to a specific type of tumor when it is actually present in other types too but it is not detected due to the low tumor content of some samples 7.1. For this reason not all the literature available about liquid biopsies is reliable: lack of normalization leads to completely wrong conclusions. This applies to all kinds of assays: from microarrays to the sequencing of extracellular vesicles.

7.2. ISSUES IN THE INTERPRETATION OF CFDNA DATA

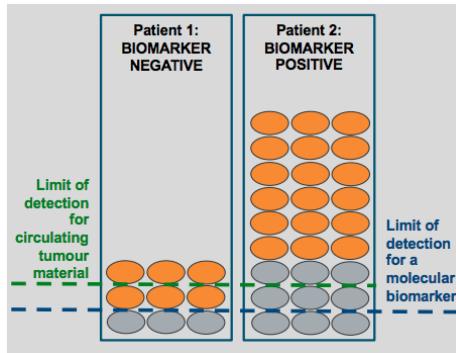


Figure 7.1: The two samples have the same percentage of tumor cells, however the first one results negative for the marker because of the low tumor content

7.2.2 Quantity of input material

Another source of errors in the interpretation of cfDNA data is the amount of input material: if the patient's tumor content is high the results could be obtained with a limited amount of extracted nucleic acid, but if the tumor content is low, too little material can lower the chances of detecting tumor cells 7.2. The problem is that in most cases the tumor content is unknown before the analysis and this must be considered when designing an experiment. Usually the standard procedure is to begin with 2ml of plasma. If no tumor is detected, one should repeat the assay with more material (or sequence another vial and combine the results) to be sure that the tumor is not present and not just undetected. In some cases some information about the state of the patient is available: for example if a patient is in remission more material is required.

Keep in mind that if the sample is pure, 10 ng of DNA should correspond to around 1500 diploid tumor genomes.

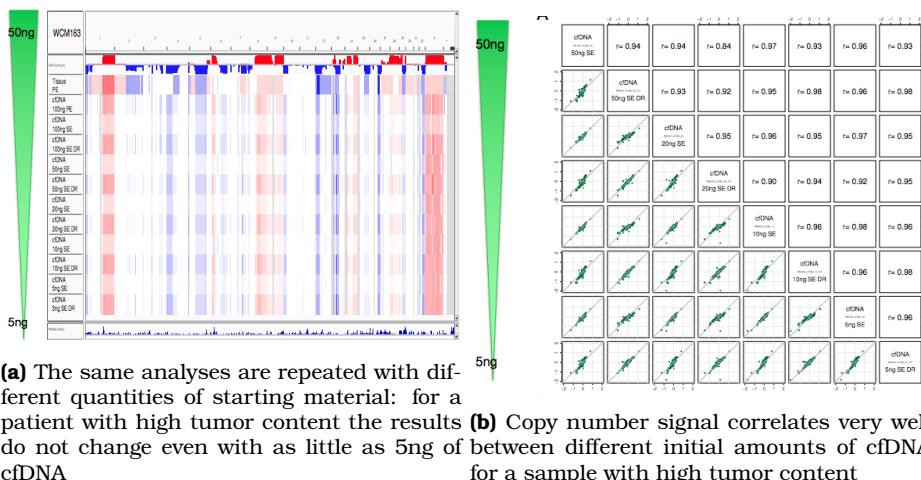


Figure 7.2: Patient with high tumor content: very different results would be obtained if the tumor content was low

7.3 SNV detection in liquid biopsies

Technical problems:

- PCR artifacts
- Sequencing errors: one mutation should be validated by multiple reads to be confirmed
- Problems related to the depth of coverage: the required coverage should be estimated considering the expected tumor content of the sample and deeper sequencing may be required

Biological problems:

- Low tumor content: ctDNA/cfDNA ratio
- Clonal hematopoiesis (when a hematopoietic stem cell starts making cells with the same genetic mutation): to distinguish the signal coming from clonal hematopoiesis, compare it with what has been sequenced before from solid tumors. It is rare to observe something in liquid biopsy that has never been noticed in solid ones.
- Copy number variations and ploidy: with a whole genome duplication and a SNV only present on one allele, the signal corresponding to the mutation is only 25% and has to be correctly interpreted.
- Intra-patient tumor heterogeneity: very low allelic fractions for SNVs that are not clonal can be difficult to observe

Multiple **tools** are available to detect SNVs. Each tool will probably give different results (or partially concordant ones). Each tool can be tuned to favour some types of calls, so the tuning parameters should be carefully selected.

7.4 Requirements depend on the application

Application	Requirements
<ul style="list-style-type: none"> • Early tumor detection • MRD detection • Recurrence detection 	Tumor quantity is low so a low signal is expected: higher quantity of starting material is required but there needs to be a balance between the number of false positives (with too much material) and false negatives (with too little) that can be produced
<ul style="list-style-type: none"> • Tumor dynamics • Treatment response • Mechanisms of resistance 	The assay should be designed in order to be able to distinguish between different clones (sub clonality analysis)
Single biomarker assessment	The only important thing is to detect whether one point mutation is present or not, so in this case tumor content is not important. A targeted assay is used and specific locations associated with the SNV are sequenced as deep as possible to detect the mutation

7.5 Whole genome vs targeted sequencing

Whole genome sequencing has higher computational cost, while targeted assays have higher sample preparation time. The sequencing cost is higher for whole genomes but it does not decrease evenly 7.3.

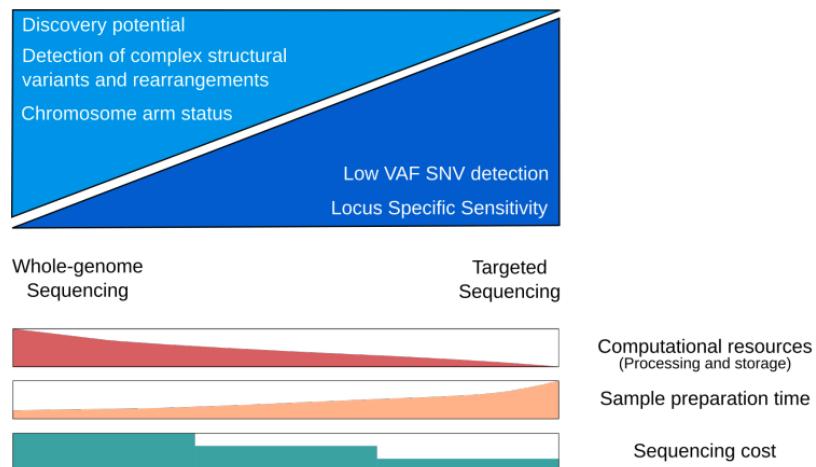


Figure 7.3: Whole genome vs targeted sequencing

7.6. TAKE-HOME MESSAGE

7.6 Take-home message

Possible exam question: what type of assay should be run and which are the requirements for a specific situation.

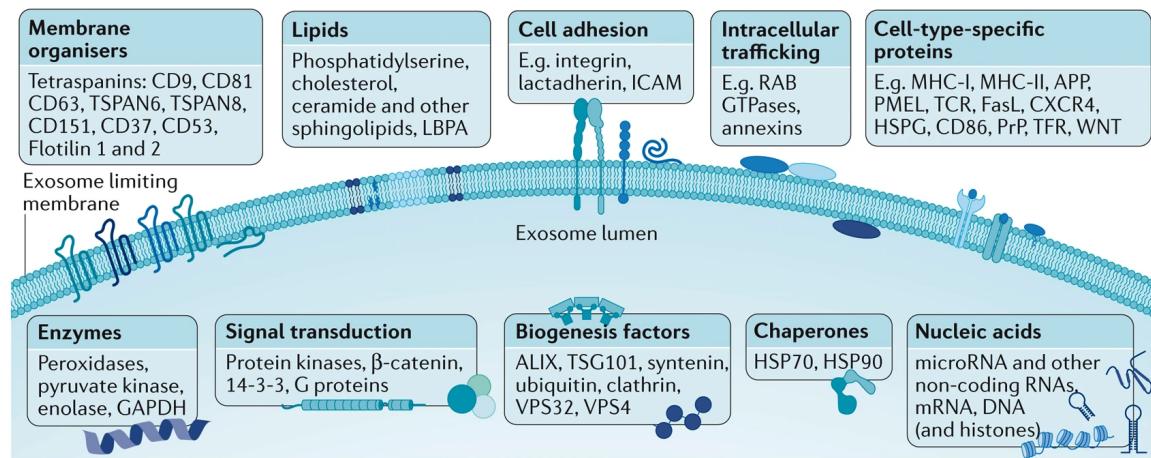
Chapter 8

Extracellular vesicles

8.1 Introduction to EVs

"Extracellular vesicles are **membrane-enclosed nanoscale particles** released from essentially all prokaryotic and eukaryotic cells that **carry proteins, lipids, RNA and DNA**." ("RNA delivery by extracellular vesicles in mammalian cells and its applications", O'Brien et al, 2020 - *Nature Reviews Molecular Cell Biology*). The content of an extracellular vesicle (EV), including membrane proteins, is usually referred to as **cargo**.

EVs present many different surface proteins, mainly **tetraspanins** (commonly used markers to identify them) but also receptors, adhesion molecules and immune system ligands; these molecules are responsible of the targetting function, meaning that they define which cells the EV should interact with.



The cargo of an EV tends to reflect the state of the cell that produced it; this way EVs become a way to transport material from a cell to another, but mostly to communicate even at long distances (since EVs can enter the blood stream).

Different types of EVs exist (different cargo, dimensions, genesis...). In older literature EVs were classified based on the cells that produced them and/or their size and/or func-

8.1. INTRODUCTION TO EVS

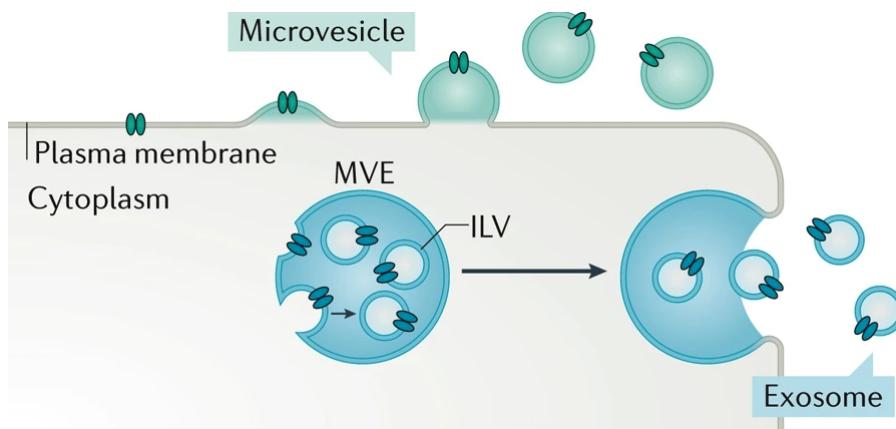
tion (e.g. large oncosomes); now the International Society for Extracellular Vesicles (ISEV) suggests to classify EVs into three categories, **exosomes**, **microvesicles** and **apoptotic bodies**. The respective characteristics are summarized in the following table.

	Exosomes	Microvesicles	Apoptotic Bodies
Origin	Endocytic pathway	Plasma membrane	Plasma membrane
Size	40-120 nm	50-1,000 nm	500-2,000 nm
Function	Intercellular communication	Intercellular communication	Facilitate phagocytosis
Markers	Alix, Tsg101, tetraspanins (CD81, CD63, CD9), flotillin	Integrins, selectins, C D40	Annexin V, phosphatidylserine
Contents	Proteins and nucleic acids (mRNA, miRNA and other non-coding RNAs)	Proteins and nucleic acids (mRNA, miRNA and other non-coding RNAs)	Nuclear fractions, cell organelles

Notice that size is not enough to subdivide them since there are overlaps. A way better way of classifying EVs is by their genesis:

- Exosomes originate from **multivesicular bodies**. Multivesicular bodies are organelles whose membrane buds inward creating **intraluminal vesicles**; then when the multivesicular bodies merge with the plasmatic membrane their content is released into the extracellular environment and intraluminal vesicles become exosomes.
- Microvesicles originate from outward budding of the plasmatic membrane.
- Apoptotic bodies are generated from apoptotic cells through various mechanisms.

8.2. MEDICAL APPLICATIONS OF EVS



The EV genesis defines the cargo and the surface markers, which define the target cell for the EV. Surface markers can be used in **flow cytometry** to divide these three populations. It is important to note that the content of exosomes and microvesicles is not just a portion of the cytoplasm of the cell generating them, but it is also enriched in specific molecules thanks to **selective sorting** mechanisms (only some of which are known), especially for proteins and RNAs.

EV uptake by target cells can occur through different modalities (depending on target cell, vesicle size and others):

- Phagocytosis
- Macropinocytosis
- Clathrin dependent endocytosis
- Receptor mediated endocytosis
- Fusion with the plasmatic membrane

8.2 Medical applications of EVs

Due to the aforementioned facts that:

- Almost all cells produce EVs, cancer and other diseased cells included
- The cargo reflects the functional status of the secreting cell
- EVs can convey signal through long distances

EVs are being studied more and more for clinical applications, mainly to understand disease pathogenesis (and eventually how to act on it) and to potentially use them in early-screening assays. The fact that tumor derived EVs play a major role in reorganizing the surrounding environment has already been proven: tumor derived EVs induce endothelial proliferation and thus neoangiogenesis, fibroblast differentiation and extracellular matrix remodeling. This last aspect is especially relevant since EVs can create niches that facilitate the metastatic process.

8.2. MEDICAL APPLICATIONS OF EVS

One other significant advantage of EVs is the fact that they can be obtained through liquid biopsy, together with circulating tumor cells (CTCs), cell free DNA (cfDNA) and ribolipoproteins. Liquid biopsy generally refers to blood samples, therefore a non invasive technique to study the disease. Integrating transcriptomic data from EVs with other techniques could provide efficient biomarkers for early detection and biomarkers to easily measure overtime to track tumor evolution or response to a treatment.

In order to use EVs in clinical applications, a consistent and reproducible way of extracting EVs from the liquid biopsy is needed. In the context of the PRIME project (PRostate cancer plasma Integrative Multi-modal Evaluation consortium), different methods to extract EVs from plasma (from prostate cancer and healthy patients) have been tested and compared, those methods being:

- **Nickel-based isolation (NBI)**, which utilizes the fact that EVs are negatively charged, hence they are attracted by positively charged nickel beads; the beads are then retrieved using magnets and the EVs are eluted from their surface.
- **Size exclusion chromatography (SEC)**, which is a chromatographic technique in which the retention time of an object in the column depends on their size (the bigger the object, the fewer the pores of the stationary phase it can enter into, the faster the object is eluted). This technique requires knowing the elution times of the EVs, which can be difficult considering their high heterogeneity.
- **Ultracentrifugation (UCFG)**, meaning centrifugation at around 22000 RPM for some hours. EVs are too small to sediment using regular benchtop centrifuges.

The total amount of RNA obtained from each extraction was measured using SMARTseq kit.

The result was that different isolation methods were highly reproducible on homogeneous samples (EVs from prostate cancer cell lines culture medium), while the different methods showed higher variability on heterogeneous samples (EVs from blood). This shows that different isolation methods isolate better different EV populations, especially in samples such as blood which contains a myriad of EVs with different origin and characteristics, since all healthy tissues produce EVs that mix with tumor derived ones in the blood. This leads to the need to identify which method is the best in order to enrich tumor derived EVs rather than healthy tissue derived ones; no standard protocol for the isolation of EVs and the analysis of their transcriptome is available.

Another challenge, deriving from the use of EVs, is the fact that RNA signal deriving from multiple populations is difficult to interpret. Ideally one would need some way to deconvolute the signal into the components associated with each cell population; in order to do so, two potential approaches are possible:

- **Supervised deconvolution**, which means using known cell line signatures to split the signal and compute the fraction of contribution for each population (one tool that does this is CIBERSORT). The main problem with this method is that it is not possible to get the signal for unknown cell populations; moreover it is difficult to define cell population specific signatures since we do not have pure EVs populations obtained from blood.
- **Unsupervised deconvolution**, which means using unsupervised clustering algorithms to subdivide the signals into populations; the problem is that these ap-

8.3. EVS CONFERENCE

proaches tend to be less sensitive and the identification of the clusters is not simple. Still, this allows to identify not previously known cell populations.

Deconvolution approaches are therefore plausible but not well established.

8.3 EVs conference

Notes from the conference "*Extracellular vesicles as diagnostic and therapeutic tools for kidney diseases, Benedetta Bussolati, Dept. of Molecular Biotechnology and Health Sciences, University of Torino, 12 May 2022*".

DISCLAIMER: they might not be perfect but they should suffice for a general idea of the concept

EVs are part of the secretome produced by stem cells in order to try and induce tissue regeneration after damage, since they do not act directly in the regeneration process by differentiating. For this reason stem cell derived EVs (scdEVs), are subject of study for potential tissue therapies. Most studies have been performed on mesenchymal stem cell derived EVs, but some studies have shown that, despite the great heterogeneity of EVs produced by stem cells, little to no difference was found between mesenchymal stem cells derived EVs and other scdEVs. Of this heterogeneous population (especially regarding the expressed tetraspanins), small EVs seem to be the ones which are more associated to tissue regeneration; moreover small EVs are the safer ones for potential medical applications since they do not display HLA or tissue factor, that are sometimes present in bigger vesicles and that could lead to immune response and coagulation respectively. A very big spectrum of genes is regulated simultaneously through EVs; to support the role of EV content in the regenerative process, DROSHA KO models (which have impaired miRNA loading into EVs) lose most of their therapeutic effect.

One way to analyse EVs is MACSPlex, a cytofluorimetric tool with beads and detection antibodies for tetraspanins; that being said, especially for medical purposes, better and more standardized ways to quantify, identify and test the potency of EVs are still needed. Notice that the potency of an EV, meaning its ability to induce a certain response, is highly application dependent.

Regarding the activity of EVs on kidney diseases, there have been different studies:

- Renal damage markers in kidney injury model decrease overtime in presence of stem cells or just scdEVs; the responses in the two cases are basically the same, suggesting that most of the therapeutic effect of stem cells in kidney diseases is due to EVs.
- Repeated administrations of scdEVs to diabetic nephropathy models reduce inflammation and fibrosis
- In healthy patients most vesicles reach liver and spleen, while in diseased patients one can find more EVs than usual in the damaged site; this supports both a specific and an aspecific targeting of EVs to the damaged area. This holds true even in kidney disease models, where administered intravenous EVs reach the kidneys within 15 minutes.
- A phase 1 study has been conducted on the use of scdEVs in kidney diseases.
- Urine derived EVs are comparable in potency with MSCs EVs. Urine derived EVs are all generated from kidney cells, since no EVs from the blood stream can pass

8.3. EVS CONFERENCE

the glomerular filtration membrane; this also means that urine derived EVs could be used as a diagnostic tool for kidney diseases.

- Klotho, a recently discovered hormone, is produced mostly by the kidney both in a transmembrane and in a soluble form. Klotho KO murine models have a significantly shorter lifespan and a faster aging process. Klotho can be found both in urine and blood. Moreover klotho has been found coexpressed with tetraspanins, confirming its presence in EVs. By providing recombinant klotho EVs to a klotho KO mouse model, the healthy phenotype is rescued; moreover providing klotho through EVs rather than by direct injection is more efficient.
- Autologous urinary EVs seem to have a beneficial effect in kidney injury model.
- CD133+ is a marker for regenerative kidney cells in adult humans; studies have tried to test if its expression on urinary EVs correlates with the outcome of kidney transplant. Healthy human urinary EVs display very high levels of CD133+. Bad responders to kidney transplant displayed lower levels of CD133+ compared to good responders, but both had significantly lower levels of expression compared to healthy individuals. Blood and urine samples of kidney transplant patients were collected at various timepoints during a period of one year. Samples were centrifuged to remove bigger debris, analyzed using MACSPlex and then normalized for the number of identified tetraspanins. Some of the patients did not recover while others did; by analyzing samples from 10 days after the transplant was possible to predict which patients would recover and which would not. Both in blood and urine EVs were found different biomarkers (among which CD133+) whose concentration was significantly higher in individuals that would recover.

Chapter 9

Epigenetic profiling of cell-free DNA

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9.1 Introduction

All the cells of the human organism present the same genetic information but they give rise to different types of tissues and cells. This happens mostly thanks to epigenetics. The main epigenetic modifications are:

- **DNA methylation:** in humans they are mainly found on CpG islands (genomic regions with high CG content)
- **Histone post translational modifications (PTMs)**
- The **chromatine architecture**
- ...and many others

All levels of epigenetic controls are often dysregulated in cancer: these variations usually go in favor of cancer cells survival. For this reason, epigenetic reprogramming has recently been added to the hallmarks of cancer.

The epigenetic landscape is very different from the genetic one. DNA mutations are directional: they cannot be reverted so they accumulate with subsequent cells generations. The epigenome is plastic, so it can be reverted (possibly through therapy but this can happen physiologically). Moreover, the human epigenome is tissue/cell specific while the genome is unique.

9.2 DNA methylation

DNA methylation is the addition of methyl-groups to cytosines in CpG islands. It is regulated by enzymes that are responsible for regulating the cell-specific transcriptional state. These enzymes can be:

9.2. DNA METHYLATION

- Cis-factors: local control
- Trans-factors: genome-wise control

CpG islands are spread through the genome and when they are in a promoter they regulate gene expression through transcriptional silencing of the corresponding gene if they are methylated. The mechanisms are multiple and still not completely clear: DNA methylation could for example impair the binding of transcription factors or recruit repressing proteins. This methylation landscape is highly regulated and tissue-specific.

In cancer tissue, hypomethylated and hypermethylated regions are often observed, leading to an abnormal regulation of gene expression. In addition to that, hypermethylation of pericentromeric heterochromatin in cancer can lead to mitotic recombination and thus genomic instability 9.1.

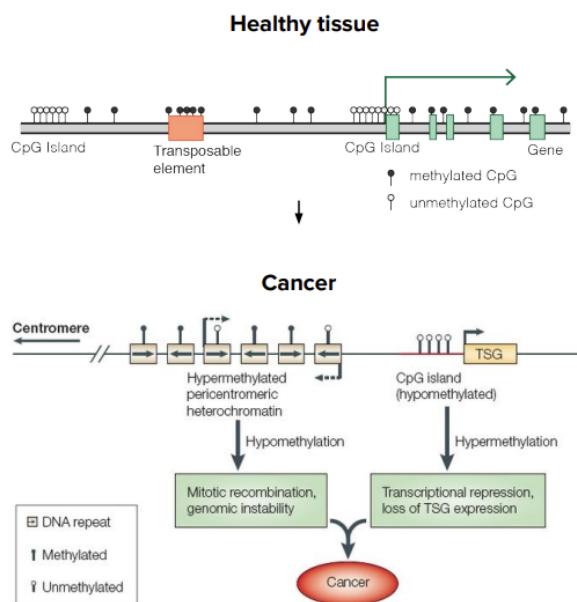


Figure 9.1: Methylation patterns altered in cancer

This landscape of regulation is very complex: DNA methylation can regulate gene expression but it is not the only regulating factor, some histone modifications also contribute for example.

DNA methylations are not inherited across generations, so there is no accumulation of methylation variants, as it happens with regular DNA mutations. Each individual is born with a brand new methylation landscape that is then disrupted during life (not only due to cancer or disease). Interestingly, it could be possible to exploit variations in the DNA methylome to measure age by computing how many cell divisions led to that specific methylation state.

9.3. HOW IS DNA METHYLATION MEASURED?

9.3 How is DNA methylation measured?

The first step is the **bisulfite conversion**: thanks to bisulfate ions, unmethylated Cs are converted into Us. With some particular alignment algorithms that are aware of these modifications one can detect the errors and thus methylations. Both array-based and shotgun-sequencing-based assays are used to this aim. The result of such an assay is a series of **beta values**: the fraction of reads corresponding to one genome site that is methylated.

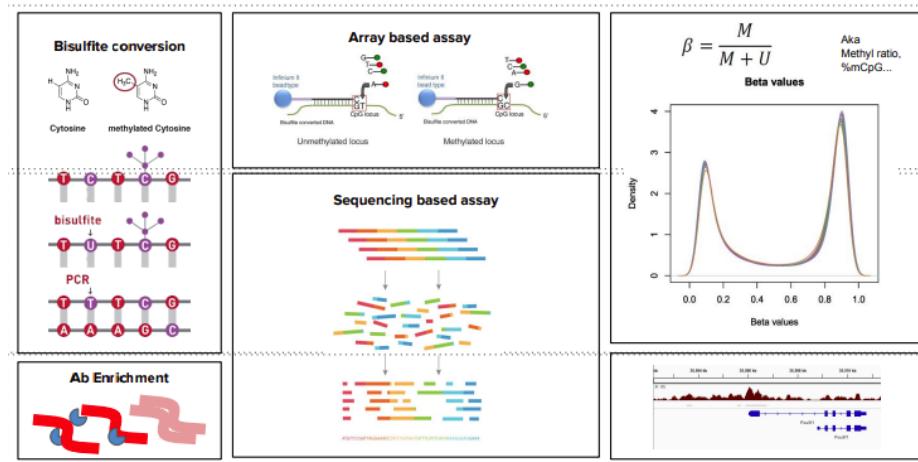


Figure 9.2: Main methods for DNA methylation measurement

Immunoprecipitation-based methods are also available, but the most frequently used methods are based on whole genome profiling.

It is useful to analyze the sequencing result at the **single-read level**: different methylation configurations can lead to the same global methylation level but have different biological interpretations. For example one methylation level of 0.5 can be the result of one completely methylated allele with the other one unmethylated or two half-methylated alleles. This kind of information is important in order to determine, for example, if the sample contains different types of cells or if there is some disrupting pathological situation.

9.4 Tissue-specific vs disease-specific DNA markers

- **Aspecific** → most of the genome
- **Tissue-specific** → methylations that regulate gene expression to activate the tissue-specific functions of cells
- **Disease-specific** → CpG hypermethylation, genome-wide hypomethylation and other modifications usually correlated with cancer
- **Tissue+cancer-specific** → methylation patterns specific of cancer in a certain tissue. These markers allow to discriminate between different tumor types.

9.5 DNA methylation based liquid biopsy

When a cancer cell dies, its DNA is released in circulation and it is potentially possible to get it with a liquid biopsy. The goal is to analyze methylations of cfDNA to retrieve information about the state of the patient, and possibly detect early-stage tumors.

For this purpose, when compared to genomic DNA, the analysis of the methylation landscape has some positive and some negative aspects. For genomic DNA, the percentage of actually informative signal on the whole information that is obtained can be small and difficult to observe, on the other hand, for DNA methylations it is difficult to discriminate between what is aberrant and what is not because the modifications are tissue-specific and it is difficult to obtain clear background references to make a comparison.

	Genomic DNA	DNA methylation
Molecular signal	Signal is limited to genomic alterations, and thus might be low for SNVs or quiet tumors	Extended and multi-facet signal, amenable to genome wide detection
Background/reference	A single well known background: the normal human genome, as profiled by the control germline sample	Multiple cell populations with distinct profiles, each contributing to the DNA methylation signal
Variability	Low rate of biological variability, discrete signal and overall acceptable technical errors	Discrete degree of biological variability, continuous signal with variable confidence (* coverage, platform, experimental approach...)
Information content	Limited to genomic information (SNV, SCNA...) but possible fragmentomics applications	Could potentially capture transcriptional state of cancer cells, offering a snapshot of processes such as lineage switching
State of the art	Highly characterized and interpretable, extended literature and high quality samples are available to aid interpretation	Fewer datasets available, but promising results in the past few years. Currently a mostly uncharted territory

Figure 9.3: Comparison of genomics vs methylation for cancer detection

9.5.1 Workflow

First, the data is sequenced from solid and liquid samples: the methylation profiles from solid samples are needed as reference. The reference profiles for liquid biopsies analysis are derived from white blood cells and from the cancer type of interest. White blood cells are the background reference for cfDNA, since the most frequent genomic material in circulation originates from this type of cells. If a methylation pattern different from the one of blood cells is found in cfDNA it means that cells of some other tissue are dying and their material is going into circulation and it is not a positive signal. With these patterns as reference, the goal is to discover biomarkers and perform feature selection. Subsequently, a model is fitted and optimized to perform predictions on new data. The last step is performance evaluation.

9.5. DNA METHYLATION BASED LIQUID BIOPSY

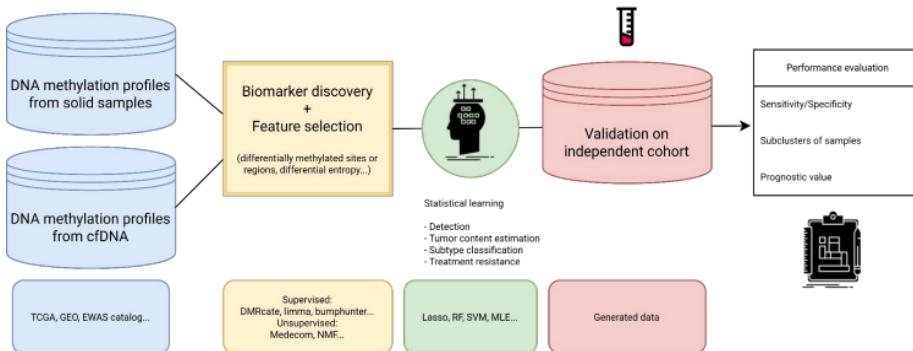


Figure 9.4: Common analysis workflow

9.5.2 CCGA study

The Circulating Cell-free Genome Atlas (CCGA) is a study conducted by Grail designed to characterize the landscape of genomic cancer signals in the blood of people with and without cancer. The study enrolled approximately 15,000 participants. Their goal is early and simple detection of cancer from analysis of methylations on cell-free DNA.

They performed whole genome methylation profiles after choosing between three different independent methods (the other two were targeted sequencing and whole genome sequencing for CNVs but they decided to further develop the methylation path). In the second phase they developed an assay for a targeted methylation study: the best features to discriminate between the two classes (cancer vs non-cancer) were selected in order to sequence the areas with these modifications without whole genome analysis. A model for this classification was developed, trained and validated. The last step is a large-scale clinical validation with a 5 years follow-up that is still in progress.

The results are great but not for all cancer types: sensitivity is better for cancers of highly-vascularized tissues and metastatic tumors, while some types of cancer produce a lot of false negative results. Moreover, detection is obviously better when cancer progresses but the goal is early detection.

9.5.3 Deconvolution approaches

Deconvolution of cell-free DNA is another task to be performed on DNA methylation other than classification. The goal is to explain the observed signal with a combination of pure signals: discover the main contribution that led to a specific methylation landscape, one example is tumor profiling.

From liquid biopsies, it is possible to detect which are the main contributors to the cfDNA. These results can be compared with the ones obtained from cancer patients to determine which are the contributors to the difference in cfDNA that is observed and to infer data for tumor diagnosis or treatment resistance detection.

In order to perform deconvolution, **high-quality reference atlases** are needed: one was built with the contribution of Grail. They sorted healthy donor cells with FACS and profiled them. Cell type specific methylation profiles were built, so it is possible to use this atlas to select biomarkers, like a reference genome. They generated specific methylation patterns for 39 human cell types from 207 methylomes.

9.6 Targeted panel approaches for tumor content estimation

Demichelis' group study

Their interest is detection of treatment resistance in prostate cancer. The goal is to know when the tumor becomes resistant, in order to be able to change or calibrate the therapy. A sequencing panel was developed to detect the amount of cancer-derived DNA in circulation, and interestingly only 50 regions are sufficient to get a satisfying estimation. A model is built to know how much ctDNA is expected after treatment and it is possible to get a score that estimates the level of resistance.