Chapter 8
Role of Next-Generation Sequencing Technologies in Personalized Medicine

Stefania Morganti, Paolo Tarantino, Emanuela Ferraro, Paolo D'Amico, Giulia Viale, Dario Trapani, Bruno Achutti Duso, and Giuseppe Curigliano

1 Historic Background of DNA Sequencing

Cancer is a genetic disease. Decades of research has led to this knowledge, showing that it is the accumulation of molecular alterations that is the key element of tumorigenesis, directing the acquisition of the malignant phenotype (Vogelstein et al. 2013). Genes involved in oncogenesis are classified in "oncogenes," whose activation is responsible for tumor transformation and oncosuppressors, whose inactivation leads to cellular proliferation. Mutations of oncogenes (gain of function) or oncosuppressors (loss of function) can be genetically inherited (germline), but they are mostly acquired and caused by DNA replication errors and/or exposure to carcinogens (Kinzler and Vogelstein 1996).

The understanding of cancer as a genetic disease, though multifactorial and non-Mendelian in the majority of the cases, has led researchers to focus on cancer cells genome, looking for the leading cause(s) of the pathological proliferation that ultimately cause cancer. The identification of specific driver genomic alterations allowed the development of targeted therapies, more effective and less toxic compared to standard chemotherapies. Trastuzumab (approved in 1998) and imatinib (approved in 2001) were the first two drugs to show the potential of targeted therapy, followed by many molecules nowadays approved for the treatment of several types of cancer (Fischer et al. 2003). Interestingly, the US Food and Drug Administration (FDA) granted an accelerated approval for imatinib for the dramatic sustained response of chronic myelocytic leukemia (CML) patients treated with the novel tailored approach (Johnson et al. 2003), in 2001; today, both imatinib and

S. Morganti · P. Tarantino · E. Ferraro · P. D'Amico · G. Viale · D. Trapani
B. A. Duso · G. Curigliano (✉)
Division of Early Drug Development for Innovative Therapy, IEO,
European Institute of Oncology IRCCS, Milan, Italy

Department of Oncology and Hemato-Oncology (DIPO), University of Milan, Milan, Italy
e-mail: giuseppe.curigliano@ieo.it

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trastuzumab are enlisted as "essential medicines" by the World Health Organization, for the treatment of CML and breast cancer, available as a generic and biosimilar, respectively.

Genetic testing soon became a standard in oncological care: in the 2000s we have started to stratify patients according to their tumor mutational profile, tailoring therapies according to the genetic signature. However, we only aimed genetic testing at those few mutations known to be targetable in each specific tumor type, thus limiting the information acquired in a strict disease-oriented manner. In order to better understand the relevance of cancer mutations across different tumour types and easier identify new actionable targets, a reference "normal" genome sequence was needed to compare with the abnormal ones. The Human Genome Project provided such a feature in 2003, thanks to an international effort lasting almost 15 years, the project was accomplished using the Sanger sequencing technique to determine the exact sequence of nucleotide base pairs of the human genome (Green et al. 2015). During the same years, researchers kept studying the basic mechanisms of cancer growth, identifying new oncogenes and oncosuppressors. With a complete human genome reference in hand, it finally became possible to confirm the pathogenic alterations and to discover new genetic variants linked to human diseases. Large-scale cancer sequencing projects, such as the American TCGA (The Cancer Genome Atlas) and the British Cancer Genome Project were born with this purpose, giving birth to the "genomic era" of cancer research, thus promoting the progressive evolution of sequencing methods: in 2004, 454 Life Sciences showcased a paralleled form of sequencing called pyrosequencing, decreasing sequencing expenses at six-fold compared with Sanger sequencing. This technological implementation led to the birth of the first of many NGS platforms, which allowed a faster and simpler sequencing by employing microscopic, spatially separated DNA templates to massively parallelize the capture of data. With such platforms in hand, it became possible to sequence all the coding exons of a genome (Whole Exome Sequencing, WES) and even a full genome (Whole Genome Sequencing, WGS) in a short time and at an affordable price, providing a huge amount of data. Analyzing and interpreting this data promises to be the challenge of the next decades (Fig. 8.1).

1.1 The NGS Revolution in the Context of Precision Medicine

Besides improving our understanding of cancer, NGS promoted the birth of a new way of treating cancer patients, which we today call Precision Medicine (PM). With this term, we refer to the suiting of medical therapy to the individual characteristics of each subject and its condition (cfr. Chap. 5). In cancer care, this means tailoring oncological treatments to each patient's features and each cancer genomic alterations. It is not a new concept, but the use of NGS and the consequent availability of large-scale human genome databases have created an opportunity for significant onward movement of this approach.

We have already moved from a One-size-fits-all Medicine to a progressive stratification of patients according to their disease subtype, clinical features, and bio-

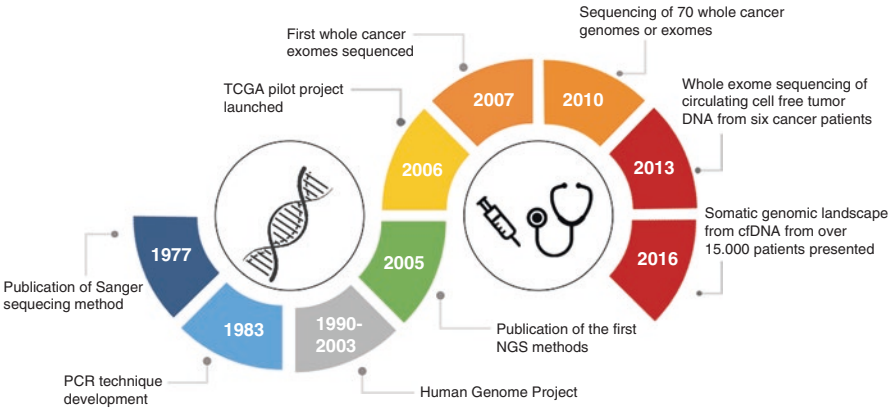

Fig. 8.1 Timeline of major achievements in sequencing technologies

markers (Stratified Medicine). NGS promises to lead the shift toward Precision Medicine, taking into account a wide set of patient features and the cancer mutational scenario to select the best therapeutic approach in oncological care (Shin et al. 2017) (Fig. 8.2).

PM in oncology involves identifying mutations in cancer genomes predicting response or resistance to therapies. In the pre-NGS era, Sanger sequencing and PCR (polymerase chain reaction)-based techniques allowed to obtain a limited amount of information on cancer mutational status; with NGS panels it is now possible instead to screen a broad set of genes in one comprehensive test, able to identify alterations even in the scarce biopsy tissue often available in the everyday practice. And in those frequent cases where collecting tissue for molecular testing is unsafe (e.g., brain, lung, peritoneal lesions), NGS allows to obtain extensive genetic information from simple blood draws (see "Liquid Biopsy" below). In fact, it is possible to obtain genetic material for sequencing from circulating tumor cells (CTCs) and circulating cell-free tumor DNA (ctDNA), which represents a unique instrument to capture the intratumoral heterogeneity, to identify prognostic and predictive factors and imminent resistance mechanisms (Ignatiadis and Dawson 2014). It was recently proposed to incorporate this instrument into cancer staging, shifting to a TNM-B cancer staging system to be assessed in the diagnosis of every cancer and at every successive stage of the disease (Yang et al. 2017).

2 Technical Aspects: From Sanger Sequencing to NGS

In 1977, Frederick Sanger and colleagues first developed a technique to sequence DNA (Sanger et al. 1977). Also known as "chain-termination method," it can be described as a DNA replication reaction during which the random incorporation of dideoxynucleotides (ddNTP) causes the termination of chain elongation. This generates DNA strands of various lengths that are later separated by electrophoresis.

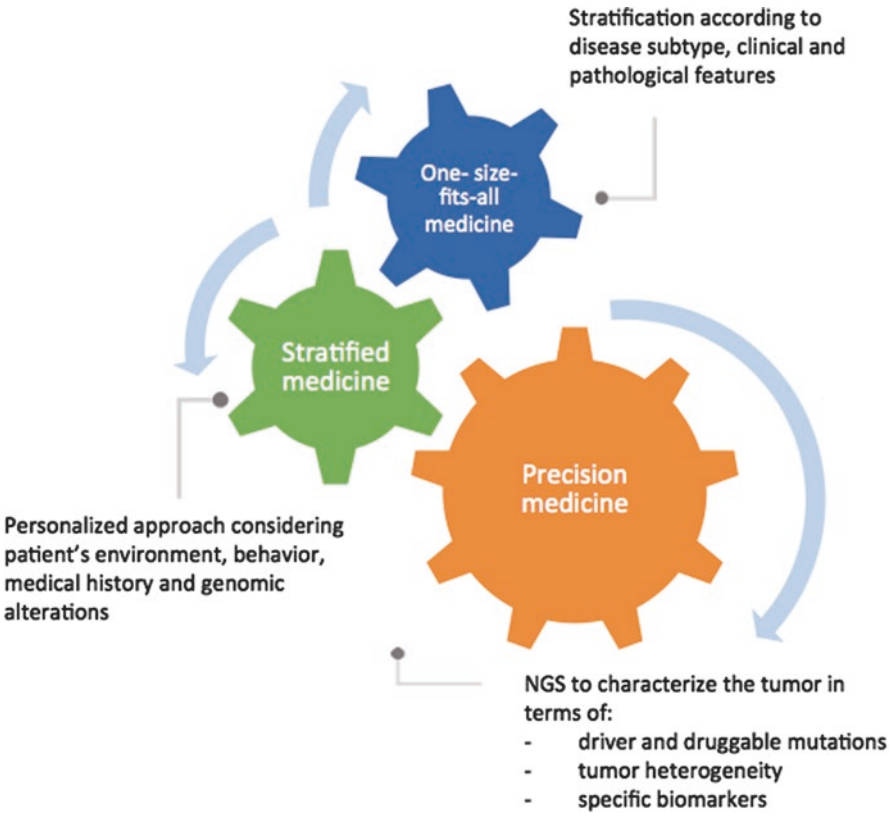

Fig. 8.2 Comprehensive approach in cancer care

Elements required for a classic chain-termination reaction are illustrated in Table 8.1.

The Sanger process is a very accurate sequencing method, giving high-quality sequence for relatively long fragments of DNA (up to 900 base pairs). On the other hand, it is a very expensive process with a low data output.

The need for simpler and faster sequencing processes led to the development of new technologies for DNA reading, collectively named "next-generation sequencing" (NGS). In 2005, the 454 Life Science launched on market the first NGS platform (Margulies et al. 2005), and since then many other companies developed NGS platforms that allow for high-throughput sequencing in a cost- and time-effective way.

Despite the platform used, every NGS process can be summarized in three phases: library preparation (± amplification), sequencing, and data analysis.

Table 8.1 Basic elements of a Sanger sequencing reaction

Table	A single-strand DNA sample that is previously amplified by PCR to generate many identical copies of a DNA sequence of interest.
DNA polymerase	The enzyme that sequentially adds nucleotides into the elongating chain. It catalyzes the reaction: dNTP (or ddNTP) + DNAn ⇌ diphosphate + DNAn+1.
Primers	Short sequences of nucleotides (almost 20) that bind to the DNA template and act as a starter for the DNA polymerase.
Deoxynucleotides (dATP, dCTP, dGTP, dTTP)	Monomers that compose a DNA sequence. Each of them consists of a nitrogenous base, a deoxyribose sugar, and a phosphate group.
Dideoxynucleotides (ddATP, ddCTP, ddGTP, ddTTP)	ddNTP are special, artificial nucleotides analogous to dNTP, but lacking the –OH group at 3' carbon position. They act as chain-elongating inhibitors of DNA polymerase.
	To permit automate reading, ddNTP are usually labeled.

dNTP deoxynucleotides, *ddNTP* dideoxynucelotides, *PCR* polymerase chain reaction

2.1 Library Preparation and Amplification

The sequencing library is created by random fragmentation of a DNA template. Fragments are then linked to platform-specific adapters and amplified by PCR (polymerase chain reaction) or alternative techniques (solid-phase bridge amplification or rolling circle amplification).

2.2 Sequencing

NGS technology can be categorized into short- and long-read sequencing. The difference intuitively lies on read length: 100–600 bp for the first technique, up to 900 Kb for the second one.

Short-read sequencing approach is the most frequently used today—it is cheaper and has a higher accuracy. However, the short-read length limits its capability to resolve complex regions with repetitive or heterozygous sequences, for which a long-read technique is more suitable.

Illumina, Ion Torrent, 454 Life Science, and SOLiD are the major platforms created using a short-read technology. The first three platforms use a technique called sequencing "by synthesis," whereas the SOLiD system is based on sequencing "by ligation."

The MinION system, based on nanopore sequencing, and the PacBio sequencer, which uses a "Single Molecule, Real-Time (SMRT)" sequencing approach, represent instead the main long-read technologies available on the market. A technical comparison of all these NGS platforms is given in Table 8.2.

2.3 Data Analysis

The large amount of raw data generated is then inserted into bioinformatics workflows in order to convert these nucleotide sequences into meaningful biological results.

A typical NGS data analysis pipeline can be divided into four main operations: base calling, read alignment, variant identification (SNVs, indels, CNAs, SVs), and variant annotation. Table 8.3 briefly describes these steps.

3 NGS Methods: Genomics, Transcriptomics, and Epigenomics

3.1 Genomics

Next-generation sequencing was first applied to genomics research, mainly to detect variants in DNA sequence in terms of single nucleotide variations (SNVs), insertion-deletions (indels), structural variations (SVs), and copy number alterations (CNAs).

NGS methodology applied to an entire genome is called "whole genome sequencing," in which both coding and non-coding regions are sequenced. WGS generates huge amounts of data per sample, but usually low depth of coverage. A typical WGS experiment assures a 30X coverage, enough to detect most germline variants in human genome, but inadequate to identify all rare somatic mutations present in cancer genomes.

"Whole exome sequencing" is instead specifically designed to sequence only coding DNA. These regions are isolated before sequencing by an enrichment step, which targets only the exons inside the library of interest. By sequencing only 2% of a genome, a single region can be read many more times, ensuring a coverage of 100X with a cheaper and faster process. WES is therefore more suitable to analyze cancer genome; however, the capability to detect SVs and CNVs is much lower when excluding non-coding regions.

An even more selective genome analysis is given by "targeted sequencing," in which specific regions of interest are isolated and sequenced. Many gene panels have been designed specifically for this purpose, allowing to focus time and resources on selected genes usually sequenced with a 500–1000X coverage.

Table 8.2 Comparison between commercially available NGS platforms

Platform	Sequencing	Maximum read lenght (bp)	Reads per run	Run time	Maximum output	Error rate
First generation						
Sanger	NA	900	96	20 min–3 h	2.1 Mb	0.3%
Second generation						
454						
GS Junior+	Pyro	700	0.1 M	18 h	70 Mb	1% indels
GS FLX Titanium XL+	Pyro	700	1 M	23 h	700 Mb	1% indels
Illumina						
Hi Seq[a]	SBS	36 (SE)	Up to 4 B (SE)	<1–3.5 h (Hi Seq 3000/4000)	1500 Gb	0.1%
		125 (PE)	Up to 8 B (PE)	7 h – 6 d (Hi Seq 2500)		substitution
MiniSeq[b]	SBS	150 (PE)	25 M	4–24 h	7.5 Gb	<1%
						substitution
NextSeq 550[b]	SBS	75 (SE)	Up to 400 M (SE)	12–30 h	120 Gb	<1%
		150 (PE)	Up to 800 M (PE)			substitution
MiSeq (v3)	SBS	75 (PE)	25 M (PE)	4–55 h	15 Gb	0.1%
		300 (PE)				substitution
Hi SeqX[a]	SBS	150 (PE)	5.3-6 B	<3 d	1800 Gb	0.1%
						substitution
NovaSeq6000[c]	SBS	150 (PE)	20 B	36–44 h	6000 Gb	NA
Ion Torrent						
PGM	SBS	400 (SE)	400000–5.5 M	2.3–7.3 h	2 Gb	1% indels
Proton	SBS	Up to 200 (SE)	60–80 M	2–4 h	Up to 10 Gb	1% indels
S5	SBS	600 (SE)	2–130 M	2.5–4 h	25 Gb	1% indels
SOLiD (Sequencing by Oligonucleotide Ligation and Detection)						
5500xl	SBL	75 (SE)	~1.4 B	10 d	240 Gb	0.01%
		50 (PE)				A-T bias
Third generation						
PacBio (Pacific Bioscience)						
RS II	SMRT	>15000 (average)	Up to 55000	30 min–4 h	1 Gb	15% indels
Sequel	SMRT	30000 (average)	~400000	30 min–20 h	10 Gb	15%

(continued)

Table 8.2 (continued)

Platform	Sequencing	Maximum read lenght (bp)	Reads per run	Run time	Maximum output	Error rate
Oxford Nanopore						
MinION	SMRT	Up to 900 kb	Up to 1 M	Up to 48 h	20 Gb	5–10%

A-T adenine-thymine, *B* billion, *bp* base pairs, *d* days, *Gb* gigabase pairs, *h* hours, *indels* insertions-deletions, *Kb* kilobase pairs, *M* million, *Mb* megabase pairs, *min* minutes, *NA* not applicable, *PE* pair-end, *Pyro* pyrosequencing, *SBL* sequencing by ligation, *SBS* sequencing by synthesis, *SE* single-end, *SMRT* single-molecule-real-time
[a]Dual flow cells; [b]high output; [c]dual S2 flow cells

Table 8.3 Basic steps of NGS data processing

Base calling	Signals provided during sequencing are translated into a sequence of bases, removing the noisy signals.
Read alignment	DNA of the sequenced sample is compared/aligned to a reference genome. Given that NGS generally produces millions of short reads, each read needs to find the corresponding part on reference genome.
Variant identification/ calling	Variants from sequence data are identified in this step. Four main classes of sequence variants exist (SNVs, indels, CNAs, and SVs), each requiring a different computational approach for sensitive and specific identification.
Variant annotation	Real variants are distinguished from sequencing artefacts, trying to identify which ones are potentially pathogenic and have a real clinical value.

SNVs single nucleotide variations, *indels* insertion/deletion, *CNAs* copy number alterations, *SVs* structure variants

3.2 Transcriptomics

The transcriptome can be defined as "the complete set of transcripts in a cell or a population of cells for a specific developmental stage or physiological condition" (Wang et al. 2009). Transcriptomics studies have a pivotal role in cancer research, providing a unique focus of what happens in neoplastic cells after DNA transcription.

RNA-sequencing (RNA-seq) is a relatively new application of NGS, which is gradually replacing microarrays as favorite technology for transcripts analysis. Differently from arrays, RNA-seq is not designed as a targeted test and does not require species- or transcript-specific probes. It can be used both to quantify gene expression and to detect novel transcripts, gene fusions, SNV, and indels at the same time.

Besides gene expression analysis, NGS has also been applied to small non-coding RNA (ncRNA) discovery and profiling through dedicated small RNA-seq platforms. Small non-coding RNAs are short sequences of nucleotides (≈20 bp) not translated into proteins. Several classes of small ncRNA exist, like transfer RNA (tRNA), ribosomal RNA (rRNA), microRNA (miRNA), small interfering RNA (siRNA), and Piwi-interacting RNA (piRNA). Between them, miRNA and siRNA are of major interest to transcriptomic research in oncology because of their role in

gene expression regulation of cancer cells (Gomes et al. 2013). Through a cellular process called RNA interference (RNAi), both miRNA and siRNA interact with the so-called RNA-induced silencing complex (RISC) to block and silence target mRNAs.

This powerful gene-silencing process is object of study also from a therapeutic point of view. RNA-based therapeutics represents a new class of anticancer drugs, inhibiting molecules targets that were inaccessible until now. None of these drugs is approved by FDA to date, but many are currently under investigation in clinical trials (Barata et al. 2016).

3.3 Epigenomics

The term epigenetics refers to "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" (Wu and Morris 2001). DNA methylation, histone-modification, and altered DNA–protein interactions are three major epigenetic alterations involved in cancer development and progression.

In the past years, epigenomics studies were essentially conducted through microarrays technologies. The arrival of NGS signed a paradigm shift in this field, dramatically increasing the chance to survey epigenetic markers genomewide with high-throughput data output at single nucleotide resolution. Methylation sequencing (or bisulfite sequencing) (Lister et al. 2008) and ChIP-seq (Chromatin Immunoprecipitation Sequencing) (Barski et al. 2007) are the NGS-based assays commonly employed for epigenetics studies.

4 NGS Applications for a Personalized Oncology

4.1 Detection of Driver Alterations and Resistance

The availability of next-generation sequencing technologies had literally revolutionized the comprehension of cancer biology during the last decades. Massive genome sequencing of thousands of tumors from all major cancer types has become feasible, leading to identification and classification of many genetic and epigenetic alterations potentially involved in tumorigenesis.

By the time a cancer is diagnosed, it comprises billions of these genomic alterations. Some are responsible for malignant transformation, others are acquired along the way. The pivotal work of Greenman and coworkers defined these two categories of mutations as "driver" and "passenger" (Greenman et al. 2007). The term "driver" is reserved for somatic mutations that, directly or indirectly, confer a selective growth advantage to malignancies bearing them. The term "passenger" is instead

referred to alterations that arise in somatic cancer genome during the progression of a tumor, but do not contribute to its growth.

Detection of driver alterations that results in oncogene addiction is currently the primary application of NGS in oncology research and discriminating between driver and passenger alterations is a challenge point of translational research. Several statistical and computational techniques to characterize these mutations have been described, including variant effect prediction, recurrence/frequency assessment, and pathway/network analysis. These techniques provide alternative strategies to filter the long list of somatic mutations, thus identifying an enriched subset of subclonal carriers who may undergo further functional validation (Gonzalez-Perez et al. 2013; Raphael et al. 2014; Ding et al. 2014). Given that driver mutations are responsible for oncogenic addiction, any targeted therapy must be based on their identification. The implementation of this "lock-and-key" model led to the approval of several specific biologic agents, targeting specific driver alterations in different cancer types.

Here we present the example of NGS application in clinical practice for identification of driver and resistance mutations in lung cancer, breast cancer, and cancer of unknown primary origin.

4.1.1 Lung Cancer

Lung cancer represents, by far, the disease in which pathways of oncogenic addiction have been characterized the most. There are, on average, more than 300 nonsynonymous mutations per lung cancer, but only a minority of these genes can promote tumorigenesis, resulting in driver mutations. Large-scale genomic studies have recognized a variety of potential therapeutic targeting, including:

- Established targets: EGFR, ALK, ROS-1, BRAF
- Emergent target: MET, RET, NTRK, HER2, PI3KCA, AKT1, MAP 3K1, FGFR, DDR2
- Elusive targets: KRAS, TP53

International guidelines recommend molecular testing for these established targets in everyday clinical practice.

Detection of EGFR and BRAF mutations are classically carried out using RT-PCR (Real Time-PCR) or Sanger sequencing, whereas ALK and ROS1 rearrangements are identified through FISH (fluorescence in situ hybridization) or IHC (immunohistochemistry) methods. In recent years, NGS panels implementation is gradually replacing these techniques in clinical laboratories, allowing the analysis of several genes at the same time. The last MAP (Molecular Analysis for Personalised Therapy) consensus (Swanton et al. 2016) recommends the use of NGS panels in the context of clinical trials. For non-small-cell lung cancer (NSCLC), at least 20 genes should be tested in molecular screening programs to drive patients onto therapeutic trials (EGFR, BRAF, HER2, KRAS, PI3KCA, NTKR, ALK, MET, AKT1, BRCA1/BRCA2, HRAS, NRAS; rearrangement status of ALK, ROS1, NTRK;

amplification of RET, MET, and EGFR; aberrations (mutations or amplifications) in FGFR1/2/3, NOTCH1/NOTCH2).

Profiling of EGFR, ALK, ROS1, and BRAF defines as many "subtypes" of NSCLC, for which specific algorithm of treatment exists. Activating EGFR mutations in the tyrosine kinase (TK) domain of the EGFR gene, most frequently exon 19 deletion mutations and the single-point substitution mutation L858R in exon 21, are predictive for response to the EGFR TK Inhibitors (EGFR-TKIs) gefitinib, erlotinib, afatinib, osimertinib, and dacomitinib. ALK rearrangement-positive NSCLC are instead candidate to frontline therapy with ALK-inhibitors alectinib, crizotinib, or ceritinib. The last two of them are also the referred targeted drugs for ROS1-rearranged NSCLC, whereas cancers positive for BRAF V600E can receive the combination dabrafenib-trametinib (www.nccn.org/professionals/physician_gls/pdf/nscl.pdf).

Unfortunately, almost all patients treated with targeted therapies develop secondary resistance. NGS can be useful to identify the implicated mechanisms of resistance and to aid on following treatment choices. For instance, T790M mutation has been found in almost 50% of patients that progress during treatment with first- and second-generation EGFR-TKIs. This finding led to the development of osimertinib, a third-generation EGFR TKI that inhibits T790M as well as the common activating mutations. The AURA 3 trial (Mok et al. 2017) demonstrated the great superiority of osimertinib to platinum-based chemotherapy in EGFR-TKIs pretreated patients with T790M mutation, reporting a PFS of 10.1 months in osimetinib group versus 4.4 months in the control group. The introduction of osimertinib has allowed prolonging as far as possible the chemo-free interval in EGFR-positive population.

Interestingly, the T790M mutation was documented using the Cobas EGFR Mutation Test v2 on ctDNA on blood and urine samples. Osimertinib is currently approved only for T790M-positive NSCLC, and this mutation can be indifferently assessed on tissue sample or liquid biopsy. In this common clinical scenario, preferring blood- over tissue-sampling is clinical practice.

4.1.2 Breast Cancer

The estrogen receptor (ER) and the HER2 signaling pathways are the dominant drivers of oncogenesis in breast cancer. The available arsenal of hormonal agents and anti-HER2 drugs has dramatically changed the natural history of metastatic breast cancer (MBC) during last decades, achieving a twofold increase in 5-year relative survival rate (Mariotto et al., 2017).

Unfortunately, ER-expression and/or HER2-amplification can well predict but are not secure guarantee of response to targeted therapy with endocrine therapy and HER2-signaling blocking agents. Many patients are resistant *ab initio* (de novo resistance), whereas others become resistant after an initial phase of therapeutic efficacy (acquired/secondary resistance).

ESR1 mutations in ER-positive breast cancer is a recognized cause of resistance to endocrine therapy, more commonly as acquired resistance. First described in cell

models in 1996 (Weis et al. 1996), ESR1 mutations were found to confer ER constitutive activation and resistance to endocrine agents. Nevertheless, these alterations were rarely found in subsequent studies (0.5% of cases), and their potential role remained underappreciated for several years (Koboldt et al. 2012).

With NGS technology applications, several studies renewed interest in ESR1 mutations by demonstrating high prevalence in ER-positive MBC after aromatase inhibitor (AIs) therapy, suggesting a role in the endocrine resistance, both as predictive and prognostic biomarker (Schiavon et al. 2015; Jeselsohn et al. 2014; Merenbakh-Lamin et al. 2013; Robinson et al. 2013).

The SOFEA trial compared exemestane alone with fulvestrant-containing regimens (fulvestrant+anastrozole or fulvestrant +placebo) in patients with MBC pretreated with AIs (Johnston et al., 2013). In a retrospective analysis of this trial, detection of ESR1 mutations (39% of patients) correlated with an improved PFS after taking fulvestrant compared with exemestane, whereas wild-type patients had similar outcomes with both treatments (Fribbens et al. 2016).

Additionally, a retrospective analysis of the BOLERO-2 trial, evaluating the benefit of incorporating everolimus to AI therapy, showed longer PFS with everolimus only in the subgroup of patients harboring D538G ESR1 mutations (21.1%), with similar outcomes when compared to wild-type patients. This benefit was not observed for patients with Y537S mutation (alone or with D538G mutation). Despite the treatment arm, all patients ESR1-mutated had a worse overall survival (OS). The authors concluded that ESR1 mutations are not predictive of benefit with everolimus, but ESR1 keeps a negative prognostic value (Chandarlapaty et al. 2016).

In the PALOMA3 trial, pre- and postmenopausal patients failing a prior ET within 12 months in the adjuvant and 1 month in the metastatic setting were randomized to fulvestrant plus palbociclib or fulvestrant and placebo (Cristofanilli et al. 2016). ESR1 mutations were detected in 25% of patients, at baseline, as a finding related to the endocrine resistance mechanism. A significant PFS benefit was reported for patients treated with fulvestrant/palbociclib versus patients receiving fulvestrant alone, and this benefit was maintained in patients harboring an ESR1 mutation. This evidence confirms a conserved selective sensitivity to fulvestrant for ESR1-mutant cancers, even if these mutations are commonly associated with a worse prognosis (Turner et al. 2016). In conclusion, the suggestion is to select the combination fulvestrant +/- palbociclib over AIs when ESR1 mutations are detected.

Prospective trials are needed to understand if ESR1 mutations analysis could impact on treatment choice and final outcome of ER-positive MBC. Specific inhibitors are under investigation like AZD9496, in a refined targeted approach to endocrine therapy (Hamilton et al. 2018).

4.1.3 Carcinoma of Unknown Primary Site

Management of carcinoma of unknown primary (CUP) site is another field that made considerable steps forward since NGS availability. CUP accounts for 3–5% of all malignancies, the seventh for incidence and the fourth cause of cancer death

(Massard et al. 2011). ESMO guidelines recommend a platinum-based regimen for the majority of CUP patients (85–90% of cases), defined as "poor-risk" subset because of lacking any clinico-pathological features that provide a favorable outcome. Their prognosis is dismal despite chemotherapy (median OS of 9 months) (Fizazi et al. 2015).

Thanks to NGS profiling, CUP management has radically changed. First of all, it has been shown that gene expression profiling can predict the tissue of origin and consequently allow treatment optimization, in a histology-oriented approach. A prospective trial conducted by Hainsworth and colleagues at the Sarah Cannon Research Institute found that the primary tissue can be predicted in 98% of cases. Patients in this trial were subsequently treated with a site-specific regimen, reaching a median survival of 12.5 months (Hainsworth et al. 2012). Considering the modest benefit achieved with a platinum-based empiric regimen, the identification of the putative primary may substantially change the management and outcome of patients with CUP, particularly if a tumor more responsive to the best site-specific therapy is recognized.

Detection of actionable mutation is another promising application of the genome sequencing for a molecular-oriented approach to CUP management. Performing a sequencing panel encompassing 410 cancer-associated genes (the MSK-IMPACT panel), Varghese et al. analyzed 150 tissue samples of CUP. A targetable genomic alteration was found in 30% of cases (45 patients), and 10% of them (13 patients) received a targeted drug. The most common putative driver alterations detected were: ERBB2 amplification, BRAF V600E mutation, and PIK3CA mutations (Varghese et al. 2017).

"CUPISCO" is a randomized, phase II study designed to compare efficacy and safety of targeted therapy or immunotherapy versus platinum-based chemotherapy in CUP (NCT03498521). After three cycles of platinum-based induction CT, patients are randomized 3:1 to targeted therapy/immunotherapy or chemotherapy. A comprehensive genomic profiling is performed on all patients enrolled before receiving the induction CT, allowing a subsequent choice of the best targeted therapy in the experimental arm. This trial is actually recruiting and the first results are expected in 2022. If positive, their results could dramatically change the management of CUP in everyday clinical practice.

4.2 Biomarkers

In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Atkinson et al. 2001). A biomarker has a "prognostic" value when it gives information about disease outcome irrespective of treatment, whereas a "predictive" biomarker indicates the likely benefit from a specific treatment.

In the era of targeted therapy, predictive biomarkers and related targeted drugs are commonly validated and approved in parallel. HER2 amplification/trastuzumab in breast cancer, BCR-ABL translocation/imatinib in chronic myeloid leukemia, EGFR mutation/EGFR TKI in NSCLC, and BRAF mutation/melanoma are only few examples of "predictive biomarker/targeted drug" pairs that commonly guide the therapeutic choice.

Beside the well-known biomarkers for cancer treatment response prediction, relatively new and more complex models are emerging. Microsatellite instability (MSI), homologous recombination deficiency (HRD), and tumor mutation burden (TMB) have the most robust data so far and will probably soon impact on clinical practice as predictive of response to DNA-disrupting agents, DNA repair targeting compounds, and immunotherapy.

4.2.1 Homologous Recombination Deficiency

Homologous recombination (HR) is a genetic recombination mechanism essential for repair of DNA double-strand breaks (DSBs) (Jasin and Rothstein 2013; Szostak et al. 1983). BRCA1 and BRCA2 genes are essential components of HR-mediated DNA repair, and mutations of these genes cause HR pathway failure (Moynahan et al. 1999; Moynahan et al. 2001). In HR-deficient cells, other mechanism of DNA repair must take over, such as non-homologous end joining (NHEJ) or base-excision repair (BER) (Hustedt and Durocher 2017). Specific agents, like poly-(ADP ribose) polymerase (PARP) inhibitors, have been designed to target these alternative pathways (Fong et al. 2009; Robson et al. 2017; Swisher et al. 2017). This therapeutic strategy, called "synthetic lethality," sentences HR-deficient cells to die by apoptosis.

The singularity of HRD as predictive biomarker lies on its complexity. BRCA1/2 are only two of many proteins involved in this pathway, and all of these need to be analyzed in parallel to make HRD a reliable biomarker. Many panels based on NGS sequencing are currently available to test HRD in different cancers, providing a quantitative score that reveals if the HR pattern is impaired or not (O'Kane et al. 2017).

4.2.2 Microsatellite Instability

Microsatellite instability refers to hypermutability of short nucleotide sequences tandemly repeated (microsatellites) (Thibodeau et al. 1993). This condition is essentially due to impairment of DNA mismatch repair (MMR) pathway, because of mutation of MMR genes (e.g., MLH1, MSH2, MSH3, MSH6, and PMS2).

MSI is observed in 15% of sporadic colorectal tumors (Vilar Gruber 2010), and has been reported in tumors of endometrium, ovaries, urothelium, stomach, small intestine, hepatobiliary tract, brain, and skin. If instead a germline mutation is

found, the MSI phenotype identifies a genetic disease called "hereditary non-polyposis colorectal cancer" (HNPCC), or Lynch syndrome (Lynch et al. 1993).

A potential role of MSI as predictive biomarker has recently been investigated, following the evidences that high levels of MSI seem to predict a good response to immune-checkpoint inhibitors (ICPI), whereas MSI stable tends not to (Le et al. 2017). This result led to the FDA approval of pembrolizumab for MSI-H cancers in May 2017, the first tumor-agnostic drug approval in history.

MS instability is usually analyzed through PCR and IHC assays. Nevertheless, dedicated NGS panels have been recently implemented, showing feasibility and reliability if compared to "old" techniques (Vanderwalde et al. 2018). The main advantages of NGS methodology over IHC and PCR are the unnecessity of normal tissue (unlike PCR), a quantitative result (instead of IHC, which is a qualitative test) and obviously the availability of many additional information for a therapy personalization at its best.

4.2.3 Tumor Mutational Burden

Tumor mutational burden is defined as the number of somatic mutations within the coding region of a tumor genome. A high mutational load is typically found in tumor associated to environmental DNA damage, like lung cancer (i.e., tobacco smoking, environmental pollution) or melanoma (i.e., sun exposure) (Chalmers et al. 2017).

TMB has recently been identified as predictive biomarker of immunotherapy efficacy. The rationale lies in the principle of immunotherapy itself: a TMB correlates with expression of multiple neoantigens by cancer cells, and consequently to potential efficacy of ICPI in reactivating immunity against cancer cells.

Major evidences about a role of TMB as predictive biomarker of response to ICPI come from retrospective analysis of different studies, including melanoma, NSCLC, and urothelial cancer (Rosenberg et al. 2016; Rizvi et al. 2015; Snyder et al. 2014). A prospective validation in phase III trials is awaited; however, early phase trials suggest a predictive role for TMB. Both in NSCLC and SCLC, the first-line combination therapy nivolumab + ipilimimab has shown to be more effective in patients with high TMB, as respectively outlined in Checkmate 227 (Hellmann et al. 2018a, b) and 032 (Hellmann et al. 2018a, b) trials. Similar evidences come from trials with atezolizumab in first- (B-F1RST study (Velcheti et al. 2018) and second-line (POPLAR and OAK trials (Gandara et al. 2017) treatment for NSCLC. Quantification of TMB was classically carried out through whole exome sequencing. This approach is accurate, but expensive and not suitable for routine use in clinical practice. For this reason, major biotechnology companies have designed specific targeted panels to quantify TMB in a simple and cost-effective way. Many independent trials have already proved their reliability if compared to WES (Johnson et al. 2016). Prospective trials are now necessary to validate their implementation in clinical practice to identify which patients are more likely to respond to immunotherapy.

4.3 Liquid Biopsy

Liquid biopsies are noninvasive blood tests that detect circulating tumor cells (CTCs) and fragments of tumor DNA (cell-free tumor DNA – ctDNA) released into the bloodstream from the primary tumor and from metastatic sites.

Collection of fluid instead of classic tissue sample is gradually spreading from research laboratories to clinical practice. A liquid biopsy consists of a simple blood sampling, overcoming the issues related to the feasibility of invasive biopsy procedures. For the same reason it can be repeated many times without risks or side effects, providing a picture of tumor evolution over time. Finally, analysis of ctDNA may allow a better representation of tumor heterogeneity, possibly detecting different clones at once. Many potential applications of liquid biopsy are object of ongoing clinical trials. The most promising are briefly presented below.

4.3.1 Early Diagnosis of Primary Disease

Early detection of cancer through a validated screening assay is probably the most ambitious purpose of liquid biopsy. Like every screening test, high sensitivity, specificity, and cost-effectiveness are essential requirements. Despite recent development of very sensitive technologies, a reliable test for early cancer detection remains a challenge.

Cohen et al. (2018) launched very recently the CancerSEEK panel, developed for detection of the eight most common cancers (ovary, liver, stomach, pancreas, esophagus, colorectum, lung, and breast). This method combines the evaluation of eight blood biomarkers with sequencing of 16 cancer-related genes from ctDNA. On a sample of 1005 individuals with clinically detected non-metastatic cancers, the authors reported a median sensitivity of 70% (ranging from 98% in ovarian cancers to 33% in breast cancers), with a specificity ≥99%. Despite these encouraging results, some limitations to this study must be noted. Firstly, the experimental cohort was composed by patients with clinically detected cancers. In a real-world screening population most individuals would have less advanced disease, probably determining a minor test sensitivity. Secondly, the control cohort included only health individuals, without all the comorbidities that could augment the number of false positive results.

4.3.2 Early Detection of Relapse

Several studies have demonstrated that CTCs detection is associated with unfavorable prognosis in various types of solid tumors, in particular for early-stage diseases.

Early breast cancer (EBC) is the setting for which more evidences exist. The largest trial realized so far has been published by Rack and colleagues in 2014

(Rack et al. 2014). The authors used the CellSearch System to analyze CTCs in patients with EBC: 2026 women were tested before adjuvant CT and 1492 after the treatment. CTCs detection before CT was associated with poor outcome both in terms of disease-free survival and overall survival. The persistence of CTCs after receiving adjuvant CT was analogously linked to a negative prognosis.

Beside breast cancer, CTCs count has been evaluated as prognostic marker for metastatic relapse in many other tumor types, like colorectal (Yokobori et al. 2013), bladder (Rink et al. 2012), liver (Schulze et al. 2013), head and neck (Gröbe et al. 2014), and testicular germ cell tumors (Nastaly et al. 2014).

Cell-free tumor DNA profiling has been similarly performed to assess its value in predicting metastatic relapse. In two different studies published in 2015, ctDNA was serially assessed for earlier detection of metastasis in patients with EBC. In both cases, mutation tracking in serial samples has been shown to accurately predict metastatic relapse, in several instances months before clinical relapse (8–11 months on average) (Olsson et al. 2015; Garcia-Murillas et al. 2015). Reinert et al. (2016) conducted a similar trial on patients with early colorectal cancer, with analogous final evidences: using an NGS approach on ctDNA it was possible to detect metastatic recurrence with a 10 months' lead time compared to conventional follow-up.

Taken together, these evidences suggest that implementation of liquid biopsy for screening of patients with high risk, early-stage cancer may create a therapeutic window for interventions before the development of clinical metastasis.

4.3.3 Detection of Driver/Resistance Mutations and Real-Time Monitoring of Therapies

As previously mentioned, detection of driver- and resistance mutations is a key application of NGS. DNA profiling is performed on a tissue sample from a biopsy or a surgical specimen, usually from the primary tumor and sometimes from a metastatic site. These samples are then archived in pathological labs, always available for additional analysis. Nevertheless, they may represent a "static" picture unable to reflect the temporal evolution under drug pressure. Moreover, metastatic relapse frequently happens several years after primary tumor resection, and the information obtained from that specimen might be outdated. Serial tissue biopsies of both primary tumors and metastatic sites are unfeasible in clinical practice. On the contrary, liquid biopsy allows repeated analyses over the course of treatment, providing a dynamic and reliable picture of tumor genome that can be used for monitoring therapies in real time.

Treatment choice in metastatic breast cancer is determined by ER-expression and HER2-amplification. ER-positive MBCs are eligible for hormonal treatment; that is, commonly continued until development of resistance and disease progression. A common cause of acquired resistance to endocrine therapy is tumor heterogeneity: patients with ER-positive BC can harbor ER-negative CTCs, as demonstrated by Paoletti et al. (2015).

Mutation of ER itself is a common cause of resistance. Chu and coworkers proved that somatic mutations in the ER gene (ESR1) can be readily identified in ctDNA, and they correlate with failure of endocrine therapy (Chu et al. 2016). Liquid biopsy has been also successfully applied for analysis of ESR1 methylation, known to be responsible for epigenetic silencing of ESR1 (ER downregulation) and development of secondary endocrine resistance (Mastoraki et al. 2018).

The HER2 oncogene is another key target in MBC treatment. Also, for HER2 status a discrepancy between CTCs and primary tumors has been found in up to 30% of cases (Fehm et al. 2010). This evidence inspired the development of dedicated interventional trials, where patients HER2-negative at primary assessment can receive anti-HER2 agents on the basis of HER2-status on CTCs (DETECT III study—NCT01619111, Treat CTC trial—NCT01548677). In colorectal cancer, NRAS, KRAS, and BRAF status are essential requirements for therapy optimization. Many studies have reported a high level of concordance between mutational analysis on tissue samples and ctDNA (Mouliere et al. 2013; Siravegna et al. 2015). Moreover, liquid biopsy has shown to provide a better picture of tumor heterogeneity, detecting RAS mutation not found on tissue sample (Siravegna et al. 2015).

Mutational analysis of KRAS status during treatment with anti-EGFR can also predict disease progression several months before radiologic assessment (Misale et al. 2012). Longitudinal ctDNA profiling has even demonstrated that these mutant KRAS clones decline following anti-EGFR withdrawal, indicating that clonal evolution is a continuous process (Siravegna et al. 2015).

Lung cancer is the prototype of therapy personalization based on mutational status. Once again, liquid sequencing has proved to be a reliable surrogate of tissue biopsy (Kuang et al. 2009; Taniguchi et al. 2011; Nakamura et al. 2012; Douillard et al. 2014; Reck et al. 2016). On June 1, 2016, FDA approved "Cobas EGFR Mutation Test v2" as first liquid biopsy test available in clinical practice. It is licensed for the detection of exon 19 deletions or exon 21 substitutions in EGFR gene. If negative, guidelines recommend a routine test using tissue sample to be performed (www.nccn.org/professionals/physician_gls/pdf/nscl.pdf).

EGFR profiling through liquid biopsy is a useful tool also during treatment with TKIs, allowing for detection of EGFR mutations responsible for therapy resistance. Oxnard et al. analyzed plasma ctDNA in 9 patients with EGFR-mutated NSCLC treated with erlotinib. All patients were negative for mutation T790M before starting treatment, but in 2/3 of them serial ctDNA profiling showed an increasing in T790M EGFR mutant levels up to 16 weeks before radiologic progression, anticipating the clinical–radiological progression (Oxnard et al. 2014).

Androgen blockade represents the cornerstone for treatment of prostate cancer. Unfortunately, progression to castration-resistant prostate cancer (CRPC) occurs virtually in all patients. Genomic and transcriptomic alterations of androgen receptor, essentially in terms of AR amplification and AR splice variants, are primarily responsible for progression to castration resistance.

AR-v7 is a splicing variant of AR, a truncated form of the receptor that is constitutively active because of lacking the ligand-binding domain. When detected, it is responsible not only for resistance to classical first-line androgen-deprivation ther-

apy, but also to second-generation anti-androgen agents commonly applied in CRPC (i.e., enzalutamide and abiraterone). AR-v7 is commonly tested analyzing mRNA from CTCs (Antonarakis et al. 2014).

A recent clinical audit published by Johns Hopkins University has confirmed the potentiality of AR-v7 as predictive biomarker, revealing that its knowledge can influence the clinical decision making in 53% of patients (Markowski et al. 2017). Nevertheless, (it must be pointed out that) the last St. Gallen prostate cancer conference has discouraged a routine use of AR-v7 testing in clinical practice, mainly because only single-center experiences are available, and a prospective, external validation is still lacking (Gillessen et al. 2018). Moreover, AR-V7 positivity is 3% (Scher et al. 2016) in patients naive to abiraterone, enzalutamide, or taxane exposure, increasing only after progression on second-generation anti-androgen agents (19–39%) (Antonarakis et al. 2014). For this reason, the panel concluded for its limited value both in first-line setting, for its low-rate detection, and in second line, where chemotherapy is already the treatment of choice.

4.3.4 Characterization of Tumor Heterogeneity

Genetic diversity exists between individuals with the same tumor type (intertumor heterogeneity), but also within a single tumor (intratumor heterogeneity). Intratumor heterogeneity (ITH) is both spatial, comprising different subclones inside a unique lesion and in distinct sites, and temporal, emerging during the evolution of a malignancy.

The "trunk and branch" model is commonly used to represent ITH. Into the trunk are found driver somatic alterations that arise very early during the natural history of a tumor. Since indispensable for neoplastic growth, they are detectable in every subclone and tumor region. Conversely, subclonal mutations that occur later during cancer evolution are not homogeneously localized, but present in only a subset of cancer cells. They make up the branches of the tree (Yap et al. 2012).

In a pivotal paper published on Science almost 40 years ago, Peter Nowell firstly postulated this theory of cancer as a process of clonal evolution, in which successive rounds of clonal selection give rise to tumor heterogeneity (Nowell 1976). However, this theory could find a clinical application outside the preclinical experiments only years later, with the emergence of NGS techniques. Serial extensive tissue sampling of both primary and metastatic lesions is unfeasible in clinical practice, and sampling bias may occur because only limited geographical regions are analyzed.

The advent of next-generation sequencing has dramatically improved our understanding over tumor evolution, starting to resolve the complexity of ITH at single-nucleotide level. Given that ctDNA is a reliable noninvasive surrogate for tissue biopsies, massive parallel sequencing of ctDNA is likely to be the most powerful tool available to investigate ITH.

In a proof-of-concept study, De Mattos-Arruda et al. sequenced the genome of a primary cancer, a liver metastasis, and plasma ctDNA from a single patient with synchronous ER+/HER2- metastatic breast cancer. Using a targeted panel of 300

cancer genes, they found in ctDNA all the mutations present in the primary tumor and/or the liver metastasis. Conversely, not all mutated genes detected in the metastasis were reliably identified in the primary. The authors successfully proved that ctDNA sequencing is clearly a powerful tool for heterogeneity investigation, providing an accurate representation of the complete repertoire of mutations detected in all tumor sites (De Mattos-Arruda et al. 2014).

Many other studies conducted on different cancer types outlined analogous results (Siravegna et al. 2015; Landau et al. 2013; Lebofsky et al. 2015). Based on this assumption, ongoing trials have been conceived to monitor disease evolution prospectively, from early-stage diagnosis through the different stages of tumor progression and metastatic spreading.

The TRACERx is a pioneering project in this research field. Consisting of four parallel observational studies (lung, renal, melanoma, prostate), it is built on the ambitious aim of understanding the relationships between cancer genomic evolution in metastases, immune evasion, adaptation, and clinical outcome (http://tracerx.co.uk/).

4.3.5 CTCs and ctDNA Analysis

CTCs and ctDNA are cancer biomarkers with complementary roles. Outlining different information, they can be more or less useful with regard to specific research needs and clinical contexts. CTCs can be isolated by several methods, using physical, immunologic, molecular, or functional assays [98]. Several platforms for CTCs detection are commercially available, but CellSearch® system is the only FDA-approved for clinical use. It is an antibody-based assay, by which CTCs are isolated through a double check of positive and negative selection. A cell is identified as CTC by CellSearch if EpCAM (Epithelial Cell Adhesion Molecule)-positive, cytokeratins-positive, and CD45-negative.

For many years, CTCs count has been used alone as a prognostic tumor biomarker. Recent advantages in isolation and sequencing technologies changed this perspective, paving the way to DNA, RNA, and protein analysis at single-cell level (Heitzer et al. 2013; Lohr et al. 2014; Perakis and Speicher 2017). However, some limitations exist. CTCs' detection remains challenging, especially because of their very low concentration in blood. Both detection and enrichment steps require sensitive and specific analytic methods, made possible only with expensive technologies (Pantel and Alix-Panabières 2013; Lowes et al. 2016).

Main technologies available for ctDNA analysis are droplet digital PCR (ddPCR) and next-generation sequencing. The first is a targeted-approach, mainly used for detection of selected mutations. It is most sensitive and cost-effective, and it allows for an absolute quantification of mutant and wild-type copies. Conversely, NGS can be both targeted (gene panels) and untargeted (WES, WGS). It is complex and expensive, but it has a higher throughput that renders a more comprehensive detection of all known and unknown genomic alterations (SNVs, indels, CNAs, SVs), without preventive selection of any gene (Perakis and Speicher 2017). Very recently,

the FoundationACT® assay, a 70-gene panel designed by Foundation Medicine, granted a Breakthrough Device designation by the FDA, likely to become the first liquid biopsy NGS panel to achieve regulatory approval (http://investors.foundationmedicine.com/news-releases/news-release-details/foundation-medicines-new-liquid-biopsy-assay-granted).

Like CTCs, ctDNA analysis has its disadvantages. Even if technically easier and cheaper than CTCs' count, a pre-analytical and analytical procedure validation is still lacking. A potential confounding factor is the presence of normal cell-free DNA that must be separated from cell-free tumoral DNA. Besides these technical considerations, a more relevant conceptual question about the biological meaning of ctDNA must be pointed out. Little is known about the origin of ctDNA (CTCs? Lytic, apoptotic tumor cells?). Assuming that they are released by dying tumor cells, how can they provide information about therapy-resistant clones?

In conclusion, liquid biopsy has demonstrated to be a valid surrogate of tissue sampling. Nevertheless, a scrupulous demonstration of analytic validity, clinical validity, and clinical utility is essential before its introduction in clinical practice.

5 NGS Implementation in Clinical Practice: Challenges and Limitations

The goal of each improvement in cancer knowledge is ultimately an improvement in patient's care. While the scientific value of NGS-based advancements is undoubtedly critical, clinical benefits deriving from them are still being discussed.

As previously mentioned, NGS allows us to obtain the entire sequence of cancer's exome or even genome at a reasonable price; in medical genetics, for example, WES and WGS represent an important tool to diagnose genetic and inherited disorders. But not all this information might have a role in determining the best diagnostic and therapeutic approach for cancer patients, for which smaller targeted panels are more often used in clinical practice (Jennings et al. 2017).

When designing an NGS cancer-panel, it is critical to distinguish between driver alterations and incidental, irrelevant genetic variants. This complex process, called variants' prioritization, is essentially made possible by large publicly accessible databases like COSMIC (Catalogue of Somatic Mutations in Cancer), the UCSC Cancer Genomics Browser, or the cBioPortal. These resources have been designed as a translational bridge between researchers and clinicians, to lower the barriers of access, and made comprehensible the complex data sets provided by large-scale genome projects like the TCGA or the CGP. In this field, the development of the GENIE project is certainly another step forward. This multiphase, multiyear, international project converges on a regularly updated registry containing all the existing CLIA-/ISO-certified genomic data obtained during the course of routine practice at multiple international institutions. The information provided is certainly useful for variants' prioritization, but also available for powering clinical and translational research, validating biomarkers, expanding drug labels or identifying new drug targets.

Thanks to these efforts, many NGS-based cancer panels are currently available in clinical practice. One example is the 52-gene Oncomine Focus Assay, which includes most of the genes targeted by on-market oncology drugs and published evidence. The same company offers a wider panel (161 genes), and even larger panels have been recently validated, including the FDA-approved FoundationOne CDx (F1CDx), which detects mutations in 324 genes and 2 genomic signatures in any tumor type, and the also FDA-approved MSK-IMPACT, a 468-gene assay developed by Memorial Sloan Kettering Cancer Center (MSKCC).

The best panel size for clinical practice has fueled an intense debate, since for many of the identifiable driver alterations there is still no approved drug available, and performing large panels for every cancer patient is not yet affordable. However, the improvement in cancer knowledge provided by wide mutational panels performed on a large scale might encourage such effort. Particularly helpful in this sense may be the recently published ESMO Scale for Clinical Actionability of molecular Targets (ESCAT) (Mateo et al. 2018), which proposes a classification system for molecular aberrations, dividing them between six levels of actionability and clinical usefulness, based on the strength of evidence from clinical studies. Such a scale might help prioritize some alterations, in order to design "pragmatic" and affordable panels for everyday oncology practice. Besides the size of the panel, discussion is still open regarding the clinical benefit deriving from these molecular characterizations. Large prospective studies have been conducted and are still ongoing to address this question. The SHIVA trial (Le Tourneau et al. 2015), for instance, studied the off-label use of targeted therapies in patients with any cancer type harboring matching molecular alterations; the study failed to show any benefit over standard treatments, arguing against the indiscriminate use of off-label molecules according to uncharacterized molecular alterations whose significance as driver mutations is unknown. This failure must be interpreted considering the aim of the study itself: it was not powered to evaluate if a specific drug would have any antitumor activity in a selected subgroup of patients but was only able to evaluate the efficiency of the treatment algorithm used to allocate drugs on the basis of molecular profiling. It is not a failure for precision medicine, but a demonstration of inefficacy of that treatment algorithm in improving patients' outcome.

The MOSCATO Trial (Massard et al. 2017), instead, showed an interesting benefit in terms of PFS in a subgroup of patients with hard-to-treat advanced cancers where an actionable alteration was found and a targeted therapy was available. It must be noted that the MOSCATO was a not-randomized, less-powerful trial, where patients were taken as their own controls by using the "PFS ratio" as primary endpoint. This measure is assessed by comparing the PFS reached on the targeted, experimental treatment to the PFS achieved by the most recent therapy, retrospectively assessed.

New, promising results presented during the last 2018 ASCO Congress have recently relaunched the importance of Precision Medicine in cancer care. The IMPACT (Initiative for Molecular Profiling and Advanced Cancer Therapy) trial was launched more than 10 years ago to evaluate the impact of personalized therapy in patients with hard-to-treat cancers. Among 3743 patients tested, 1307 had at least one druggable genomic alteration and received a specific matched therapy. The

authors reported a median OS significantly longer in the matched-therapy group versus the nonmatched-therapy group (9.3 vs. 7.3 months), and a better median PFS (4 vs. 2.8 months). Interestingly, in the multivariate analysis the matched-targeted therapy was found to be an independent factor of longer OS, whereas mutations in the PI3K/AKT/motor pathway were an independent factor of shorter OS if compared to other alterations (NCT00851032 (Tsimberidou et al. 2018)).

A prospective validation of these results is expected by the IMPACT 2 trial, a randomized phase II study comparing the PFS achieved by patients receiving molecular-matched targeted therapy to PFS reached by patients treated with a molecular-unselected strategy (NCT02152254).

Beside this great potential, the implementation of Precision Medicine in the real-world of cancer care has several limitations. First of all, costs of NGS-based tests are still prohibitive and largely not reimbursed, representing a patient's effort as out-of-pocket expense. Costs of targeted gene panels vary widely, mainly depending on the numbers of genes sequenced. For example, a recently published nationwide French study reported a cost ranging between €376 and €968 (Marino et al. 2018), whereas the cost-effective analysis conducted on 10 studies by Tan et al. calculated an average cost of $1609 USD per sample (range: $488–$3443 USD) (Tan et al. 2018). The authors observed that cost of sequencing is generally lower if performed in-house compared to outsourcing to a service provider.

Many concerns have been raised about the impact of these costs in terms of clinical benefit. Even if evidences for cost-effectiveness are still lacking for many cancer types, in NSCLC an upfront mutational analysis based on NGS demonstrated to be less costly and faster than a single-gene test approach. Presented at ASCO 2018, this economic model showed a saving of 2 billion dollars for US Medicare reimbursement (Pennell et al. 2018).

Accessibility to tests and drugs is another obstacle that needs to be overcome. In recent years, many national projects have been launched to facilitate test accessibility. "France Medecine Genomiques 2025" (https://www.gouvernement.fr/sites/default/files/document/document/2016/06/22.06.2016_remise_du_rapport_dyves_levy_-_france_medecine_genomique_2025.pdf) and the "100k Genomes Project from UK" (https://www.genomicsengland.co.uk/the-100000-genomes-project/) are two such examples, born to transfer resources and results of genomic medicine from clinical trials to clinical care. On the other hand, even if a patient is found to harbor a druggable mutation, the accessibility to a specific target therapy is not guaranteed outside clinical trials. Targeted agents are approved by regulatory agency more often in histology-oriented settings, being the tumor-agnostic approval of pembrolizumab is still an exception for microsatellite-unstable tumors. To solve this question, predicting biomarkers, molecular tests, and targeted drugs should be ideally developed and approved in parallel. Innovative and clever study designs have emerged with this purpose: basket, umbrella, and adaptive enrichment are state-of-the-art approaches conceived for a personalization of treatment at its best.

During the last 2018 ASCO Congress, Otis Brawley, MD and ASCO chief medical officer said: "Precision medicine has given us some things, but it has promised a lot, which it has yet to deliver." Instead of interpreting this sentence as a criticism, we want to read it as a promise. The best is yet to come.

References

Antonarakis, E. S., Lu, C., Wang, H., Luber, B., Nakazawa, M., Roeser, J. C., et al. (2014). AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. *New England Journal of Medicine, 371*(11), 1028–1038.

Atkinson, A. J., Colburn, W. A., Degruttola, V. G., Demets, D. L., Downing, G. J., Hoth, D. F., et al. (2001). Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. *Clinical Pharmacology & Therapeutics, 69*(3), 89–95.

Barata, P., Sood, A. K., & Hong, D. S. (2016). RNA-targeted therapeutics in cancer clinical trials: Current status and future directions. *Cancer Treatment Reviews, 50*, 35–47.

Barski, A., Cuddapah, S., Cui, K., Roh, T. Y., Schones, D. E., Wang, Z., et al. (2007). High-resolution profiling of histone methylations in the human genome. *Cell, 129*(4), 823–837.

Chalmers, Z. R., Connelly, C. F., Fabrizio, D., Gay, L., Ali, S. M., Ennis, R., et al. (2017). Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. *Genome Medicine, 9*(1), 34.

Chandarlapaty, S., Chen, D., He, W., Sung, P., Samoila, A., You, D., et al. (2016). Prevalence of ESR1 mutations in cell-free DNA and outcomes in metastatic breast cancer: a secondary analysis of the BOLERO-2 clinical trial. *JAMA Oncology, 2*(10), 1310–1315.

Chu, D., Paoletti, C., Gersch, C., Van Den Berg, D. A., Zabransky, D. J., Cochran, R. L., et al. (2016). ESR1 mutations in circulating plasma tumor DNA from metastatic breast cancer patients. *Clinical Cancer Research, 22*(4), 993–999.

Cohen, J. D., Li, L., Wang, Y., Thoburn, C., Afsari, B., Danilova, L., et al. (2018). Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science, eaar3247*.

Cristofanilli, M., Turner, N. C., Bondarenko, I., Ro, J., Im, S. A., Masuda, N., et al. (2016). Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy (PALOMA-3): final analysis of the multicentre, double-blind, phase 3 randomised controlled trial. *The Lancet Oncology, 17*(4), 425–439.

De Mattos-Arruda, L., Weigelt, B., Cortes, J., Won, H. H., Ng, C. K. Y., Nuciforo, P., et al. (2014). Capturing intra-tumor genetic heterogeneity by de novo mutation profiling of circulating cell-free tumor DNA: A proof-of-principle. *Annals of Oncology, 25*(9), 1729–1735.

Ding, L., Wendl, M. C., McMichael, J. F., & Raphael, B. J. (2014). Expanding the computational toolbox for mining cancer genomes. *Nature Reviews Genetics, 15*(8), 556.

Douillard, J. Y., Ostoros, G., Cobo, M., Ciuleanu, T., Cole, R., McWalter, G., et al. (2014). Gefitinib treatment in EGFR mutated caucasian NSCLC: circulating-free tumor DNA as a surrogate for determination of EGFR status. *Journal of Thoracic Oncology, 9*(9), 1345–1353.

Fehm, T., Müller, V., Aktas, B., Janni, W., Schneeweiss, A., Stickeler, E., et al. (2010). HER2 status of circulating tumor cells in patients with metastatic breast cancer: A prospective, multicenter trial. *Breast Cancer Research and Treatment, 124*(2), 403–412.

Fischer, O. M., Streit, S., Hart, S., & Ullrich, A. (2003). Beyond herceptin and gleevec. *Current Opinion in Chemical Biology, 7*(4), 490–495.

Fizazi, K., Greco, F. A., Pavlidis, N., Daugaard, G., Oien, K., & Pentheroudakis, G. (2015). Cancers of unknown primary site: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology, 26*(suppl_5), v133–v138.

Fong, P. C., Boss, D. S., Yap, T. A., Tutt, A., Wu, P., Mergui-Roelvink, M., et al. (2009). Inhibition of poly (ADP-ribose) polymerase in tumors from BRCA mutation carriers. *New England Journal of Medicine, 361*(2), 123–134.

Foundation Medicine's New Liquid Biopsy Assay Granted Breakthrough Device Designation by U.S. Food and Drug Administration | Foundation Medicine, Inc. http://investors.foundationmedicine.com/news-releases/news-release-details/foundation-medicines-new-liquid-biopsy-assay-granted

FRANCE MÉDECINE GÉNOMIQUE. (2025). https://www.gouvernement.fr/sites/default/files/document/document/2016/06/22.06.2016_remise_du_rapport_dyves_levy_-_france_medecine_genomique_2025.pdf

Fribbens, C., O'Leary, B., Kilburn, L., Hrebien, S., Garcia-Murillas, I., Beaney, M., … & Jiang, J. (2016). Plasma ESR1 mutations and the treatment of estrogen receptor-positive advanced breast cancer.

Gandara, D. R., Kowanetz, M., Mok, T. S. K., Rittmeyer, A., Fehrenbacher, L., Fabrizio, D., et al. (2017). 1295OBlood-based biomarkers for cancer immunotherapy: Tumor mutational burden in blood (bTMB) is associated with improved atezolizumab (atezo) efficacy in 2L+ NSCLC (POPLAR and OAK). *Annals of Oncology, 28*(suppl_5).

Garcia-Murillas, I., Schiavon, G., Weigelt, B., Ng, C., Hrebien, S., Cutts, R. J., et al. (2015). Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. *Science Translational Medicine, 7*(302), 302ra133-302ra133.

Gillessen, S., Attard, G., Beer, T. M., Beltran, H., Bossi, A., Bristow, R., et al. (2018). Management of patients with advanced prostate cancer: The report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. *European Urology, 73*(2), 178–211.

Gomes, A. Q., Nolasco, S., & Soares, H. (2013). Non-coding RNAs: Multi-tasking molecules in the cell. *International Journal of Molecular Sciences, 14*(8), 16010–16039.

Gonzalez-Perez, A., Mustonen, V., Reva, B., Ritchie, G. R., Creixell, P., Karchin, R., et al. (2013). Computational approaches to identify functional genetic variants in cancer genomes. *Nature Methods, 10*(8), 723.

Green, E. D., Watson, J. D., & Collins, F. S. (2015). Human Genome Project: Twenty-five years of big biology. *Nature News, 526*(7571), 29.

Greenman, C., Stephens, P., Smith, R., Dalgliesh, G. L., Hunter, C., Bignell, G., et al. (2007). Patterns of somatic mutation in human cancer genomes. *Nature, 446*(7132), 153.

Gröbe, A., Blessmann, M., Hanken, H., Friedrich, R. E., Schön, G., Wikner, J., et al. (2014). Prognostic relevance of circulating tumor cells in blood and disseminated tumor cells in bone marrow of patients with squamous cell carcinoma of the oral cavity. *Clinical Cancer Research, 20*(2), 425–433.

Hainsworth, J. D., Rubin, M. S., Spigel, D. R., Boccia, R. V., Raby, S., Quinn, R., & Greco, F. A. (2012). Molecular gene expression profiling to predict the tissue of origin and direct site-specific therapy in patients with carcinoma of unknown primary site: a prospective trial of the Sarah Cannon research institute. *Journal of Clinical Oncology, 31*(2), 217–223.

Hamilton, E. P., Patel, M. R., Armstrong, A. C., Baird, R. D., Jhaveri, K., Hoch, M., et al. (2018). A first in human study of the new oral selective estrogen receptor degrader AZD9496 for HR+/HER2− advanced breast cancer. *Clinical Cancer Research, clincanres-3102*.

Heitzer, E., Auer, M., Gasch, C., Pichler, M., Ulz, P., Hoffmann, E. M., et al. (2013). Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing. *Cancer Research*.

Hellmann, M. D., Callahan, M. K., Awad, M. M., Calvo, E., Ascierto, P. A., Atmaca, A., et al. (2018a). Tumor mutational burden and efficacy of nivolumab monotherapy and in combination with ipilimumab in small-cell lung cancer. *Cancer Cell, 33*(5), 853–861.

Hellmann, M. D., Ciuleanu, T. E., Pluzanski, A., Lee, J. S., Otterson, G. A., Audigier-Valette, C., et al. (2018b). Nivolumab plus ipilimumab in lung cancer with a high tumor mutational burden. *New England Journal of Medicine*.

Hustedt, N., & Durocher, D. (2017). The control of DNA repair by the cell cycle. *Nature Cell Biology, 19*(1), 1.

Ignatiadis, M., & Dawson, S. J. (2014). Circulating tumor cells and circulating tumor DNA for precision medicine: Dream or reality? *Annals of Oncology, 25*(12), 2304–2313.

Jasin, M., & Rothstein, R. (2013). Repair of strand breaks by homologous recombination. *Cold Spring Harbor Perspectives in Biology, a012740*.

Jennings, L. J., Arcila, M. E., Corless, C., Kamel-Reid, S., Lubin, I. M., Pfeifer, J., et al. (2017). Guidelines for validation of next-generation sequencing–based oncology panels: A joint consensus recommendation of the Association for Molecular Pathology and College of American Pathologists. *The Journal of Molecular Diagnostics, 19*(3), 341–365.

Jeselsohn, R., Yelensky, R., Buchwalter, G., Frampton, G., Meric-Bernstam, F., Gonzalez-Angulo, A. M., et al. (2014). Emergence of constitutively active estrogen receptor-α mutations in pretreated advanced estrogen receptor–positive breast cancer. *Clinical Cancer Research, 20*(7), 1757–1767.

Johnson, D. B., Frampton, G. M., Rioth, M. J., Yusko, E., Xu, Y., Guo, X., et al. (2016). Targeted next generation sequencing identifies markers of response to PD-1 blockade. *Cancer Immunology Research*.

Johnson, J. R., Bross, P., Cohen, M., Rothmann, M., Chen, G., Zajicek, A., et al. (2003). Approval summary: Imatinib mesylate capsules for treatment of adult patients with newly diagnosed Philadelphia chromosome-positive chronic myelogenous leukemia in chronic phase. *Clinical Cancer Research, 9*(6), 1972–1979.

Johnston, S. R., Kilburn, L. S., Ellis, P., Dodwell, D., Cameron, D., Hayward, L., et al. (2013). Fulvestrant plus anastrozole or placebo versus exemestane alone after progression on non-steroidal aromatase inhibitors in postmenopausal patients with hormone-receptor-positive locally advanced or metastatic breast cancer (SoFEA): a composite, multicentre, phase 3 randomised trial. *The Lancet Oncology, 14*(10), 989–998.

Kinzler, K. W., & Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. *Cell, 87*(2), 159–170.

Koboldt, D. C., Fulton, R. S., McLellan, M. D., Schmidt, H., Kalicki-Veizer, J., McMichael, J. F., et al. (2012). Comprehensive molecular portraits of human breast tumours. *Nature, 490*(7418), 61–70.

Kuang, Y., Rogers, A., Yeap, B. Y., Wang, L., Makrigiorgos, M., Vetrand, K., et al. (2009). Noninvasive detection of EGFR T790M in gefitinib or erlotinib resistant non–small cell lung cancer. *Clinical Cancer Research, 15*(8), 2630–2636.

Landau, D. A., Carter, S. L., Stojanov, P., McKenna, A., Stevenson, K., Lawrence, M. S., et al. (2013). Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell, 152*(4), 714–726.

Le, D. T., Durham, J. N., Smith, K. N., Wang, H., Bartlett, B. R., Aulakh, L. K., et al. (2017). Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science, 357*(6349), 409–413.

Le Tourneau, C., Delord, J. P., Gonçalves, A., Gavoille, C., Dubot, C., Isambert, N., et al. (2015). Molecularly targeted therapy based on tumour molecular profiling versus conventional therapy for advanced cancer (SHIVA): A multicentre, open-label, proof-of-concept, randomised, controlled phase 2 trial. *The Lancet Oncology, 16*(13), 1324–1334.

Lebofsky, R., Decraene, C., Bernard, V., Kamal, M., Blin, A., Leroy, Q., et al. (2015). Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. *Molecular Oncology, 9*(4), 783–790.

Lister, R., O'Malley, R. C., Tonti-Filippini, J., Gregory, B. D., Berry, C. C., Millar, A. H., & Ecker, J. R. (2008). Highly integrated single-base resolution maps of the epigenome in Arabidopsis. *Cell, 133*(3), 523–536.

Lohr, J. G., Adalsteinsson, V. A., Cibulskis, K., Choudhury, A. D., Rosenberg, M., Cruz-Gordillo, P., et al. (2014). Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. *Nature Biotechnology, 32*(5), 479.

Lowes, L. E., Bratman, S. V., Dittamore, R., Done, S., Kelley, S. O., Mai, S., et al. (2016). Circulating tumor cells (CTC) and cell-free DNA (cfDNA) workshop 2016: Scientific opportunities and logistics for cancer clinical trial incorporation. *International Journal of Molecular Sciences, 17*(9), 1505.

Lynch, H. T., Smyrk, T. C., Watson, P., Lanspa, S. J., Lynch, J. F., Lynch, P. M., et al. (1993). Genetics, natural history, tumor spectrum, and pathology of hereditary nonpolyposis colorectal cancer: an updated review. *Gastroenterology, 104*(5), 1535–1549.

Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., et al. (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature, 437*(7057), 376.

Marino, P., Touzani, R., Perrier, L., Rouleau, E., Kossi, D. S., Zhaomin, Z., et al. (2018). Cost of cancer diagnosis using next-generation sequencing targeted gene panels in routine practice: a nationwide French study. *European Journal of Human Genetics, 26*(3), 314.

Mariotto, A. B., Etzioni, R., Hurlbert, M., Penberthy, L., & Mayer, M. (2017). Estimation of the number of women living with metastatic breast cancer in the United States. *Cancer Epidemiology and Prevention Biomarkers*.

Markowski, M. C., Silberstein, J. L., Eshleman, J. R., Eisenberger, M. A., Luo, J., & Antonarakis, E. S. (2017). Clinical utility of CLIA-grade AR-V7 testing in patients with metastatic castration-resistant prostate cancer. *JCO Precision Oncology, 1*, 1–9.

Massard, C., Loriot, Y., & Fizazi, K. (2011). Carcinomas of an unknown primary origin—Diagnosis and treatment. *Nature Reviews Clinical Oncology, 8*(12), 701.

Massard, C., Michiels, S., Ferté, C., Le Deley, M. C., Lacroix, L., Hollebecque, A., et al. (2017). High-throughput genomics and clinical outcome in hard-to-treat advanced cancers: Results of the MOSCATO 01 trial. *Cancer Discovery*.

Mastoraki, S., Strati, A., Tzanikou, E., Chimonidou, M., Politaki, E., Voutsina, A., et al. (2018). ESR1 methylation: A liquid biopsy–based epigenetic assay for the follow-up of patients with metastatic breast cancer receiving endocrine treatment. *Clinical Cancer Research, 24*(6), 1500–1510.

Mateo, J., Chakravarty, D., Dienstmann, R., Jezdic, S., Gonzalez-Perez, A., Lopez-Bigas, N., et al. (2018). A framework to rank genomic alterations as targets for cancer precision medicine: the ESMO Scale for Clinical Actionability of molecular Targets (ESCAT). *Annals of Oncology, 29*(9), 1895–1902.

Merenbakh-Lamin, K., Ben-Baruch, N., Yeheskel, A., Dvir, A., Soussan-Gutman, L., Jeselsohn, R., et al. (2013). D538G mutation in estrogen receptor-α: A novel mechanism for acquired endocrine resistance in breast cancer. *Cancer Research*.

Misale, S., Yaeger, R., Hobor, S., Scala, E., Janakiraman, M., Liska, D., et al. (2012). Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature, 486*(7404), 532.

Mok, T. S., Wu, Y. L., Ahn, M. J., Garassino, M. C., Kim, H. R., Ramalingam, S. S., et al. (2017). Osimertinib or platinum–pemetrexed in EGFR T790M–positive lung cancer. *New England Journal of Medicine, 376*(7), 629–640.

Mouliere, F., El Messaoudi, S., Gongora, C., Guedj, A. S., Robert, B., Del Rio, M., et al. (2013). Circulating cell-free DNA from colorectal cancer patients may reveal high KRAS or BRAF mutation load. *Translational Oncology, 6*(3), 319–328.

Moynahan, M. E., Chiu, J. W., Koller, B. H., & Jasin, M. (1999). Brca1 controls homology-directed DNA repair. *Molecular Cell, 4*(4), 511–518.

Moynahan, M. E., Pierce, A. J., & Jasin, M. (2001). BRCA2 is required for homology-directed repair of chromosomal breaks. *Molecular Cell, 7*(2), 263–272.

Nakamura, T., Sueoka-Aragane, N., Iwanaga, K., Sato, A., Komiya, K., Kobayashi, N., et al. (2012). Application of a highly sensitive detection system for epidermal growth factor receptor mutations in plasma DNA. *Journal of Thoracic Oncology, 7*(9), 1369–1381.

Nastaly, P., Ruf, C. G., Becker, P., Bednarz-Knoll, N., Stoupiec, M., Kavsur, R., et al. (2014). Circulating tumor cells in patients with testicular germ cell tumors. *Clinical Cancer Research*, clincanres-2819.

National Comprehensive Cancer Network. Non-small cell lung cancer (Version 6.2018). https://www.nccn.org/professionals/physician_gls/pdf/nscl.pdf

Nowell, P. C. (1976). The clonal evolution of tumor cell populations. *Science, 194*(4260), 23–28.

O'Kane, G. M., Connor, A. A., & Gallinger, S. (2017). Characterization, detection, and treatment approaches for homologous recombination deficiency in cancer. *Trends in Molecular Medicine*.

Olsson, E., Winter, C., George, A., Chen, Y., Howlin, J., Tang, M. H. E., et al. (2015). Serial monitoring of circulating tumor DNA in patients with primary breast cancer for detection of occult metastatic disease. *EMBO Molecular Medicine, 7*(8), 1034–1047.

Oxnard, G. R., Paweletz, C. P., Kuang, Y., Mach, S. L., O'Connell, A., Messineo, M. M., et al. (2014). Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. *Clinical Cancer Research*.

Pantel, K., & Alix-Panabières, C. (2013). Real-time liquid biopsy in cancer patients: Fact or fiction? *Cancer Research*.

Paoletti, C., Muniz, M. C., Thomas, D. G., Griffith, K. A., Kidwell, K. M., Tokudome, N., et al. (2015). Development of circulating tumor cell-endocrine therapy index in patients with hormone receptor–positive breast cancer. *Clinical Cancer Research, 21*(11), 2487–2498.

Pennell, N. A., Mutebi, A., Zhou, Z. Y., Ricculli, M. L., Tang, W., Wang, H., … & Otterson, G. A. (2018). Economic impact of next generation sequencing vs sequential single-gene testing modalities to detect genomic alterations in metastatic non-small cell lung cancer using a decision analytic model.

Perakis, S., & Speicher, M. R. (2017). Emerging concepts in liquid biopsies. *BMC Medicine, 15*(1), 75.

Rack, B., Schindlbeck, C., Jückstock, J., Andergassen, U., Hepp, P., Zwingers, T., et al. (2014). Circulating tumor cells predict survival in early average-to-high risk breast cancer patients. *JNCI: Journal of the National Cancer Institute, 106*(5).

Raphael, B. J., Dobson, J. R., Oesper, L., & Vandin, F. (2014). Identifying driver mutations in sequenced cancer genomes: Computational approaches to enable precision medicine. *Genome Medicine, 6*(1), 5.

Reck, M., Hagiwara, K., Han, B., Tjulandin, S., Grohé, C., Yokoi, T., et al. (2016). ctDNA determination of EGFR mutation status in European and Japanese patients with advanced NSCLC: The ASSESS study. *Journal of Thoracic Oncology, 11*(10), 1682–1689.

Reinert, T., Schøler, L. V., Thomsen, R., Tobiasen, H., Vang, S., Nordentoft, I., et al. (2016). Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. *Gut, 65*(4), 625–634.

Rink, M., Chun, F. K., Dahlem, R., Soave, A., Minner, S., Hansen, J., et al. (2012). Prognostic role and HER2 expression of circulating tumor cells in peripheral blood of patients prior to radical cystectomy: A prospective study. *European Urology, 61*(4), 810–817.

Rizvi, N. A., Hellmann, M. D., Snyder, A., Kvistborg, P., Makarov, V., Havel, J. J., et al. (2015). Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. *Science, 348*(6230), 124–128.

Robinson, D. R., Wu, Y. M., Vats, P., Su, F., Lonigro, R. J., Cao, X., et al. (2013). Activating ESR1 mutations in hormone-resistant metastatic breast cancer. *Nature Genetics, 45*(12), 1446.

Robson, M., Im, S. A., Senkus, E., Xu, B., Domchek, S. M., Masuda, N., et al. (2017). Olaparib for metastatic breast cancer in patients with a germline BRCA mutation. *New England Journal of Medicine, 377*(6), 523–533.

Rosenberg, J. E., Hoffman-Censits, J., Powles, T., Van Der Heijden, M. S., Balar, A. V., Necchi, A., et al. (2016). Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. *The Lancet, 387*(10031), 1909–1920.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the national academy of sciences, 74*(12), 5463–5467.

Scher, H. I., Lu, D., Schreiber, N. A., Louw, J., Graf, R. P., Vargas, H. A., et al. (2016). Association of AR-V7 on circulating tumor cells as a treatment-specific biomarker with outcomes and survival in castration-resistant prostate cancer. *JAMA Oncology, 2*(11), 1441–1449.

Schiavon, G., Hrebien, S., Garcia-Murillas, I., Cutts, R. J., Pearson, A., Tarazona, N., et al. (2015). Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. *Science Translational Medicine, 7*(313), 313ra182-313ra182.

Schulze, K., Gasch, C., Staufer, K., Nashan, B., Lohse, A. W., Pantel, K., et al. (2013). Presence of EpCAM-positive circulating tumor cells as biomarker for systemic disease strongly correlates to survival in patients with hepatocellular carcinoma. *International Journal of Cancer, 133*(9), 2165–2171.

Shin, S. H., Bode, A. M., & Dong, Z. (2017). Precision medicine: the foundation of future cancer therapeutics. *NPJ Precision Oncology, 1*(1), 12.

Siravegna, G., Mussolin, B., Buscarino, M., Corti, G., Cassingena, A., Crisafulli, G., et al. (2015). Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. *Nature Medicine, 21*(7), 795.

Snyder, A., Makarov, V., Merghoub, T., Yuan, J., Zaretsky, J. M., Desrichard, A., et al. (2014). Genetic basis for clinical response to CTLA-4 blockade in melanoma. *New England Journal of Medicine, 371*(23), 2189–2199.

Swanton, C., Soria, J. C., Bardelli, A., Biankin, A., Caldas, C., Chandarlapaty, S., et al. (2016). Consensus on precision medicine for metastatic cancers: a report from the MAP conference. *Annals of Oncology, 27*(8), 1443–1448.

Swisher, E. M., Lin, K. K., Oza, A. M., Scott, C. L., Giordano, H., Sun, J., et al. (2017). Rucaparib in relapsed, platinum-sensitive high-grade ovarian carcinoma (ARIEL2 Part 1): an international, multicentre, open-label, phase 2 trial. *The Lancet Oncology, 18*(1), 75–87.

Szostak, J. W., Orr-Weaver, T. L., Rothstein, R. J., & Stahl, F. W. (1983). The double-strand-break repair model for recombination. *Cell, 33*(1), 25–35.

Tan, O., Shrestha, R., Cunich, M., & Schofield, D. J. (2018). Application of next-generation sequencing to improve cancer management: A review of the clinical effectiveness and cost-effectiveness. *Clinical Genetics, 93*(3), 533–544.

Taniguchi, K., Uchida, J., Nishino, K., Kumagai, T., Okuyama, T., Okami, J., et al. (2011). Quantitative detection of EGFR mutations in circulating tumor DNA derived from lung adenocarcinomas. *Clinical Cancer Research*.

The 100,000 Genomes Project | Genomics England. https://www.genomicsengland.co.uk/the-100000-genomes-project/

Thibodeau, S. N., Bren, G., & Schaid, D. (1993). Microsatellite instability in cancer of the proximal colon. *Science, 260*(5109), 816–819.

TracerX | Tracking Cancer Evolution through study. http://tracerx.co.uk/

Tsimberidou, A. M., Hong, D. S., Wheler, J. J., Falchook, G. S., Naing, A., Fu, S., … & Nogueras-Gonzalez, G. M. (2018). Precision medicine: Clinical outcomes including long-term survival according to the pathway targeted and treatment period–The IMPACT study.

Turner, N. C., Jiang, Y., O'Leary, B., Hrebien, S., Cristofanilli, M., Andre, F., … & Bartlett, C. H. (2016). Efficacy of palbociclib plus fulvestrant (P+ F) in patients (pts) with metastatic breast cancer (MBC) and ESR1 mutations (mus) in circulating tumor DNA (ctDNA).

Vanderwalde, A., Spetzler, D., Xiao, N., Gatalica, Z., & Marshall, J. (2018). Microsatellite instability status determined by next-generation sequencing and compared with PD-L1 and tumor mutational burden in 11,348 patients. *Cancer Medicine, 7*(3), 746–756.

Varghese, A. M., Arora, A., Capanu, M., Camacho, N., Won, H. H., Zehir, A., et al. (2017). Clinical and molecular characterization of patients with cancer of unknown primary in the modern era. *Annals of Oncology, 28*(12), 3015–3021.

Velcheti, V., Kim, E. S., Mekhail, T., Dakhil, C., Stella, P. J., Shen, X., … & Phan, S. C. (2018). Prospective clinical evaluation of blood-based tumor mutational burden (bTMB) as a predictive biomarker for atezolizumab (atezo) in 1L non-small cell lung cancer (NSCLC): Interim B-F1RST results.

Vilar, E., & Gruber, S. B. (2010). Microsatellite instability in colorectal cancer—the stable evidence. *Nature Reviews Clinical Oncology, 7*(3), 153.

Vogelstein, B., Papadopoulos, N., Velculescu, V. E., Zhou, S., Diaz, L. A., & Kinzler, K. W. (2013). Cancer genome landscapes. *Science, 339*(6127), 1546–1558.

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics, 10*(1), 57.

Weis, K. E., Ekena, K., Thomas, J. A., Lazennec, G., & Katzenellenbogen, B. S. (1996). Constitutively active human estrogen receptors containing amino acid substitutions for tyrosine 537 in the receptor protein. *Molecular Endocrinology, 10*(11), 1388–1398.

Morris, J. R. (2001). Genes, genetics, and epigenetics: a correspondence. *Science, 293*(5532), 1103–1105.

Yang, M., Forbes, M. E., Bitting, R. L., O'neill, S. S., Chou, P. C., Topaloglu, U., et al. (2017). Incorporating blood-based liquid biopsy information into cancer staging: time for a TNMB system? *Annals of Oncology, 29*(2), 311–323.

Yap, T. A., Gerlinger, M., Futreal, P. A., Pusztai, L., & Swanton, C. (2012). Intratumor heterogeneity: seeing the wood for the trees. *Science Translational Medicine, 4*(127), 127ps10-127ps10.

Yokobori, T., Iinuma, H., Shimamura, T., Imoto, S., Sugimachi, K., Ishii, H., et al. (2013). Plastin3 is a novel marker for circulating tumor cells undergoing the epithelial-mesenchymal transition and is associated with colorectal cancer prognosis. *Cancer Research*, canres-0326.