

## Cancer Reviews 2021 series

## Cancer evolution: Darwin and beyond

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## Abstract

Clinical and laboratory studies over recent decades have established branched evolution as a feature of cancer. However, while grounded in somatic selection, several lines of evidence suggest a Darwinian model alone is insufficient to fully explain cancer evolution. First, the role of macroevolutionary events in tumour initiation and progression contradicts Darwin's central thesis of gradualism. Whole-genome doubling, chromosomal chromoplexy and chromothripsis represent examples of single catastrophic events which can drive tumour evolution. Second, neutral evolution can play a role in some tumours, indicating that selection is not always driving evolution. Third, increasing appreciation of the role of the ageing soma has led to recent generalised theories of age-dependent carcinogenesis. Here, we review these concepts and others, which collectively argue for a model of cancer evolution which extends beyond Darwin. We also highlight clinical opportunities which can be grasped through targeting cancer vulnerabilities arising from non-Darwinian patterns of evolution.

**Keywords** cancer; cancer evolution; cancer therapy; tumour heterogeneity

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## Introduction

In his revolutionary work (Darwin, 1859), Darwin provided an evolutionary framework which enabled the understanding of somatic selection, diversification and extinction through the application of three key concepts: variation, heredity and selection. More than a 100 years later, the observation of heterogeneity in advanced malignancies led Peter Nowell to hypothesise that tumorigenesis is also an evolutionary process, whereby the same Darwinian principles could be applied to elucidate the mechanisms responsible for cancer formation and development (Nowell, 1976). Owing to Nowell's seminal work, a Darwinian framework has been historically adopted to develop models of tumour evolution and therapy resistance (Michor *et al*, 2004; Gatenby & Vincent, 2008; Pepper *et al*, 2009; Greaves & Maley, 2012) (see Box 1). While gene-centric Darwinian principles have been shown to explain tumour

evolutionary trajectories in multiple instances (Gerlinger & Swanton, 2010; Purushotham & Sullivan, 2010; Gillies *et al*, 2012), recent studies have suggested additional evolutionary concepts beyond Darwin's are required to reconcile the full spectrum of evolutionary behaviours in cancer. Specifically, increasing evidence now supports macroevolutionary jumps as a feature of cancer (Stephens *et al*, 2011; Baca *et al*, 2013; Sottoriva *et al*, 2015), which are likely interspaced by phases of microevolutionary gradualism. Furthermore, evidence of discordant inheritance patterns between cells (Decarvalho *et al*, 2018), and the role of neutral evolution (Ling *et al*, 2015; Williams *et al*, 2016; Wu *et al*, 2016), cell plasticity (Pogrebniak & Curtis, 2018; Mills *et al*, 2019; Boumahdi & de Sauvage, 2020) and the tumour microenvironment (Coussens & Werb, 2002; Lin & Karin, 2007; Laconi *et al*, 2020) in cancer demand consideration of a broader set of evolutionary models. Understanding how tumour evolution influences disease progression and how such processes are shaped by environmental factors and treatment remains critical.

With this review, we discuss our understanding of tumour evolution as a process grounded in Darwinian selection but argue that in light of recent data, we must now incorporate these concepts into a larger conceptual framework inclusive of alternative approaches to fully understand, predict and better respond to cancer evolution and to improve patient outcome.

## Darwinian selection as the basis of subclonal diversity

Cancer has been historically viewed from a Darwinian gene-centric perspective (Greaves & Maley, 2012). Indeed, tumours are frequently typified as a large population of genetically diverse cells giving rise to distinct subpopulations. Subclones will compete with one another for a limited set of nutrients and metabolites and face ever-shifting selective pressures driven by both endogenous (*i.e.* microenvironmental pressures and geographical barriers) and exogenous (*i.e.* therapy) factors (Merlo *et al*, 2006). The outcome of this competition is the survival of clones adapted to grow under very specific conditions, as Darwinian selection is highly contextual and blind to the future. Many clones that were dominant at one point in time may reach evolutionary dead ends and disappear, while only a minority may be able to persist. Quoting Darwin "One general law, leading to the advancement of all organic beings, namely, multiply, vary, let the strongest live and the weakest die" (Darwin, 1859).

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### Box 1. An historical perspective of tumour evolution and intratumour heterogeneity

One hundred years have passed since Theodor Boveri set the foundations for much of our understanding of the origins of cancer. He not only hypothesised that cancers arise from normal cells as a consequence of genetic alterations, but also postulated that most tumours and their metastases originate from one cell, a concept that shaped today's understanding of clonal expansion (Boveri, 2008). The application of evolutionary concepts to understand cancer formation and development can be attributed to Peter Nowell, who pioneered the hypothesis of tumour evolution. Nowell's model stated that most cancers originate from a single neoplastic cell and evolve through a process of selection for somatic alterations, leading to the proliferation and survival of the most aggressive clones (Nowell, 1976). Despite this conceptual advance, Nowell's view was largely overlooked, and tumour evolution was traditionally viewed as a linear succession of clonal cell divisions. Under the linear model, alterations accrue in progenitor cells in a stepwise fashion and endow cells with a strong selective advantage, hence enabling previous clones to be outcompeted (Fig 1). As a result, tumours would be composed of clonally identical cells resulting from continuous selective sweeps (Davis *et al*, 2017). Gloria Heppner challenged this view by demonstrating that tumours are comprised of genetically different subclones exhibiting fundamentally distinct behaviours (Dexter *et al*, 1978). By applying concepts of population genetics, she described tumours as "societies highly adapted for survival" and recognised that tumours "survive natural and artificial (therapeutic) selection through heterogeneity by producing new variants to "outflank" it." (Heppner, 1984). These observations supported a model whereby tumours grow in a non-linear, branched fashion, with multiple subclones derived from a common ancestor eventually diverging and expanding simultaneously with differing fitness (Greaves & Maley, 2012; Swanton, 2012) (Fig 1).

A consequence of branched tumour evolution is intratumour heterogeneity (ITH)—that is the coexistence of molecularly and phenotypically distinct subclones within a tumour. Tumour morphological heterogeneity has been long recognised by pathologists such as Johannes Muller and Rudolf Virchow in their pioneering studies in the 19<sup>th</sup> century as well as Theodor Boveri (Muller, 1973; Boveri, 2008; Parquet, 2014; Wright, 2014). Those initial discoveries were substantiated by several groups with orthogonal techniques, which provided evidence for intratumour diversity across multiple cancer types (Shapiro *et al*, 1981; Teixeira *et al*, 1996; Takahashi *et al*, 1998; Maley *et al*, 2006; Cottu *et al*, 2008). However, it was only with the recent technological innovations (see Box 2), particularly next-generation sequencing (NGS), that the full breath of ITH could start to be fully resolved (Campbell *et al*, 2010; Navin *et al*, 2010; Gerlinger *et al*, 2012; Nik-Zainal *et al*, 2012b; Walter *et al*, 2012; Patel *et al*, 2014; Sottoriva *et al*, 2015; Jamal-Hanjani *et al*, 2017; Naxerova *et al*, 2017; Turajlic *et al*, 2018; Fittall & Van Loo, 2019; Rambow *et al*, 2019; Reiter *et al*, 2019; Gerstung *et al*, 2020; Machnik & Oleksiewicz, 2020; Marine *et al*, 2020).

In the one to two decades, direct evidence to support Darwinian evolution in cancer has been reported, principally from studies using next-generation sequencing (NGS) to perform detailed characterisation of genetic tumour evolution (see Box 2). One of the earliest studies was from Shah *et al* (2009), where matched primary and metastatic tissue from a lobular breast tumour were sequenced revealing extensive mutational heterogeneity with ~80% of the non-synonymous mutations in the metastasis absent from the primary site (Shah *et al*, 2009). The finding of pervasive heterogeneity in breast cancer has additionally been reported by the extensive

work of Kornelia Polyak, which demonstrated that breast tumours were composed of a variety of cell types with distinct morphologies and behaviours, with the source of heterogeneity arising in part from clonal evolution (Campbell & Polyak, 2007). Early evidence of abundant, genetically diverse subpopulations of cells was also revealed through single-cell sequencing (see Box 2) studies in breast cancer by Nick Navin and others (Navin *et al*, 2011). Regarding haematological malignancies, Anderson *et al*. were among the first to show branching evolutionary trajectories in acute lymphoblastic leukaemia (Anderson *et al*, 2011). Our own work from Gerlinger *et al* (2012) profiled 30 tumour samples from four renal cell carcinoma patients and revealed that 63 to 69% of all somatic mutations were not detectable across every tumour region (Gerlinger *et al*, 2012). These observations demonstrated the extent and relevance of branched evolution. Furthermore, evidence of parallel evolution was demonstrated for multiple tumour suppressor genes (*SETD2*, *PTEN*, *KDM5C*), suggesting selective pressures drive inactivation of the same gene multiple times within a single tumour. This report was followed by work from Nik-Zainal *et al* (2012b), who studied the life history of 21 breast tumours, identifying extensive genetic variation within individual breast tumours (Nik-Zainal *et al*, 2012b). This study also showed further evidence of selection, with each tumour containing a dominant subclonal lineage, representing more than 50% of tumour cells. Extending into further detail on the metastatic process, work from Gundem *et al* (2015) utilised autopsy sampling in 10 prostate cancer patients to identify metastasis to metastasis seeding as a common event (Gundem *et al*, 2015). This study emphasised not only the extent of subclonal diversification, but also the complexity of seeding routes to and between metastatic sites. However, these early studies were limited by small sample sizes. Furthermore, the diverse range of cancer types studied meant the nature of specific evolutionary patterns, as generalisable across all tumour types or histology specific, remained undetermined. Despite the limitations, these early NGS studies gave the first direct evidence of extensive genetic subclonal diversification, hence supporting a model of cancer growth as a branched evolutionary process (Fig 1). Furthermore, the demonstration of branched evolution as a feature of solid tumour growth spurred a change in thinking across the community to recognise the importance of Darwinian selection in cancer. Branched evolution has also been shown to be applicable to relatively homogeneous primary tumours and/or metastases, whereby particularly aggressive subclones that may achieve a clonal sweep and present clinically with a homogeneous profile (Reiter *et al*, 2018) (Fig 1). Clear examples of this are described in pancreatic cancer, where virtually all major driver gene alterations (*KRAS*, *CDKN2A*, *TP53*, *SMAD4*) are present in the most recent common ancestor and limited evidence of mutational heterogeneity is observed across metastases (Makohon-Moore *et al*, 2017). Similar examples are observed in some aggressive renal cell carcinomas, where ~10–20% of tumours exhibit multiple clonal driver mutations, limited heterogeneity and poor clinical outcome (Turajlic *et al*, 2018). It is proposed the variation in heterogeneity between tumours may reflect differences in the inherent biology of a given tumour and impact upon the process of metastatic dissemination and clinical outcome (Iacobuzio-Donahue *et al*, 2020).

However, models of accumulating genetic changes subject to selective pressure are not fully sufficient to explain the full spectrum of cancer evolutionary histories, and increasing evidence points to

## Box 2. Sequencing technologies to assess tumour evolution

The advent of next-generation sequencing (NGS) has revolutionised biology. Its high-throughput, scalability, speed, and cost efficiency enabled researchers to study the genomic and transcriptomic profiles of many human diseases, including cancer, with unprecedented resolution. Studies of cancer evolutionary dynamics and ITH have particularly benefited from these technological advances.

### Bulk sequencing

To date, most studies of tumour evolutionary dynamics have relied on the sequencing of bulk tumour samples. While this methodology can only provide an indirect measurement of the subclonal composition of a tumour biopsy, tumour phylogenetic architecture can still be inferred through computational approaches. By sequencing samples at sufficiently high coverage depth, variant allele frequencies (VAFs) can be quantified. VAFs can then be further corrected for tumour purity and local copy number state to derive cancer cell fraction (CCF) values, which intuitively measure what proportion of cancer cells bear a given mutation. CCFs can then be clustered using a Dirichlet process to identify clonal and subclonal populations—based on the assumption that mutations with similar CCFs fit together to form genetically distinct populations. Once the subclonal architecture of a given tumour is defined (*i.e.* the complete set of detected clones/subclones), phylogenetic techniques can be used to infer the most likely parent to child ordering relationships. This enables phylogenetic tree construction, with each tumour (sub)clone being placed on its relevant branch. Phylogenetic tree construction must adhere to basic principles, such as the pigeonhole principle, which stipulates that if there are  $m$  containers (pigeonholes) and  $n$  items (pigeons) to go within the containers and if  $n > m$ , then there must be a container with more than one item. This principle ensures that the CCF sum of child subclones cannot exceed that of its parental ancestor. Computationally, these methods are implemented within a wide number of tools, and consensus efforts are seeking to drive greater standardisation in methodologies (Schwartz & Schäffer, 2017). It should be noted that key information needed for reconstructing tumour evolution may however be lost in the process, due to confounding effects derived from dealing with mixed cell populations. For instance, alterations only present in low frequency small subclones will likely be confused as noise, posing a significant limitation for tree reconstitution (Cibulskis *et al.*, 2013). Additionally, deconvolution of bulk sequencing data is needed to infer evolutionary dynamics, an approach that may limit the identification of branching events (Malikic *et al.*, 2019).

### Single-cell approaches

The recent development of single-cell approaches (SCA) has allowed the analysis of the genomes, transcriptomes, epigenomes, proteomes and even metabolomes of individual cancer cells, as well as the TME, with unprecedented resolution (Navin *et al.*, 2011; Hiley *et al.*, 2014; Wang *et al.*, 2014; Navin, 2015). The most widely used SCA has been single-cell RNA sequencing (scRNA-seq), which has recently emerged as a valuable tool to study tumour evolutionary dynamics (Nam *et al.*, 2021). Owing to its high resolution, scRNA-seq permits detection of genes expressed even in small subclones which would be missed by bulk RNA-seq, allowing, for instance, the identification of minor treatment-resistant cell populations contributing to therapy failure (Rambow *et al.*, 2018). Additionally, copy number variation and loss of heterozygosity in individual cells can sometimes be inferred through scRNA-seq, although in most cases the resolution is not sufficient for novel SCNA discovery (Fan *et al.*, 2018). Moreover, by obtaining transcriptomic data from hundreds of individual cells in a range of different evolutionary stages, cells can be ordered in pseudotime and within evolutionary trajectories. This allows the inference of tumour evolutionary history and dynamics, albeit without providing information regarding the direction of such trajectories. To overcome this limitation, RNA velocity analyses can be used. By measuring the relative ratio between intronic and exonic reads, the rate of change in transcript abundance can be inferred, thus providing an estimate of the future transcriptional state of a cell alongside with a better understanding of the cellular transcriptional dynamics (La Manno *et al.*, 2018). Another SCA approach that has emerged in the last decade is single-cell DNA sequencing (scDNA-seq), which can provide genomic profiles of individual cells, thereby allowing the inference of genetic phylogeny without any form of deconvolution. Despite its promises, scDNA-seq still has major limitations. First, the coverage obtained with such approaches tends to be low because of the limited amount of DNA in a single cell, which typically contains a few picograms of genomic DNA, whereas NGS requires nanogram amounts of starting DNA for library preparation. Therefore, a critical step for single-cell sequencing is whole-genome amplification to generate sufficient DNA for library construction, a process which is rather error-prone (Chen *et al.*, 2018). Other frequent issues are an increase rate of false negatives due to allelic dropout and additional noise stemming from accidentally sequencing doublets (Pugh *et al.*, 2008; Lasken, 2013; Navin, 2014; Van Loo & Voet, 2014; Simonsen *et al.*, 2018; Mallory *et al.*, 2020). Interestingly, the weaknesses of scDNA-seq can be complemented by bulk DNA sequencing and vice versa and the use of both data types for inferring phylogeny of tumours has been shown to provide more accurate results and increased resolution in capturing ITH complexity and evolutionary dynamics (Salehi *et al.*, 2017; Malikic *et al.*, 2019).

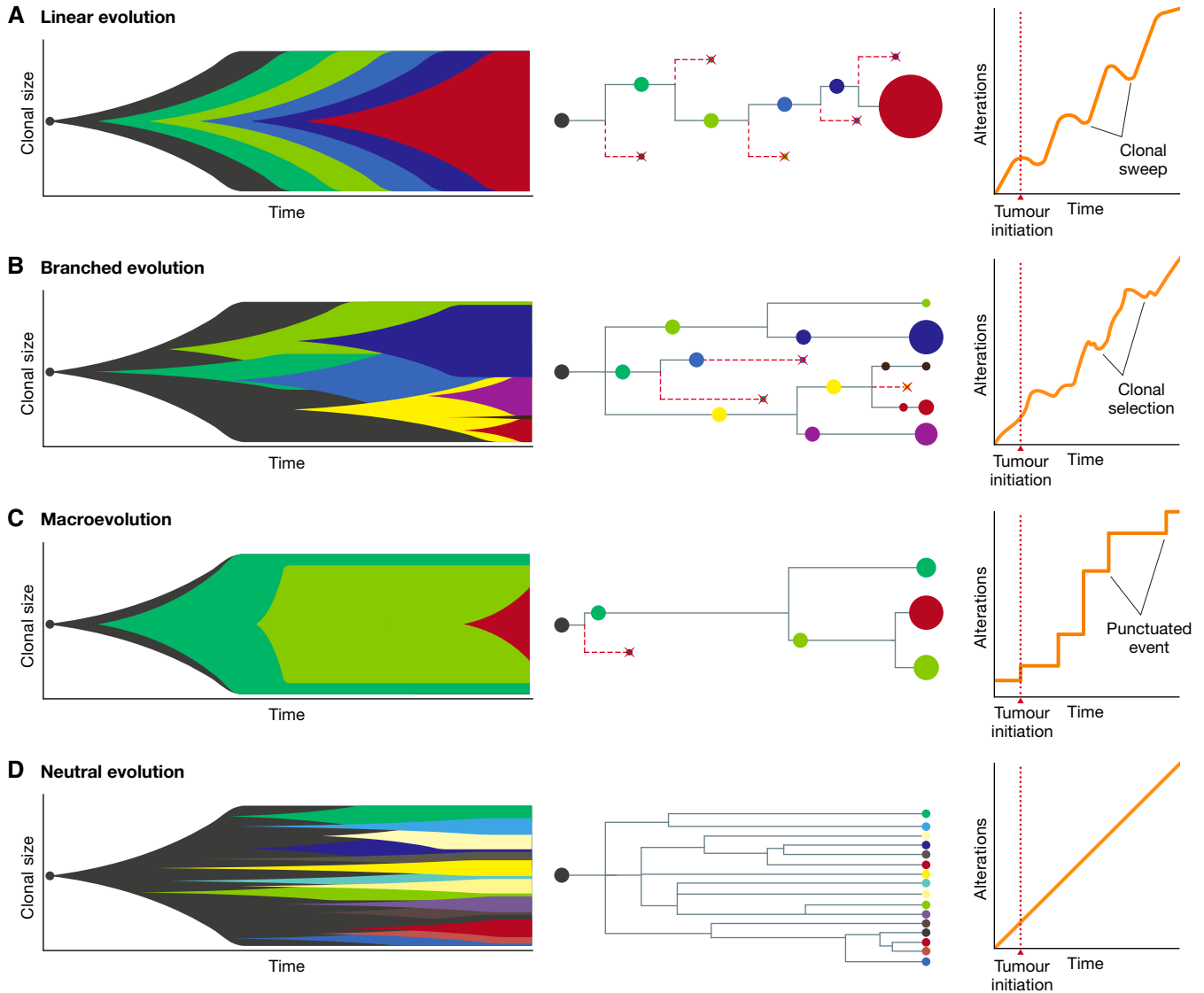
the existence of non-Darwinian mechanisms as important features of tumour evolution.

## Macroevolution and punctuated events

Neo-Darwinian models of tumour evolution generally assume that mutations are acquired sequentially in a gradual fashion over time. However, several lines of evidence suggest that in some cases, a large number of genomic aberrations may occur in short bursts of time in cancer cells (Stephens *et al.*, 2011; Baca *et al.*, 2013), as a consequence of chromosomal instability (CIN) (Bakhroum & Landau, 2017), breakage-fusion-bridge (BFB) cycles (Gisselsson *et al.*, 2000), chromoplexy (Baca *et al.*, 2013), chromothripsis (Stephens *et al.*, 2011; Notta *et al.*, 2016) and other similar catastrophic events (Fig 2). According to this model, tumour cells alternate long phases of relative mutational equilibrium with short periods of intense evolution, where tumour cells can acquire multiple strong driver

events (Cross *et al.*, 2016) (Fig 1). Such examples of saltatory evolution indicate that, at least in cancer, nature can under certain circumstances make jumps, contrary to what Darwin predicted. These observations are reminiscent of the “hopeful monsters” theorised by Richard Goldschmidt, *i.e.* organisms with a profound mutant genotype compared to their parents that hold the potential to establish a novel evolutionary lineage (Goldschmidt, 1941). Hence, through short and intense bursts of genomic change, cancer cells could potentially obtain greater fitness than would be possible through a gradual accumulation of alterations, owing to the simultaneous acquisition of multiple driver alterations (Korbel & Campbell, 2013). However, the phenotypic impact of such hereditary changes acquired through saltatory evolution, if any at all, will often be deleterious and only in rare instances will it result in an increase in cellular fitness and in the generation of viable “hopeful monsters” (Goldschmidt, 1941; Gerlinger *et al.*, 2014b).

Regarding progression from primary tumour to metastasis and death, increasing evidence implicates macroevolutionary changes as



**Figure 1. Models of tumour evolution.**

Models of linear evolution (A), branched evolution (B), macroevolution (C) and neutral evolution (D) described by Muller plots representing dynamic changes in clonal size over time (left), clonal lineages and phylogenetic trees (centre) and changes in the number of alterations over time (right). Colours indicate different clones.

important drivers of progression. For example, in the prospective TRACERx (TRacking Cancer Evolution through therapy (Rx)) study (Jamal-Hanjani *et al*, 2017), elevated copy number heterogeneity was identified as being most strongly associated with recurrence/death risk in non-small cell lung cancer (NSCLC), whereas single nucleotide variant heterogeneity was non-significant. Similarly, acquired aneuploidy was frequently detected in recurrent gliomas (Barthel *et al*, 2019), and genetic diversity alongside chromosomal complexity (characterised by high weighted genome integrity index (Endesfelder *et al*, 2014)) emerged as significant determinant of poor patient outcome in clear cell renal cell carcinoma (ccRCC) (Turajlic *et al*, 2018). In ccRCC, losses of chromosomes 9p21.3 (*CDKN2A*) and 14q31.1 (*HIF1A*) were specifically associated with

reduced survival (Turajlic *et al*, 2018). The prognostic importance of macroevolutionary changes in the form of somatic copy number alterations (SCNAs), above point mutations, is now becoming increasingly recognised as a pan-cancer phenomenon (Smith & Sheltzer, 2018). A major outstanding challenge however is minimal mapping of recurrent SCNA cytobands, to find specific causative genes. And even when clear driver genes emerge, as is likely the case for *CDKN2A* at 9p21 (Smith & Sheltzer, 2018), detailed functional work to delineate the precise mechanisms of disease progression remains to be completed. Additional evidence of punctuated evolution has been reported in prostate cancer, where intense genomic changes occurring over relatively few cataclysmic events, a process termed chromoplexy, were observed (Baca *et al*, 2013).

Similarly, ER/PR/HER2 negative breast cancers were found to undergo short periods of evolution early in tumour development and to remain relatively stable at later stages (Gao *et al*, 2016). Tumour macroevolution was also found to be driven by chromothripsis, whereby a single catastrophic mutational event is thought to be responsible for the generation of highly complex genomic rearrangements involving dozens of breakpoints (Stephens *et al*, 2011). This process has been observed in several tumour types, such as bone cancers (Stephens *et al*, 2011), colon cancers (Kloosterman *et al*, 2011), neuroblastoma (Molenaar *et al*, 2012), glioblastoma (Malhotra *et al*, 2013) and pancreatic cancer (Notta *et al*, 2016). An extreme case of punctuated evolution, caused by the aforementioned mechanisms, is the “big bang” model, whereby a single or a limited number of catastrophic events and/or genomic crises occurring early in tumorigenesis are responsible for the generation of numerous intermixed subclones that will not substantially evolve over tumour progression due to weak selective pressure (Sottoriva *et al*, 2015). Such evolutionary dynamics were observed in several cancers, including colon cancers (Sottoriva *et al*, 2015) and hepatocellular carcinoma (Ling *et al*, 2015), as well as in pan-cancer studies (Stephens *et al*, 2011).

Cancer evolution is conceptually similar to evolution in asexually reproducing organisms, whereby the impact of deleterious alterations in terms of fitness cannot be mitigated through sexual reproduction. A mechanism to alleviate the irreversible detrimental accumulation of alterations (*e.g.* extensive LOH events) may be whole genome doubling (WGD), a prevalent event in cancer (Storchova & Pellman, 2004; Zack *et al*, 2013; Dewhurst *et al*, 2014; Bielski *et al*, 2018) involving the doubling of the entire genome. The presence of additional, doubled wild-type alleles as a consequence of WGD could allow the cell to better tolerate LOH events involving essential genes (López *et al*, 2020). The early occurrence of this event therefore creates a more tolerant and permissive environment which can fuel rapid genomic diversification and CIN, while at the same time may facilitate the sub functionalisation of duplicated genes (Storchova & Pellman, 2004; Huminiecki & Conant, 2012; Dewhurst *et al*, 2014). Consequently, WGD is often associated with high rates of chromosomal aberrations (Zack *et al*, 2013; Dewhurst *et al*, 2014) and with poor prognosis and intrinsic drug resistance (McGranahan *et al*, 2012; Bielski *et al*, 2018).

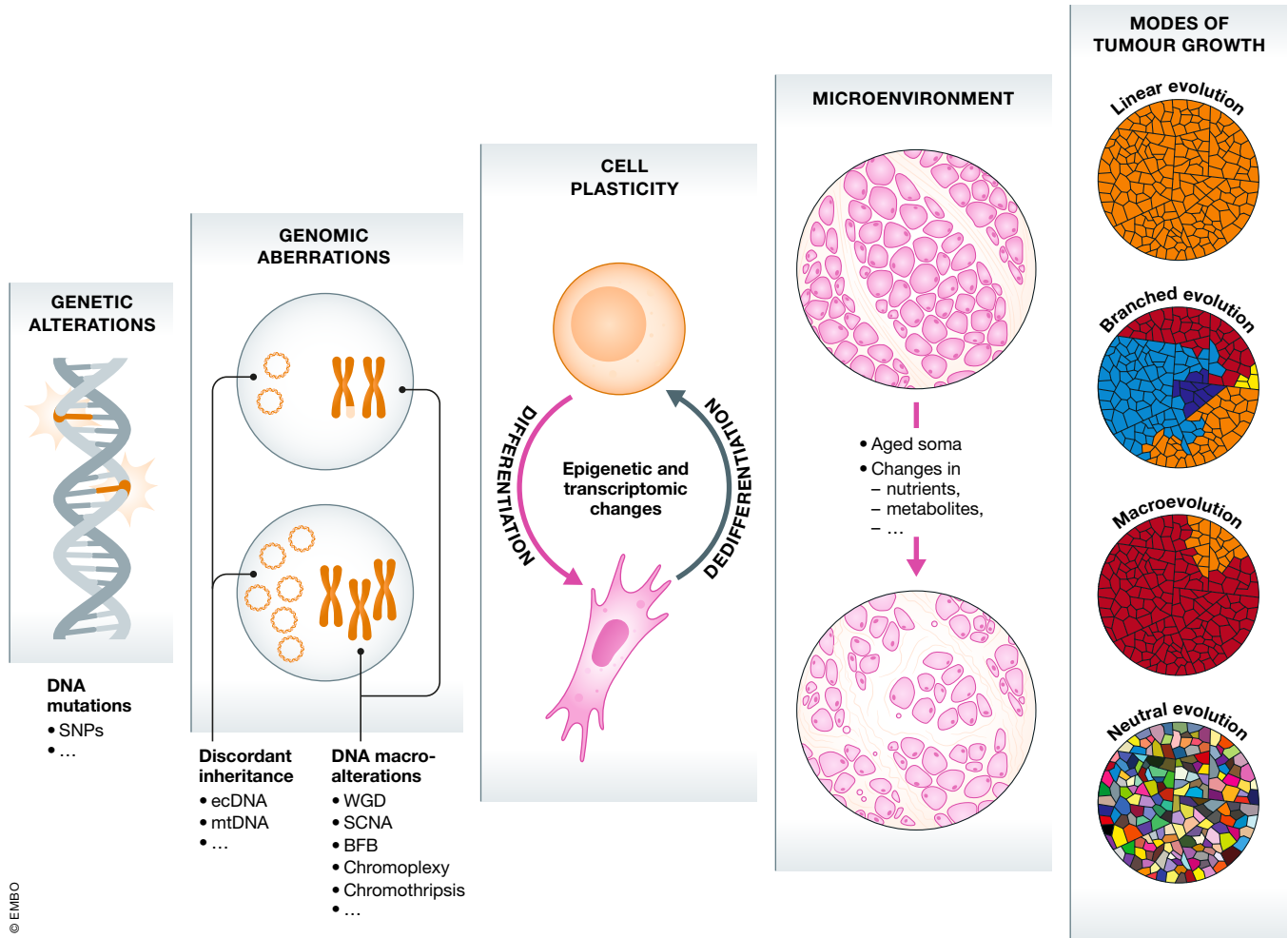
Importantly, certain classes of macroevolutionary events have been shown to be able to trigger other macroevolutionary events. For instance, chromothripsis is prone to arise in genomically unstable cells, such as those harbouring damaged telomeres or with hyperploidy (Mardin *et al*, 2015). Similarly, BFB cycles have been shown to generate increasing amounts of chromothripsis by providing free DNA ends that can engage in genomic rearrangement and by compromising centromere function (Umbreit *et al*, 2020). DNA replication stress has been shown to drive genomic instability by promoting both structural and numerical chromosomal aberrations (Burrell *et al*, 2013) and by triggering single nucleotide-level mutagenesis mediated via APOBEC3B induction (Kanu *et al*, 2016), which in turn leads to incomplete replication of genomic DNA (Venkatesan *et al*, 2021). Relatedly, regional mutational clusters (kataegis) (Stephens *et al*, 2011) and lesion segregation (Aitken *et al*, 2020) have also been shown in some instances to be associated with chromothripsis and other rearrangement architectures (Nik-Zainal *et al*, 2012a). The combination of such events rapidly

accelerates tumour evolution, causing more non-gradualism than any individual class by itself would.

### Discordant inheritance between cells

Recent studies have demonstrated that oncogene amplification within extrachromosomal DNA (ecDNA) is a frequent event in cancer (Verhaak *et al*, 2019). The existence of chromosomal material in cancer cells outside the autosomal genome has been long recognised, with the first reports of oncogenic ecDNAs going back as far as the 1980s, where sequences resembling *MYCN* were found in neuroblastoma cell lines (Kohl *et al*, 1983). However, it was only in the last few years that the frequency and functional relevance of ecDNAs in cancer started to be fully appreciated, thanks to the development of novel techniques such as long-read whole-genome sequencing and circular DNA library enrichment (Verhaak *et al*, 2019). ecDNAs are circular DNA structures located outside of chromosomes of variable size (ranging from 168 kb to 5 Mb, with a median size of 1.26 Mb) (Wu *et al*, 2019), which can contain one or more oncogenes (Bailey *et al*, 2020). ecDNAs provide cancer cells with a mechanism to achieve and maintain high copy oncogene amplification and diversity and to drive potent oncogene expression due to highly open chromatin, which allows for increased expression of genes encoded on circular DNA relative to their chromosomal counterparts (Wu *et al*, 2019; Kim *et al*, 2020). Importantly, the existence of ecDNAs defies Mendelian genetics. ecDNAs are replicated during S phase, but, owing to the lack of centromeres, they are subject to unequal segregation and are therefore randomly inherited by daughter cells during mitosis. As such, ecDNA-based oncogene amplification can accelerate tumour evolution through non-Mendelian mechanisms of inheritance and drive clonal expansion in genomically stable backgrounds (Decarvalho *et al*, 2018) (Fig 2). Importantly, the random distribution of ecDNAs fosters cell-to-cell variability in terms of both copy number and transcriptional levels of oncogenes, enabling tumours to acquire ITH more efficiently than through chromosomal amplifications (Turner *et al*, 2017; Verhaak *et al*, 2019). Several pan-cancer studies revealed a high frequency of ecDNA (albeit at highly variable numbers) across tumour types, particularly in lung, breast and prostate cancer as well as glioblastoma and neuroblastoma (Fan *et al*, 2011; Turner *et al*, 2017; Deshpande *et al*, 2019; Bailey *et al*, 2020; Koche *et al*, 2020). Key oncogenes such as *MYC*, *MYCN*, *EGFR*, *PDGFRA*, *MET*, *HER2*, *DHFR*, *CDK4* and *MDM2* have been frequently observed on ecDNAs, suggesting that ecDNA-mediated oncogene amplification is an important driver of tumour development and progression (Decarvalho *et al*, 2018; Gu *et al*, 2020). ecDNA amplification in cancer has been shown to increase cell proliferation, invasion and metastatisation and to negatively correlate with overall survival (Bailey *et al*, 2020). Consequently, ecDNA elimination has been shown to decrease oncogene amplification and to negatively affect cancer cell survival (Shimizu *et al*, 1998; Nathanson *et al*, 2014; Clarke *et al*, 2020; Oobatake & Shimizu, 2020). Furthermore, ecDNAs have been shown to enable tumour adaptation in response to changes in microenvironmental conditions and to selective pressure from therapy (Turner *et al*, 2017; Decarvalho *et al*, 2018; Kim *et al*, 2020), though in some instances ecDNA expression represents a cancer-specific vulnerability (Nathanson *et al*, 2014).





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**Figure 2. Scales of tumour evolution.**

Schematic illustration of the different determinants of tumour evolution, which influence evolutionary trajectories through highly interdependent mechanisms, from a microscopic (left) to a macroscopic (right) scale.

## Neutral evolution

Neutral evolutionary models of cancer (Ling *et al*, 2015; Williams *et al*, 2016) are based on Motoo Kimura’s work on population genetics which postulated that the vast majority of molecular alterations are not caused by selection but rather by random fixation of selectively neutral mutations through genetic drift (Kimura, 1983). According to the neutral tumour evolution model, cancer-driving alterations are selected for and accumulate in a clonal fashion prior to tumour initiation, as a consequence of ageing and carcinogenic insults. Those alterations will be sufficient for tumour formation and development, with little to no contribution of alterations occurring over the course of cancer progression (Fig 1). Therefore, the genetic ITH observed in tumours may often be entirely the result of the random fixation of (nearly) neutral alterations in the population through genetic drift and as such have no functional role in promoting tumour growth and evolution. In one study, multi-region sequencing of > 300 regions of a patient with hepatocellular carcinoma indicated that there was no particular selection for any clone

within the tumour (Ling *et al*, 2015). In another study, subclonal allele frequencies from the TCGA cohorts were used to conclude that up to one-third of all tumours do not show indications of subclonal selection but rather evolve in a neutral fashion (Williams *et al*, 2016). However, several lines of evidence suggest that these results may be an overestimation due to the low resolution of the data used for the study and may suffer from bias in the modelling, since variant abundance distributions may often not provide enough information to exclude selection (Tarabichi *et al*, 2018; Bozic *et al*, 2019).

Kimura’s neutral theory essentially states that most alterations will be neutral, especially at low population sizes and with weak purifying selection. Most variants will not have any fitness effect, and the rare ones which will have an impact on fitness will predominantly be deleterious, as predicted by mathematical modelling (Cannataro *et al*, 2017). However, Kimura never excluded the importance of occasional strong positive selection in evolution, especially as a consequence of strong purifying selection (Kimura, 1983). Hence, when applying Kimura’s neutral theory to cancer, selective pressures (*i.e.* microenvironmental changes, metastatisation, therapeutic

intervention) must be taken into consideration. Therefore, while a treatment-naïve tumour could at times evolve in a neutral fashion over the course of its progression, the emergence of strong selective forces, such as therapeutic pressure, may still drive the selection and expansion of previously neutral alterations (Almendo *et al*, 2014; Williams *et al*, 2016).

It is also worth noting that the presence of non-cell-autonomous drivers in small subpopulations may also give the false impression of neutral evolution (Marusyk *et al*, 2014). Indeed, Kornelia Polyak's group demonstrated that tumours can at times be driven by a subclone that does not have higher fitness, but instead stimulates the growth of all tumour cells through non-cell-autonomous mechanisms (Marusyk *et al*, 2014). In such a scenario, it may be misleading to assume that the absence of a predominant clone in a tumour is evidence of neutral evolution, as non-cell-autonomous driving of tumour growth can maintain clonal diversity over clinically relevant time frames while simultaneously fuelling tumour growth and evolution.

### Non-genetic determinants of evolution

There is increasing appreciation that tumour evolution is not only driven by mutations and genetic alterations but also from non-genetic—often non-heritable—determinants, such as cell plasticity and the tumour microenvironment (TME) (Caiado *et al*, 2016; Ramón y Cajal *et al*, 2020) (Fig 2).

#### Cell plasticity

The notion that cancer cells can dynamically switch from one state to another in response to environmental stresses and therapeutic pressure without genomic alterations is gaining greater recognition (Pogrebniak & Curtis, 2018; Mills *et al*, 2019; Boumahdi & de Sauvage, 2020) (discussed in other reviews of this series by Milan *et al*, 2021). This phenomenon, termed cell plasticity, is characterised by a fundamental change in the biological properties of the cell occurring as a consequence of dynamic and reversible epigenetic and transcriptional changes (in sharp contrast to genetic alterations, which have binary and largely irreversible effects) (Calabrese *et al*, 2020). One of the main advantages of cell plasticity is the ability to swiftly react to dynamic changes in the tumour and in its microenvironment and to engage finely tuned and graded adaptive responses to stressors (*i.e.* inflammation and therapy) (Rambow *et al*, 2019). A classic example of cell plasticity is the epithelial–mesenchymal transition (EMT) (Nieto *et al*, 2016) (extensively covered by Brabletz *et al* (2021) in another review of this series). Cell plasticity allows for the rapid emergence of different cell states from the same genome, giving rise to a plethora of distinct phenotypes, a process that has shown to be promoted upon therapeutic intervention (Kemper *et al*, 2014; Gunnarsson *et al*, 2020; Marine *et al*, 2020). Cell plasticity has been extensively demonstrated as an adaptive mechanism to escape therapeutic pressure. One of the first evidence of such a mechanism was the identification of drug-tolerant persisters (DTPs) cells emerging from drug-sensitive NSCLC cell lines upon exposure to EGFR tyrosine kinase inhibitor (Sharma *et al*, 2010). This drug-tolerant phenotype was shown to be transiently acquired and lost by individual cells, thereby demonstrating that cancer cells can dynamically and reversibly develop

drug resistance through non-genetic phenotype switch. Similarly, multiple phenotypically distinct—yet interdependent—drug-tolerant populations were recently shown to emerge in melanoma PDX models in response to MAPKi treatment through adaptive non-genetic mechanisms (Rambow *et al*, 2018). Importantly, although resistant phenotypes are often non-heritable, they can protect the tumour cell population from eradication thereby increasing overall tumour fitness through non-Darwinian mechanisms (Kemper *et al*, 2014; Gunnarsson *et al*, 2020; Marine *et al*, 2020). Cell plasticity can therefore enable the emergence of more permanent resistance mechanisms, as was shown in melanoma, whereby therapeutic pressure initially caused the emergence of a transient drug-tolerant transcriptional state which was later converted into a stably resistant phenotype (Shaffer *et al*, 2017).

#### Tumour microenvironment

The observation that healthy tissues often display pervasive somatic mutations, even in cancer driver genes, suggests that genetic mechanisms alone may not be sufficient to drive malignant transformation (Martincorena *et al*, 2015, 2018; Teixeira *et al*, 2019; Yizhak *et al*, 2019; Yoshida *et al*, 2020). It has been long noted that the incidence of most cancers uniformly increases after 50 years of age (Siegel *et al*, 2018b), regardless of the many differences in terms of their cellular origin, of the number of alterations required for their initiation and of the external risk factors (such as smoking, alcohol and UV light) (Rozhok & De Gregori, 2019; Laconi *et al*, 2020). To explain this discrepancy, James DeGregori's group demonstrated that cancer incidence rapidly increases with age not only as a consequence of the accumulation of alterations but also (if not predominantly) because of the age-dependent physiological decline of the soma, combined with a general weakening of the immune system which is less efficient at eliminating altered (precancerous) cells (DeGregori *et al*, 2020; Laconi *et al*, 2020). While a young healthy tissue environment may continuously and effectively prevent tumorigenesis, an aged one would provide fertile ground for cancer formation instead. Permissive environments have been shown to result in population expansion in nature, whereby relaxed constraints are known to often spur the establishment, expansion and persistence of particularly versatile species. For instance, a permissive environment was shown to have led to the expansion of two species of parasites in the Arctic as a consequence of climate change (Kutz *et al*, 2013; Kafle *et al*, 2020).

Intriguingly, the effect of mutagenic agents was also found to be influenced by the tissue type, as the mutational burden varied greatly based on tissue, suggesting tissue-specific differences in toxicokinetic, DNA repair activity or of the TME (Riva *et al*, 2020). Similar evidence comes from the observation that genetic diseases driven by highly penetrant mutations predisposing to cancer invariably lead to very specific cancer types, although the same mutation is shared between all the cells of the organism. This is the case of the *NF1* mutation in neurofibromatosis, which often leads to tumours of the nervous system (Gutmann *et al*, 2017), of the *TP53* mutations in the Li-Fraumeni syndrome, which predispose to breast cancer, adrenocortical carcinomas, central nervous system tumours, osteosarcomas and soft-tissue sarcomas (Malkin, 2011), and of the *BRCA1/2* mutations, which considerably increase the risk of breast cancer (Tung & Garber, 2018). Hence, even highly penetrant cancer-driving alterations may not be sufficient to drive cancer formation

per se. Instead, these examples suggest that oncogenic mutations may often confer a cell type-specific advantage in terms of fitness rather than a generic selective advantage on all cells and that very specific environmental conditions are needed for tumour initiation. The observation that certain cancer types or cancer subtypes metastasise to specific locations, a fact that cannot be solely explained by circulation patterns, further reinforces the idea that tumours require particular environments for their growth and survival, even in later stages of cancer evolution (Nguyen *et al*, 2009). These are only but a few examples of how the TME can affect cancer initiation and evolution, as this topic is amply discussed in other reviews of this series (see Kepp *et al*, 2021; Parker *et al*, 2021).

### Clinical opportunities: leveraging evolutionary principles

Advances over the last decade have led to a deeper understanding of the molecular pathogenesis of cancer and of its evolution. Small-

molecule inhibitors, such as BRAF and EGFR inhibitors (Bedard *et al*, 2020), and immune checkpoint inhibitors (CPI), such as anti-PD-1/L1 and anti-CTLA-4 (Waldman *et al*, 2020), have revolutionised cancer therapy (Chen *et al*, 2019). However, despite the significant benefits brought about by these approaches, many advanced tumours—even those with marked initial responses—rapidly develop resistance (Gatenby & Brown, 2020). The dynamic nature of tumour evolution, especially under the strong selective pressure exerted by anti-cancer therapies, makes longitudinal monitoring necessary (see Box 3) and warrants flexible therapeutic strategies based on the evolutionary changes within a patient's tumour.

#### Limiting tumour adaptation

Increasing evidence indicates that both Darwinian and non-Darwinian adaptive mechanisms can be activated shortly after therapy initiation to cope with the stress caused by the treatment (Hata *et al*, 2016; Rambow *et al*, 2018; Russo *et al*, 2019; Vendramin *et al*, 2021). The employment of such responses thereby fosters the

#### Box 3. Sampling techniques to study tumour evolution

##### Multiregional (longitudinal) biopsies

Over the last few years, increasing efforts have been put towards sequencing multiple geographically distinct areas of the same tumour and of its metastases and/or samples taken from the same patient at different time points (McGranahan & Swanton, 2015; Chkhaidze *et al*, 2019). These analyses have proven to be critical for the reconstruction of tumour evolutionary histories, revealing extraordinarily complex and dynamic phylogenetic architectures of cancer subclones and their variegated genetic, epigenetic and transcriptomics profiles. However, multiregional and longitudinal sampling through invasive biopsies is often deemed clinically unfeasible, since the performance status of the patient and the location of the tumour frequently prevent such an approach (Turajlic *et al*, 2018; Chkhaidze *et al*, 2019; Litchfield *et al*, 2020).

##### Rapid research autopsy

Rapid research autopsy is gaining interest as a powerful technique to assess ITH and tumour evolution through improved tissue sampling, with the potential to overcome the limitations of sampling in living patients in several ways (Duregon *et al*, 2019). First, they allow the collection of large quantities of tumour tissue, as well as the TME, from multiple body sites from individual patients, supporting a more complete representation of metastatic lesions. Second, enough material can be collected to be used for multiple orthogonal studies (Avigdor *et al*, 2017; Pisapia *et al*, 2017; Duregon *et al*, 2019). Third, these samples can be used for the development of patient-derived xenografts, organoids and primary cell lines, thus allowing mechanistic validation of the data generated through orthogonal analyses (Grasso *et al*, 2012; Xie *et al*, 2015; Krook *et al*, 2019b). Last, the samples obtained are typically composed of therapy-resistant cells and can inform about the evolution of drug resistance (Dagogo-Jack & Shaw, 2018). Such an approach offers an ethical alternative to multiregional biopsy sampling of living patients and can be effectively used to overcome its limitations. Albeit limited in number, the studies performed through rapid research autopsies have substantially increased our understanding of ITH and tumour evolution, and how both processes are affected by therapeutic selective pressure (Campbell *et al*, 2010; Haffner *et al*, 2015; Hoadley *et al*, 2016; Brown *et al*, 2017; Siegel *et al*, 2018a; De Mattos-Arruda *et al*, 2019). For instance, rapid research autopsies have allowed tracing of the origins of metastases to individual subclones, delineating a precise time course from an initial founder cell to the development of metastases (Yachida *et al*, 2010; Gerlinger *et al*, 2012; Aryee *et al*, 2013; Gerlinger *et al*, 2014a). It is worth noting that, apart from some notable exceptions (Embuscado *et al*, 2005; Yachida *et al*, 2010), the majority of these studies have been performed on therapy-resistant lesions and might therefore not fully recapitulate the evolutionary processes occurring in drug-naïve cells (Dagogo-Jack & Shaw, 2018; Duregon *et al*, 2019; Krook *et al*, 2019b). Moreover, the execution of such studies requires interdisciplinary collaboration across different medical and scientific disciplines, which can be difficult to be put in place, and necessitate the consent and close involvement of cancer patients and their families.

##### Blood-based tests

High frequency monitoring of tumour evolution, especially over the course of therapeutic intervention, is required for the precise understanding of the mechanisms leading to drug resistance. Shedding light on this dynamic process could provide direct evidence of clonal selection and readily warn about the emergence of drug-resistant clones. Evidently, these types of information could not be obtained through longitudinal multiregional biopsies, both for practical and ethical reasons, nor through rapid research autopsies. In recent years increasing attention has therefore been directed at detecting cancer-derived components, such as circulating tumour cells (CTCs), as well as tumour-derived components such as circulating tumour DNA (ctDNA), cell-free RNAs, extracellular vesicles (EVs), proteins and metabolites (Ramalingam & Jeffrey, 2018; Rossi & Ignatiadis, 2019; Ignatiadis *et al*, 2021), in patients' bodily fluids. Provided that tumour cells and their components detected in the circulation represent their clonal frequency, blood-based tests could allow the detection of changes occurring within a patient's tumour in real time. As such, blood-based tests have the potential to revolutionise personalised medicine and guide clinical decision-making through constant assessment of ITH and tumour evolutionary dynamics via non-invasive and cost-effective monitoring of tumour growth, minimal residual disease (MRD), metastatisation and response to therapy. Growing evidence supports the feasibility of blood-based tests to monitor tumour evolution across several tumour types (Misale *et al*, 2012; Dawson *et al*, 2013; Aceto *et al*, 2014; Scher *et al*, 2015; Siravegna *et al*, 2015; Abbosh *et al*, 2017, 2018; Anagnostou *et al*, 2019; Vidal *et al*, 2020). Additionally, blood-based tests were shown to have prognostic value at the time of primary diagnosis and surgery in various solid tumours including breast, bladder, colorectal, head and neck and testicular cancer (Pantel & Alix-Panabières, 2019; Chen *et al*, 2020) as well as melanoma (Lee *et al*, 2019) and NSCLC (Abbosh *et al*, 2017).



emergence of a reservoir of drug-tolerant cellular populations from which therapy-resistant clones can evolve from. Therefore, therapeutic strategies targeting the mechanisms responsible for drug tolerance may prevent, or at least delay, the evolution of acquired resistance (Fig 3). This strategy was tested in a recent study where the selective pressure exerted by EGFR inhibitors in lung cancer was shown to induce APOBEC3B overexpression, leading to increased cancer cell mutability, fostering tumour adaptation to treatment (preprint: Mayekar *et al*, 2020). Similar findings were reported in colon cancer, whereby drug-tolerant cells were shown to temporarily down-regulate mismatch repair (MMR) and homologous recombination DNA repair genes in favour of error-prone polymerases as an adaptive response to overcome BRAF and EGFR inhibition (Russo *et al*, 2019). These findings suggest that, similar to bacteria (Luria & Delbrück, 1943), cancer cells can temporarily enhance their mutability and genetic instability as an adaptive response to overcome therapeutic pressure. Inhibition of these adaptive mechanisms could therefore abolish drug-driven adaptive mutagenesis and potentially prevent the emergence of drug-resistant populations.

Additionally, there is growing appreciation that non-Darwinian mechanisms can drive tumour adaptation, especially in the context of therapy resistance, where a clear genetic cause for therapy failure has only been identified in about 60% of tumours. For instance, MAPK inhibition was shown to drive transcriptional reprogramming in melanomas shortly after treatment initiation which resulted in drug tolerance (Smith *et al*, 2016). Importantly, this response was reversible and non-mutational in nature. Targeting of a master regulator of this adaptive mechanism was shown to prevent drug tolerance and re-sensitise melanoma cells to MAPKi (Smith *et al*, 2016). Similarly, the activation of the integrated stress response (ISR) was observed to foster drug tolerance in melanoma. In this context, ISR activation was found to indirectly enhance mitochondrial translation, making these cells highly vulnerable to mitochondrial translation inhibitors, which were shown to reduce tumour heterogeneity and to prevent acquisition of resistance regardless of the tumour mutational profile or phenotype (Vendramin *et al*, 2021). The TME was also shown to promote tumour drug tolerance. For instance, paradoxical activation of the fibroblastic stroma in BRAF inhibitor treated melanomas resulted in enhanced matrix remodelling. The remodelled matrix was found to promote BRAF-independent ERK activation through FAK signalling, thereby enabling drug tolerance and melanoma cell survival. Concomitant inhibition of BRAF and FAK prevented ERK reactivation and led to tumour volume stabilisation (Hirata *et al*, 2015). Similarly, inhibition of the microenvironment-produced morphogen EDN3 abrogated transcriptional reprogramming and phenotype switch in melanomas and reduced tumour aggressiveness and growth (Kim *et al*, 2017).

### Exploiting collateral sensitivities

The paradox of treating dynamically evolving cancers is that therapeutic strategies are typically administered in a fixed, sequential schedule in the hope of reducing tumour burden (Gatenby *et al*, 2009). The integration of evolutionary principles in the design of anti-cancer therapies may therefore bring about substantial clinical benefits. Knowledge of tumour evolutionary responses to therapy could allow the anticipation of relapse by pre-emptively targeting drug resistance mechanisms or to exploit collateral sensitivities, *i.e.*

specific weaknesses caused by the development of resistance to a particular treatment (Pluchino *et al*, 2012; Efferth *et al*, 2020) (Fig 3). For instance, combining different classes of ABL1 inhibitors with mutually exclusive profiles of resistance mutations in chronic myeloid leukaemia can prevent the emergence of drug-resistant subclones and lead to durable responses *in vivo* (Zhao *et al*, 2016; Wylie *et al*, 2017). Another example is gefitinib-resistant NSCLC, which shows collateral sensitivity for TNF $\alpha$  when compared to the non-resistant counterpart (Ando *et al*, 2005). A similar strategy is the “sucker’s gambit”, in which the initial therapy, rather than killing all cancer cells, aims to push them into an evolutionary *cul-de-sac* by forcing a phenotypic adaptation that is then exploited in second-line therapy (Merlo *et al*, 2006; Schweizer *et al*, 2015) (Fig 3). The first drug will affect most subpopulations and promote the emergence and clonal dominance of a specific clone, which would then be eliminated by the addition of a second drug (West *et al*, 2019). A limitation of this approach is that it is based on the premise that all cells will develop the same mechanism of resistance, whereas a plethora of *in vivo* experiments and autopsy studies have demonstrated that most individual tumours can achieve resistance (to chemo-, targeted and immunotherapy) via multiple distinct routes simultaneously, a process termed polyclonal resistance (Burrell & Swanton, 2014; Shi *et al*, 2014; Kemper *et al*, 2015; Faltas *et al*, 2016; Ascierto *et al*, 2017; Rambow *et al*, 2018; Razavi *et al*, 2018; Krook *et al*, 2019a; Rosenthal *et al*, 2019; Sanchez-Vega *et al*, 2019).

An alternative strategy, named “benign cell booster”, aims instead to promote the growth of benign, less aggressive tumour subpopulations at the expense of highly malignant populations (Maley *et al*, 2004). Importantly, such an approach would prevent the emergence of aggressive treatment-resistant subpopulations and have likely limited adverse effects since it would not be aimed at killing cancer cells but rather select for particularly benign tumour cell populations.

### Adaptive therapies

Another intriguing therapeutic approach based on evolutionary principles is adaptive therapy, a therapeutic strategy with the goal of maintaining a stable tumour population below a certain symptomatic threshold, rather than trying to achieve complete tumour elimination, through the use of repeated ON/OFF treatment cycles (Gatenby *et al*, 2009) (Fig 3). This strategy aims to make use of the understanding of the mechanisms of therapy resistance as well as their “cost” and to exploit the differences in terms of fitness between drug-sensitive and drug-resistant subpopulations (Cunningham, 2019). In such a scenario, the optimal dose and treatment duration is not the maximum possible but rather the minimum necessary. In essence, a treatment cycle would last until tumour response (*i.e.* regression below a certain, pre-defined threshold) is observed, after which therapy is discontinued. This approach enables minimal tumour cell proliferation to prevent the elimination of all treatment-sensitive tumour clones, which are expected to have a competitive advantage in terms of fitness over treatment-resistant populations. Upon relapse, the tumour should mostly be comprised of treatment-sensitive cells; therefore, a new treatment cycle will be initiated to bring the tumour volume below a symptomatic threshold. Despite its initial promises and successes in *in vitro* and *in vivo* models (Enriquez-Navas *et al*, 2016; Bacevic *et al*, 2017), this strategy has

not achieve notable results in the clinic, as indefinite tumour control, the goal of such an approach, has not been achieved (Crook *et al*, 2012; Hussain *et al*, 2013; Algazi *et al*, 2020). One limitation

of this approach is the lack of tools enabling constant monitoring of tumour subclonal evolution (e.g. via ctDNA/CTCs, see Box 3) over the course of treatment. Another weakness of this strategy is the

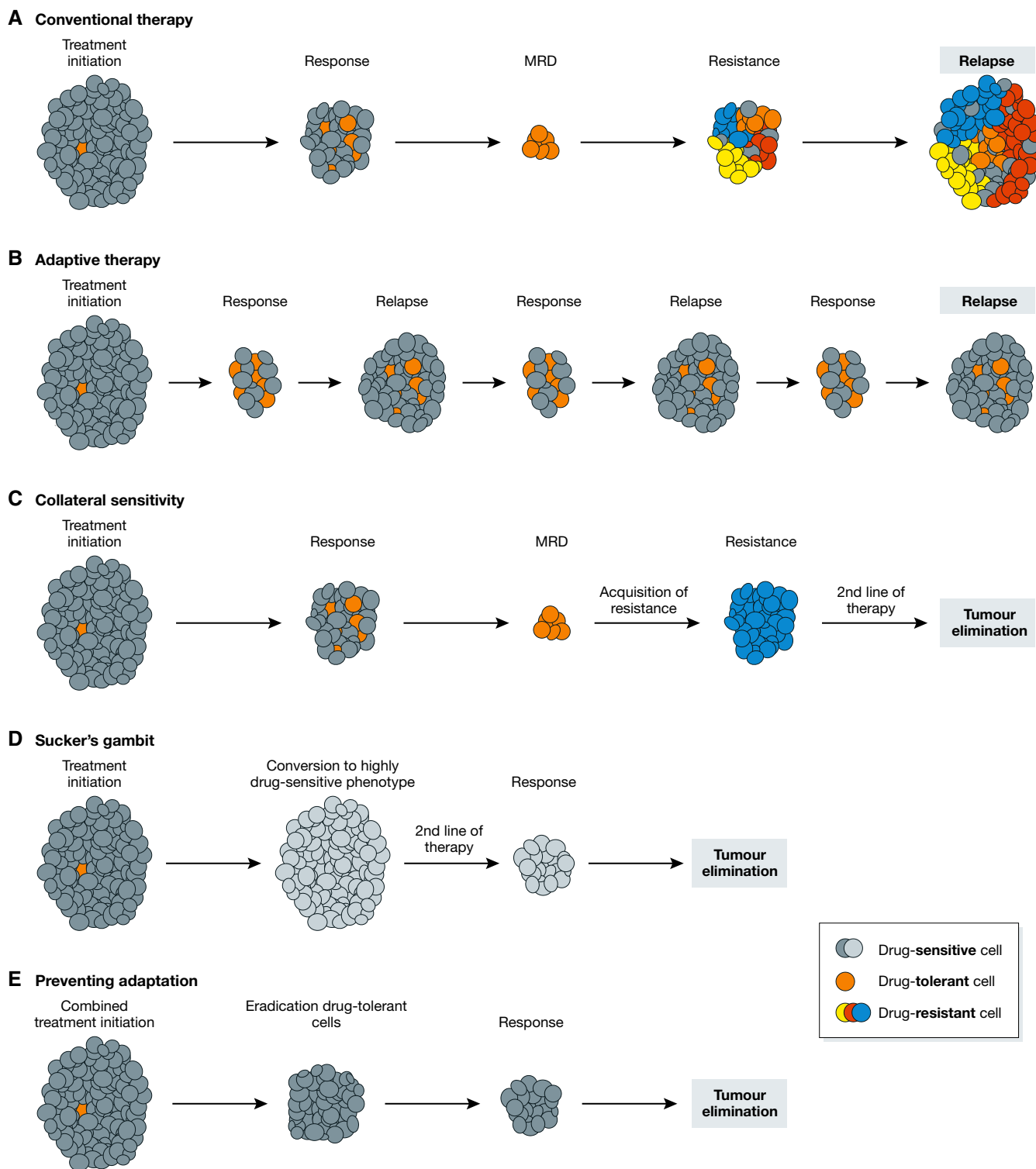


Figure 3.

**Figure 3. Evolutionary-based treatment strategies.**

(A) Most conventional therapeutic approaches will lead to marked initial responses but often fail to eliminate—or induce the emergence of—drug-tolerant cells. This population of minimal residual disease (MRD) represents a reservoir of malignant cells from which drug-resistant clone(s) may emerge and cause tumour relapse. (B) In adaptive therapy, treatment is discontinued upon reaching a pre-defined threshold so as not to eliminate all drug-sensitive cells (dark grey). These cells are then allowed to grow back and are expected to suppress the growth of drug-tolerant (or therapy-resistant) clones owing to their higher fitness upon withdrawal. (C) Collateral sensitivities, wherein acquisition of resistance to a first drug induces susceptibility to a second one, can be exploited to target cancer-specific vulnerabilities and potentially lead to tumour eradication. (D) In the Sucker's gambit, the first drug forces a phenotypic adaptation that pushes tumour cells into an evolutionary trap by increasing the fitness of a highly drug-sensitive population (leading to its clonal dominance), which is then eliminated by a second line of treatment. (E) Limiting drug adaptation may prevent the establishment of drug-tolerant (MRD) and drug-resistant populations. In this setting, a combination of drugs is used to simultaneously eradicate tumour cells and to prevent the emergence of drug-induced adaptive responses.

assumption that drug resistance always comes at a fitness “cost” for the cell, which may not be true in all cases, especially in the context of polyclonal resistance, where different resistance mechanisms may differentially impact cell fitness. Indeed, there is evidence that acquisition of resistance, at least in some instances, may not be associated with a fitness cost at all (Shah *et al*, 2007; French-Constant & Bass, 2017; Strobl *et al*, 2021).

**Early detection**

Many early-stage cancers can be cured by surgery alone if removed before metastasis has occurred, by surgery ± adjuvant therapy. Past that point, these approaches are seldomly curative (Siegel *et al*, 2017; Cohen *et al*, 2018). Therefore, until more potent and specific therapeutic agents are developed, a promising strategy for reducing cancer mortality is early tumour detection, which has been shown to reduce cancer mortality in several cancer types including lung, breast, bladder, colorectal, head and neck and testicular cancer as well as melanoma (Hawkes, 2019; Lee *et al*, 2019; Pantel & Alix-Panabières, 2019; Crosby *et al*, 2020; de Koning *et al*, 2020). Early-stage cancer detection may also allow the identification of tumours in early evolutionary stages, when they may be less resilient to treatment due to their more homogeneous nature (Jamal-Hanjani *et al*, 2015). Additionally, detecting pre-existing therapy-resistant clones before treatment initiation may allow the employment of individualised therapeutic approaches aimed at eliminating drug-tolerant/resistant populations. A major evolutionary question, that in the context of early detection remains unaddressed, is timing of tumour initiation and the lead-time between malignant transformation and clinical presentation. Evolutionary tools such as the molecular clock, originally introduced by Emile Zuckerkandl and Linus Pauling in the 1960s (Morgan, 1998), have recently provided some preliminary estimates using cancer genomics datasets. These approaches take advantage of the somatic mutations acquired by cancer cells with clocklike properties, *i.e.* a correlation with the chronological age of the person. With this knowledge, mutation counts can be used to infer the timing of biological events, for example 3p loss/5q gain events found in 36% of ccRCC patients were timed back several decades to childhood/adolescence (Mitchell *et al*, 2018). In another study, a primary colorectal tumour was timed to have likely emerged 5 to 8 years before clinical diagnosis (Lote *et al*, 2017). In another case, analysis of early NSCLC revealed WGD events and truncal driver events in ex-smokers dating back more than 20 years prior to tumour detection, indicating a prolonged tumour latency period from the first oncogenic insults to clinical manifestation (De Bruin *et al*, 2014). Recent large-scale work from the Pan-Cancer Analysis of Whole Genomes (PCAWG) consortium in 2,658 tumours also revealed that driver mutations often precede clinical diagnosis by many years, if not decades

(Gerstung *et al*, 2020). While inferred in nature, these studies still provide accumulating evidence that tumour initiation likely dates back years or even decades before clinical presentation, hence suggesting an extended window for cancer early detection, likely through the use of non-invasive blood-based tests (see Box 3) (Liu *et al*, 2020). In addition to genomic data, information on the history of prior mutagenic exposures (*i.e.* duration of smoking, age of smoking cessation) can be additionally utilised to validate and refine timing inferences (De Bruin *et al*, 2014). Lastly, as well as absolute timing, the accumulation of mutations can also be timed relative (*i.e.* before or after) macroevolutionary events such as WGD (López *et al*, 2020).

**Minimal residual disease**

Particular attention should also be given to minimal residual disease (MRD) detection and characterisation. MRD represents latent disease, *i.e.* a small population of undetectable malignant cells that persist or recur following initial surgical or systemic treatment. These cells may represent a reservoir of metastasis competent or drug-tolerant cells able to drive future tumour relapse (Luskin *et al*, 2018; Rambow *et al*, 2018; Chin *et al*, 2019). Owing to the limited number of cells, it is technically challenging to detect MRD through conventional approaches, such as computed tomography scans. To date, the most promising method to detect MRD is achieved through circulating tumour DNA assessment (see Box 3). This approach has been shown to be successful in several studies in bladder cancer (Dudley *et al*, 2019), NSCLC (Abbosh *et al*, 2017; Chaudhuri *et al*, 2017), colon cancer (Tie *et al*, 2016), breast cancer (Mounier *et al*, 2015), oesophageal cancer (Azad *et al*, 2020) and a range of other solid tumour types (Chin *et al*, 2019). Importantly, macroevolutionary changes caused by large chromosomal aberrations, WGD or CNAs that appear during metastatic formation have been detected through analysis of circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA) in recent studies performed on colon cancer patients with MRD (Gao *et al*, 2017; Joosse *et al*, 2018). Additionally, post-surgical detection of MRD is a strong predictor of impending disease relapse (Abbosh *et al*, 2018; Luskin *et al*, 2018; Azad *et al*, 2020). Hence, rational targeting of MRD is emerging as a promising strategy to prevent therapy resistance and relapse (Luskin *et al*, 2018). There are several advantages to treating patients with MRD rather than waiting for clinical relapse to initiate further therapy. First, tracking of MRD allows the identification of alterations emerging in response to treatment. Second, earlier detection of relapse via MRD allows further treatment to be implemented earlier, where a window of more favourable outcome may be achievable when tumour burden and ITH will be lower. Accordingly, the design of personalised ctDNA panels based on patient mutational status could provide a patient-specific biomarker of tumour response to

treatment. For instance, a recent study demonstrated that the use of a personalised ctDNA panel led to detection of breast cancer metastatic recurrence up to two years prior to overt clinical manifestation (Coombes *et al*, 2019). Last, this approach could allow to adapt therapeutic regimens in the adjuvant setting to eliminate all persisting cells. Regarding predictive MRD tools, the PROSPECT-C study demonstrated that time to anti-EGFR treatment failure could be reliably predicted using longitudinal ctDNA sampling in colorectal cancer, hence generating a window of opportunity for future intervention (Khan *et al*, 2018). Similarly, the IMvigor010 study (Powles *et al*, 2021) demonstrated that MRD detection through ctDNA detection, using the Signatera assay co-developed by our laboratory and Natera (Abbosh *et al*, 2017), could be used to stratify patients who will benefit from atezolizumab (anti-PD-L1) postoperatively. Evidence that targeting MRD can prevent relapse comes from the experience of treating patients with completely resected NSCLC (Winton *et al*, 2005), colon cancer (André *et al*, 2009) and breast cancer (Albain *et al*, 2012) with adjuvant (or neoadjuvant) therapy, with promising results coming from *in vivo* experiments in melanoma (Rambow *et al*, 2018; Marin-Bejar *et al*, 2021; Vendramin *et al*, 2021). A similar approach is currently being tested in the MERMAID-1 Phase III clinical trial where the effects of adjuvant treatment with durvalumab (anti-PD-L1) plus chemotherapy versus chemotherapy alone on disease-free survival (DFS) in patients with completely resected stage II-III NSCLC who show evidence of MRD.

## Conclusion and future perspectives

The longstanding assumption that cancer evolution can only be viewed through a Darwinian lens has been shown to be insufficient to explain the complex evolutionary patterns observed in human malignancies. While our understanding of tumour evolution has been built on the foundations of Darwinian selection, increasing evidence indicates that cancer evolutionary dynamics often defy Darwinian principles. The observation that tumours can evolve in a punctuated fashion, rather than in a gradual fashion as Darwin theorised when he stated that “natural selection can act only by taking advantage of slight successive variations; she can never take a leap, but must advance by the shortest and slowest step” (Darwin, 1859), demonstrates that nature does indeed make macroevolutionary jumps. It is also important to consider that the diversity within tumours is not necessarily selectively driven but can also emerge in some tumours as the result of neutral evolutionary dynamics. Evidence of discordant inheritance patterns between cells represents additional proof of the relevance of non-classical mechanisms in cancer evolution. Furthermore, neo-Darwinian evolutionary models are traditionally based on a gene-centric view of evolution, whereas increasing evidence highlights the role of non-genetic contributors, such as cell plasticity and the tumour microenvironment, as key determinants of cancer evolution. Last, it is becoming increasingly clear that tumorigenesis may in some cases reflect the physiological decline of a tissue, whereby an ageing soma provides a more permissive environment for malignant transformation. It is important to note that individual tumours may not follow a single evolutionary model. Multiple mechanisms may instead be operating either concomitantly or at different stages of progression. Hence, a coherent and unified approach encompassing Darwinian and non-

Darwinian evolutionary theories is needed to fully comprehend the breadth and the depth of cancer evolutionary dynamics.

Research output thus far has revealed that tumour evolution is highly varied in its nature, as well as evidenced how little we still know about cancer evolutionary trajectories. While the studies discussed above have provided critical clues on the driving forces of tumour evolution, they were generally based on individual time points and limited in terms of resolution and cohort size. Larger clinical studies, harnessing the full potential of recent technological innovations, such as single-cell approaches, making use of multiple longitudinal and post-mortem approaches, such as blood-based tests monitoring ctDNA/CTCs and rapid research autopsies, respectively, are thus warranted. The implementation of these approaches will allow us to shed light on the dynamics of cancer evolution and to develop evolutionary-guided therapies to impact clinical outcomes.

In conclusion, understanding cancer requires an evolutionary perspective. The study of malignant transformation within an evolutionary framework has potential to bring about a substantial improvement in clinical outcomes, by providing novel insights to support personalised cancer treatment, as well anticipating and proactively managing therapeutic resistance.

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## Conflict of interest

K.L. has a patent on indel burden and CPI response pending and speaker fees from Roche tissue diagnostics, research funding from CRUK TDL/Ono/LifeArc alliance, and a consulting role with Monopteros Therapeutics. C.S. acknowledges grant support from Pfizer, AstraZeneca, Bristol Myers Squibb, Roche-Ventana, Boehringer-Ingelheim, Archer Dx Inc (collaboration in minimal residual disease sequencing technologies) and Ono Pharmaceuticals, is an

AstraZeneca Advisory Board member and Chief Investigator for the MeRmaid1 clinical trial. C.S. has consulted for Amgen, AstraZeneca, Bicycle Therapeutics, Bristol Myers Squibb, Celgene, Genentech, GlaxoSmithKline, GRAIL, Illumina, Medixci, Metabomed, MSD, Novartis, Pfizer, Roche-Ventana and Sarah Cannon Research Institute. C.S. has stock options in Apogen Biotechnologies, Epic Biosciences, GRAIL, and has stock options and is co-founder of Achilles Therapeutics. C.S. holds patents relating to assay technology to detect tumour recurrence (PCT/GB2017/053289); to targeting neoantigens (PCT/EP2016/059401); identifying patent response to immune checkpoint blockade (PCT/EP2016/071471); determining whether HLA LOH is lost in a tumour (PCT/GB2018/052004); predicting survival rates of cancer patients (PCT/GB2020/050221); to treating cancer by targeting insertion/deletion mutations (PCT/GB2018/051893); identifying insertion/deletion mutation targets (PCT/GB2018/051892); methods for lung cancer detection (PCT/US2017/028013); and identifying responders to cancer treatment (PCT/GB2018/051912).

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