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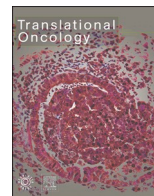
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Drug tolerance and persistence in bacteria, fungi and cancer cells: Role of non-genetic heterogeneity

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ABSTRACT

A common feature of bacterial, fungal and cancer cell populations upon treatment is the presence of tolerant and persistent cells able to survive, and sometimes grow, even in the presence of usually inhibitory or lethal drug concentrations, driven by non-genetic differences among individual cells in a population. Here we review and compare data obtained on drug survival in bacteria, fungi and cancer cells to unravel common characteristics and cellular pathways, and to point their singularities. This comparative work also allows to cross-fertilize ideas across fields. We particularly focus on the role of gene expression variability in the emergence of cell-cell non-genetic heterogeneity because it represents a possible common basic molecular process at the origin of most persistence phenomena and could be monitored and tuned to help improve therapeutic interventions.

Introduction

The phenomena of therapeutic escape are a major problem of public health. They are especially observed in the context of therapies targeting proliferating cells. Although they have been observed for a long time, their molecular and cellular bases remained largely unknown for decades[1,2]. Molecular tools allowed identifying genetic factors responsible for resistance phenotypes[3]. However, work performed within the two last decades shed light on other types of therapeutic escape that are not associated to genetically hereditary factors but rather to transient abilities of non-genetic origin to tolerate or persist in the presence of drugs[4,5]. These phenomena of tolerance and persistence are observed in various biological systems – bacteria, fungi and cancer – and there is increasing interest in identifying any common behaviors seen or molecular pathways involved, and the context-specific observations too.

Tolerance and persistence are terms widely used across species. They generally correspond to a state of dormancy allowing survival above normally inhibitory and lethal concentrations, but the terminology can be different across the fields and debated in a given field[5–7] (see Table 1 for definitions of resistance, tolerance and persistence in bacteria, fungi and cancer cell). Both tolerance and persistence are usually used indifferently in the cancer field, as shown by the common designation of Drug-Tolerant Persisters (DTPs) for those cells that transiently

survive lethal drug exposures.

Although they are also often considered superficially as similar phenomena by which growth-restricted cells survive treatment in microbial species, they correspond to specific situations. In bacteria, persistence is a transient phenomenon concerning only a fraction of the population of interest, and tolerance is the result of the acquisition of genetic mutations or environmental conditions that result in growth restriction at the whole population level[5] (Fig. 1). Despite this difference, it is often assumed that the same physiological states and molecular mechanisms underlie bacterial tolerance and persistence to antibiotics, explaining why tolerance is often studied to understand the molecular mechanisms governing persistence[5].

Similarly in fungi, tolerance is generally owed to a substantial fraction of the population (5–90%) while persistence involves rare cells (generally less than 1%) that transiently survive high concentrations of antifungal drugs (Fig. 1). But a difference with bacteria is that the term tolerance is used in fungi for cells that grow in the drug in specific assays [6]. Tolerance in fungi relies on differences in the ability to physiologically respond to environmental signals due to phenotypic heterogeneity but it is assumed to have a genetic basis, the allelic diversity between isolates explaining their variable levels of tolerance. Phenotypic heterogeneity can also have a purely non-genetic origin, originating for instance from stochastic gene expression or asymmetric molecule

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Table 1
Definitions of resistance, tolerance and persistence in bacteria, fungi and cancer cell.

	Resistance	Tolerance	Persistence
Cancer cells	Increased Minimal Inhibition Concentration (MIC) and cell growth in the presence of the anti-cancer drugs	<ul style="list-style-type: none"> - Occur when rare cells in the naïve population express a few drug-resistance genes, and thus, survive through the initial course of drug treatment - Persistent and tolerant terms are used interchangeably in the cancer literature eg. Drug-tolerant persisters (DTPs) - Able to slowly replicate - Arise due to non-genetic determinants - On prolonged drug treatment, persistent/tolerant cells acquire resistant traits through genetic mutation/evolution 	
Fungi	Reduced susceptibility of fungal cells to a drug due to heritable genetic mutations	<ul style="list-style-type: none"> - Generally owed to a substantial fraction of the population (5–90%) - Ability to survive longer times to drug exposure and to grow slowly at inhibitory drug concentrations, without an increase in MIC - Can be due to genetic and non-genetic determinants - Reservoir for genetic changes 	<ul style="list-style-type: none"> - Involves rare cells (generally less than 1%) that are able to grow at drug concentrations that are at least $8 \times$ the MIC - Can be due to genetic and non-genetic determinants - Reservoir for genetic changes
Bacteria	Increase of the MIC and the ability of bacteria to grow in presence of antibiotics	<ul style="list-style-type: none"> - Occurs when a population temporarily survives longer without growing in presence of lethal doses of bactericidal antibiotics - Characterized by a slower killing rate without a change in the MIC - Can be due to genetic determinants or growth-restrictive environments - Reservoir for genetic changes 	<ul style="list-style-type: none"> - Occurs when a small number of bacteria acquires a drug-tolerant state - Can be due to genetic and non-genetic determinants - Reservoir for genetic changes

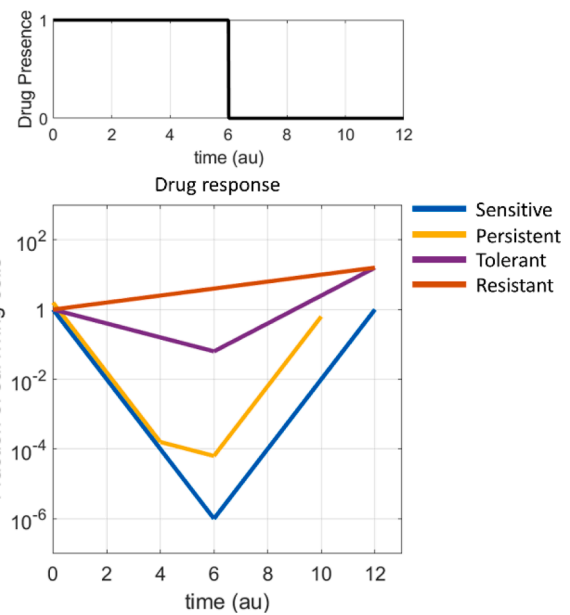


Fig. 1. Different survival modes in bacterial populations, while some modes are shared with fungi and cancer cells, some definitions are still different and less understood (Table 1). Resistant populations grow in presence of antibiotics. Antibiotic tolerance allows a population of bacteria to transiently survive longer without growing in presence of lethal doses of bactericidal antibiotics. Persistence occurs when a sub-population of cells survive for longer time under drug-treatment because of low killing rate as compared to sensitive cells. The presence and absence of drug treatment is shown by level 1 and 0, respectively.

Phenotypic heterogeneity: impact on transient resistance, tolerance and persistence in bacteria

It is well established now that isogenic bacterial populations, traditionally considered identical, are often composed of cells with a substantial phenotypic heterogeneity of non-genetic origin. Genetic identity does not always imply phenotypic identity even in constant environmental conditions (Fig. 2). In addition to stochastic variations in gene expression, the environment also plays an essential role in gene expression and, consequently, cell fate[2,10]. This heterogeneity can result due to fluctuations in transcription and translation[1]. Stochastic events associated with gene expression lead to the emergence of diverse phenotypes within isogenic populations[11]. This non-genetic heterogeneity allows a cell population to diversify so as to survive fluctuating environmental conditions. Thus, it is a bet-hedging strategy against a stressor for a finite period of time without requiring all cells to express costly genes (Fig. 2D). Interestingly, genes that show high levels of variations in expression are associated with stress response[12]. Heterogeneity in gene expression is omnipresent, varying widely across genes and biological functions. Its origin, control and selection are a rich subject of research discussed in the field[13–16]. Variations occur not only with gene function but with its regulatory network[17]. With multiple physiological consequences, heterogeneity in gene expression has been observed in bacterial metabolism, growth, substrate assimilation, compound secretion, virulence and resistance to stressors[18–23].

In this review, we discuss examples on the functional consequences that arise from existing, gradual or rapid changes in gene expression in the context of antibiotic resistance and persistence.

Antibiotic resistance and persistence

While resistance to antibiotics is defined by an increase in the minimal inhibition concentration (MIC), antibiotic tolerance and persistence enables bacteria to temporarily survive longer in the presence of

portioning during cell division for instance. We will explicitly state to which underlying mechanism we are referring when using this term.

Finally, cells that survive high drug levels were sometimes shown to proliferate in specific cases. In the cancer field, these cells are called cycling persister cells while in bacteria, such phenomena where a fraction of the population temporarily continue to grow and show a substantial reduction in susceptibility have been called transient resistance and heteroresistance [8,9]. In fungi it would correspond to the tolerant state described above.

In spite of these difficulties inherently linked to the different terminologies, we choose here to compare works on bacterial, fungal and cancer cells that transiently resist, tolerate and persist in presence of drugs with a particular focus on the role of cell-to-cell gene expression variability used to analyze the appearance and dynamics of persister cells. When available, examples of the role of non-genetic heterogeneity in gene expression in the appearance of persistence will be given. We expect this effort to cross-fertilize ideas across fields and unravel the common molecular attributes of drug-tolerant persister cells.

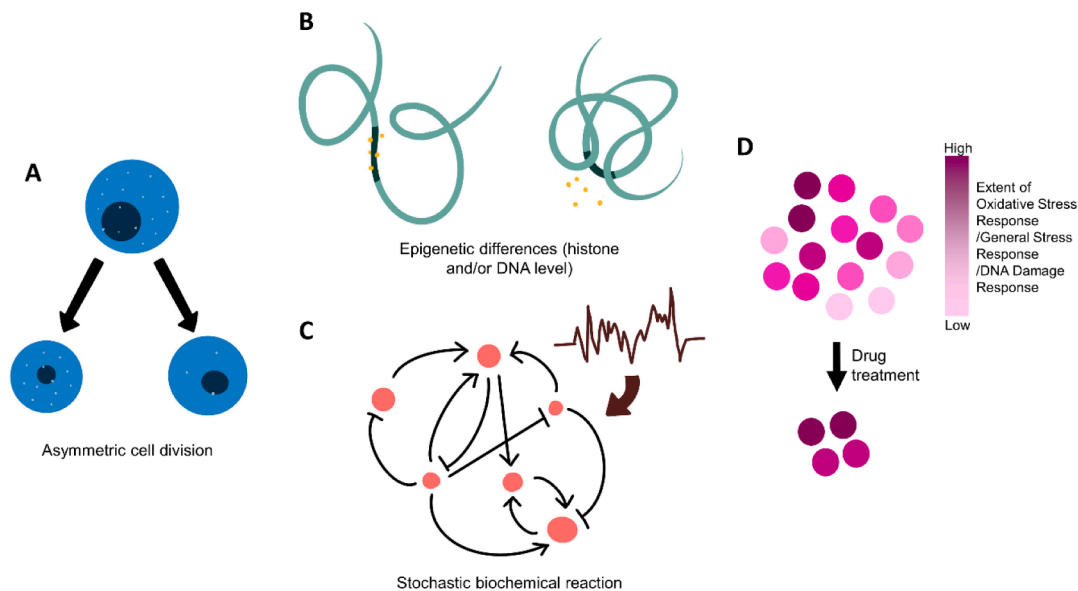


Fig. 2. Cellular processes giving rise to rare/edge cells in the population that show persistent behavior to drugs. A) Asymmetric cell division, B) Epigenetic differences (at histone and/or DNA level) among cells, and C) Stochastic biochemical reaction. A) A parent cell on division asymmetrically distributing its cellular content to the two daughter cells. B) Open and closed chromatin structure for the regulation of a gene (black) in the DNA strand (green). C) A network of biochemical species regulating each other's level and the stochasticity involved because of small molecular counts. D) Intercellular heterogeneity in the extent of oxidative stress response, general stress response and DNA damage; and survival of cells with elevated stress response upon drug treatment. Intercellular heterogeneity could be contributed by processes mentioned in panel A-C.

lethal doses of bactericidal antibiotics without exhibiting overt growth [24–26] (Fig. 1). Antibiotic tolerance allows a population to temporarily survive longer in the presence of lethal doses of bactericidal antibiotics without growing in their presence. It is associated to genetic determinants or growth-restrictive environments[5]. Growth arrest is particularly relevant as it reduces the efficacy of bactericidal antibiotics notably those targeting actively dividing bacteria[27,28].

Persistence has historically been derived from the observation of biphasic killing curves[5,26]. In scenarios involving persistence, there is a simultaneous presence of actively dividing and non-dividing bacteria in conditions that are supposed to be permissive for growth[5,26,29]. Persistence represents an interesting way in which bacteria can escape treatments. It is thought to underlie biofilm-related bacterial recurrence in medical contexts and mutants of well-known persistence genes are found in clinical isolates[30]. Persister cells can also form a long-lived reservoir of plasmid donors or recipients in environments like the gut [31]. While transient resistance is defined by the growth in presence of supposedly inhibitory concentrations of antibiotics, persistence is associated with growth arrest, slow metabolism and dormancy[5,32].

Our understanding of what governs persister formation is still partial especially because persistence was largely studied at the population level. Multiple genes involved in metabolism, growth, oxidative stress, DNA damage, and toxin/anti-toxins genes have been identified to play a role[33–36]. Moreover, due to the transient nature of persisters and their possible formation during growth, single-cell approaches emerged and have been essential in identifying this small fraction of the population and how variability in gene expression, growth and energy levels results in dormancy and antibiotic persistence.

Heterogeneity in gene expression and transient antibiotic resistance

In addition to persistence, phenotypic differences due to noise in gene expression could lead to different antibiotic resistance levels within genetically identical cells. Specifically, heterogeneity in gene expression and transient resistance allows single cells to survive antibiotic treatment without necessarily being persistent. An example is the multiple antibiotic resistance activator MarA. MarA plays an important role in

multi-drug resistance in bacteria, regulates the expression of over 60 downstream genes involved in antibiotic resistance[37], including multi-drug efflux pumps and porins regulators. While the role of MarA has been historically studied at population level, research involving single-cell techniques shows its stochastic expression among *Escherichia coli* single cells. Using *marA* promoter fused to cyan fluorescent protein gene (CFP) demonstrates the stochastic variability in MarA expression within single cell lineages. This variability correlates with single cell survival in presence of carbenicillin. Cells that had initially higher CFP levels, and thus MarA levels, are more likely to transiently survive the antibiotic exposure[38].

Another example of heterogeneity in gene expression involves a well-known resistance enzyme hydrolyzing beta-lactams, the cephalosporin hydrolase CTX-M-14. Cloning the *bla*_{CTX-M-14} gene into a sensitive *E. coli* strain leads to observable variation in resistance levels to ceftriaxone within the population. Cells that express more of this hydrolase survive and exhibit higher levels of resistance. Further, upon antibiotic exposure, cells within this population differentiated into a subpopulation of cells with a distinguishable phenotype of slowed growth and intensified hydrolase expression[39]. Further, in *Salmonella enterica*, in the absence of environmental stimuli, cell-to cell fluctuations in porins and efflux pumps expression and/or activity contribute to non-mutational resistance to kanamycin and nalidixic acid. Within a population, cells that express the porin gene *ompC* at lower levels and cells with higher efflux activity survive high doses of kanamycin and nalidixic acid respectively [40]. These examples together demonstrate that antibiotic killing does not occur in a homogeneous way within bacterial population.

Beyond antibiotic resistance genes, Rossi et al., computed the correlation between expression levels of genes with various cellular functions and the life expectancy of the cells expressing them[41]. The cellular functions included metabolism, cell structure, transport, and regulation. Unsurprisingly, the expression level varied within the reporters and their relationship with time to cell death. These promoters had different predictive power in presence of carbenicillin. Indeed, survival/death varied with their initial expression levels. Specific examples include the expression of *purA*, involved in AMP synthesis (positive relationship between cell death and fluorescence), *inaA* which

encodes a weak acid inducible protein and *gadX* which plays a role in acid resistance (negative relationship between cell death and fluorescence). The predictive power of a particular promoter varies in presence of different antibiotics. Cell growth rate is also shown to be predictive of cell death in presence of the beta-lactam carbenicillin. Furthermore, Sampaio et al., added a time scale component to quantify these expression dynamics. The authors follow the growth and gene expression of single cells in a mother machine microfluidics set-up where constant environment is flowing[42]. With a focus on stress response genes, they show several examples of genes where expression is pulsatile, with the amplitude and frequency of these pulses unique to each gene. In addition, they observed that variations in growth rates precede changes in gene expression. Importantly, the authors showed a functional consequence of this dynamic pattern where up-regulation of stress genes and slower growth affects the survival during sudden antibiotic exposure. In summary, noise is not only restricted to gene expression, but also witnessed in cellular physiology. For instance, coupling of RpoS expression and growth rate leads to the production of multi-generation RpoS pulses and higher cell survival[43].

In addition to events linked to biochemical reactions, factors linked to cell shape, cell cycle and changes in number of copies of a gene[44] can also contribute to non-genetic population heterogeneity. In *Mycobacteria*, asymmetric growth and division patterns generate a population of cells with heterogeneous cell sizes and elongation rates[45]. Non-genetic phenotypic heterogeneity contributing to single-cell survival can also arise from unequal distribution of cytosolic and membrane proteins. An example of this phenomenon is unequal partitioning of the multi-drug efflux pump AcrAB-TolC, a key determinant of membrane permeability to toxic compounds such as antibiotics. Monitoring cell division using a mother machine device shows that mother cells captured at the end channels are characterized by an increasingly old pole where AcrB accumulates in a TolC dependent manner[46]. This uneven partitioning creates a difference in drug efflux between cells and thus differences in antibiotic sensitivity.

While phenotypic heterogeneity of non-genetic origin arises from noise and random fluctuations in gene expression, the composition of the environment can certainly generate, amplify or decrease this phenomenon[10]. After stress with sub-inhibitory concentrations of the aminoglycoside antibiotic streptomycin, increased variability in growth rates and RpoH-driven heat-shock responses within individual *E. coli* cells was observed[47]. The authors proposed that an increased membrane permeability in a subset of cells serves as a positive feedback loop between stress level and reporter gene expression. Indeed, cells with high RpoH expression level prior to stress were more likely to be cleared by the antibiotic.

Furthermore, following an exposure with sub-inhibitory concentrations with trimethoprim, changes in expression of the acid resistance gene *gadBC* are observed and could predict single-cell survival probability in an acidic environment[48]. Indeed, within an *E. coli* population, noisy expression of this acid stress operon offers protection against the environmental stress of low pH. Even in a constant external environment, individual cells interact. Recently, Choudhary et al. observed a deterministic mechanism behind heterogeneity in oxidative stress response. By analyzing *E. coli* cells growing in microfluidic channels, they showed that upon H₂O₂ supplementation, the magnitude of the oxidative stress response is very sensitive to the cell's local environment and other neighboring cells[49] (Fig. 2D).

Heterogeneity in gene expression and antibiotic persistence

Fewer examples illustrate how gene expression heterogeneity is involved in antibiotic persistence[29]. Within a clonal population, most cells are killed upon treatment but a fraction enters dormancy and survives extensive treatment. An example shows that this entrance in dormancy depends on titration of the toxin HipA by the anti-toxin HipB which is a threshold-based mechanism resulting in the co-existence of

dormant and growing cells[33]. Furthermore, a recent study shows that variation in the abundance of Krebs cycle enzymes exists among *E. coli* cells and cells with a diminished Krebs cycle expression were enriched in persisters. Using an ATP sensor, the authors show a heterogeneity in ATP levels between isogenic cells and low ATP levels as well as smaller cell size predicted the ability of antibiotic survival[50]. Due to the transient nature of persisters, single-cell investigations went further to elucidate the specificity and heterogeneity even among a sub-population. Thus, heterogeneity can be seen even among the persisters, i.e. persister cells are not necessarily dormant before treatment and endure similar amounts of DNA damage compared to sensitive cells, instead, they contain specific traits during recovery in terms of SOS genes induction [51]. In addition, heterogeneity in persisters was shown under beta-lactam treatment where some cells accumulated less cytoplasmic drug. Indeed, *tolC* expression, a component of efflux pumps was correlated with bacterial persisters. This indicated a positive defense against antibiotics preceding dormancy and thus passive defense[52]. Finally, in *Mycobacterium smegmatis*, random fluctuations in expression of the catalase KatG encoding gene are important for cell survival. Thus, in presence of isoniazid, which needs to be activated by bacterial catalase, cells that express more catalase survive less[53].

Phenotypic heterogeneity: impact on permanent genetic changes and relevance in vivo

Changes in phenotypes can happen transiently but can also lead to permanent changes. Notably, persistence is shown to be linked to increasing mutations rates[54], heterogeneous expression of efflux pumps pre-disposes cells to permanent resistance and stochastic expression of DNA repair damage influences single cells mutations rates [55,56] (Fig. 2C). Additionally, upon oxidative stress, a burst of mutations happens in single cells via the transcription factor OxyR and the ROS-scavenging enzymes[57] and upon endogenous stress, sub-populations of phenotypic mutators can arise[58]. Finally, while phenotypic heterogeneity happens at the scale of individual cells on a non-genetic basis, it is clear that it can serve as a stepping stone towards permanent changes and affects a population cell fate.

Importantly, heterogeneity within populations and cell-to-cell variability in antibiotic response should be given particular attention as it is potentially relevant in clinical settings. For instance, in vivo, tolerance and persistence may be a significant contributor to infection relapse[32, 59]. Particularly, in the context of infection, the stress that bacteria encounter in different niches (eg. upon macrophage internalization, in bladder epithelial cells) can promote persistence and increase the fraction of cells that cannot be eradicated by antibiotics[60–62]. This topic is nicely reviewed by Personnic et al. and Helaine et al.[63,64]. Overall, in this section, we presented examples where bacteria take advantage of diverse mechanisms allowing heterogeneous gene expression and thus phenotypic heterogeneity of non-genetic origin in a population. Such heterogeneity can have functional consequences notably on transient resistance and persistence, two intriguing ways to cope with stress.

Fungal persisters

Fungal resistance and tolerance: definition and origins

Drug resistance in fungi is another important clinical problem[65], especially when considering that fungi resistant to all classes of agents used to treat invasive fungal infections (polyenes such as amphotericin B (AmB), azoles such as fluconazole and echinocandins such as caspofungin) have been characterized in most fungi that infect humans[66]. These resistance phenomena characterized by a higher MIC than the reference strain are of genetic origin[6] (Fig. 1). Beyond resistance, drug tolerance relies on different properties as it is defined as the ability of fungi to survive longer times to drug exposure, without an increase in MIC[67,68]. These subpopulations (5–90% of the population) grow

slowly in these conditions while the non-tolerant cells do not grow or have no detectable growth after 48 h in the drug [6] (Fig. 1). These tolerance phenomena are linked to the phenotypic heterogeneity intrinsic to an isogenic population mainly in the ability to respond to nutritional or environmental changes. The variability between cells and thus the proportion of tolerant cells depends on multiple genetic components that affect the ability to physiologically respond to environmental signals. Therefore, while tolerance is partly genetically determined, it is a consequence of phenotypic heterogeneity and relies upon several stress response pathways that allow some cells in the population to slowly grow in the drug. This phenotypic heterogeneity is clearly due to non-genetic differences between cells that can be the consequences of cell-to-cell differences in gene expression for instance [6]. It has been shown to be reversible in *Candida* species [67,69]. The mechanisms underlying tolerance are still being elucidated but some works suggest that tolerance may have to do with mitochondria as cells with dysfunctional mitochondria can sometimes grow in antifungal drugs [70]. Regulatory pathways were already shown to connect dysfunctional mitochondria and expression of multidrug resistance genes and mitochondrial function in *S. cerevisiae* [71] and mitochondrial loss is also associated with acquisition of resistance to azole derivatives in *C. glabrata* [72,73]. This is also connected to the role of the response to reactive oxygen species (ROS) in persistence (see below) (Fig. 2D). However, differences in the degree of tolerance between isolates is assumed to have a genetic basis and to be due to the allelic diversity of each isolate.

Fungal persistence: involved molecular phenomena and pathways

Besides resistance and tolerance, the term persistence is now also used in fungi to designate the survival in drug concentrations above the MIC but in that case the rare ability (<<1% of the population) to transiently survive very high concentrations of antifungal drugs [74–77]. The equivalent term that is generally used for fungi is "heteroresistance" but for the clarity of the comparison with other organisms, the term persistence will be used for fungi in this review [6]. These non-growing phenotypic variants are usually characterized in biofilms, especially for *Candida spp* [76] which are one of the most common causes of nosocomial systemic infections, in response to drugs such as AmB. They are of particular risk as these cells can repopulate the infection site after antifungal treatment and lead to chronic infections, especially in patients with compromised immune system [75,78,79]. Despite the need for treatment of persistent fungal infections, only recent research has started to decipher the factors that lead to tolerance and persistence, with an emphasis on the role of increased oxidative stress responses in mediating fungal persister cell survival [76], as for bacterial cell populations (see previous section). Antifungal drugs induce apoptosis through the production of ROS, and *Candida albicans* persister cells especially highly express superoxide dismutases (SODs) that protect against miconazole-induced ROS [80]. Other genes such as *AHP1* (alkyl hydroperoxide reductase 1) correlate with *C. albicans* persister levels in biofilms in the presence of high concentrations of AmB [81] (for a more detailed review, see [76]). Moreover, fungal persister cells activate stress-response pathways, and thus produce large amounts and accumulate glycogen and trehalose – the two protective molecules against ROS-induced apoptosis [76]. These stress protectants act as chaperones and allow survival by stabilizing proteins.

A recent review summarized data over the role of *C. albicans* stress response pathways in antifungal tolerance and resistance [82], pointing out the major role of Ca²⁺-calmodulin-activated phosphatase calcineurin, the protein kinase C cell wall integrity pathway, and the molecular chaperone heat shock protein 90 (Hsp90). Indeed, many proteins from the heat shock protein (HSP) family, especially the highly conserved ATP-dependent chaperone Hsp90, are upregulated in *C. albicans* biofilms persisters [83]. Accumulation of DNA damage also triggers fungal persistence as exemplified in *Saccharomyces cerevisiae* [84]. The

subsequent activation of the general stress response by increased DNA damage in rare cells provides protection against stress and drug environments. This phenomenon is coupled with an increased load of genetic variants in persister cells, producing genetic diversity that could be beneficial in challenging environmental conditions. As many antifungal drugs act on activating growing cells, fungal persister cells are thought to be in an inactive dormant state and be less metabolically active [76]. Interestingly, slower growing cells within a yeast population are more prone to DNA damage than their fast-growing counterparts, suggesting an increased potential of acquiring antifungal resistance mutations [85]. They also express a greater number of genes in general, suggesting a more permissive chromatin that may, in turn, allow them to explore a larger phenotypic space. These data suggest an interplay between open chromatin, DNA damage, general stress response, slower growth and persistence in fungi.

Recent data also pointed out that different other metabolic pathways can be determinant in the acquisition of the persister phenotypes. For instance, exposure of *C. albicans* to caspofungin at sub-MIC promotes persistence together with a decrease in respiratory metabolism [86]. In *Aspergillus fumigatus*, transcriptomic studies of the persister growth suggested that galactosaminogalactan and high expression of sterol biosynthetic genes and exporters are involved to establish persistence [87]. Finally, one of the major mechanisms of resistance to several classes of antifungals is the overexpression of efflux pumps, especially in *C. albicans* [88,89]. However, as AmB for instance kills the cells by the sequestration of ergosterol [90], drug efflux pumps are unlikely to be involved in persister cell survival because AmB does not need to enter into the cells.

Fungal persistence: a role for cell-to-cell heterogeneity in gene expression?

Persistence is known to rely on non-genetic mechanisms, such as gene expression noise that lead to cell-to-cell heterogeneity in drug response [91]. Of note, motifs in transcriptional regulatory networks are of particular importance as their 'wiring' modulates gene expression variability and non-genetic drug resistance, and enhanced robustness to cytotoxins and environmental stress [92–94]. In certain circumstances, high variability in gene expression allows emergence of subpopulations able to survive acute drug exposure. This subpopulation may then develop permanent resistance if the beneficial gene expression state is long enough to be passed along several generations. However, whether gene expression variability of some key proteins are responsible for phenotypic heterogeneity in drug response of non-genetic origin is poorly studied in fungi, while variability in oxidative stress response for instance is a key mechanism in fungal persistence [76]. Previous work focused on Heat shock factor 1 (Hsf1) that regulates chaperone gene expression, revealed that the increased cell-to-cell variation in Hsp90 levels generated by Hsf1 phosphorylation during heat shock contributes to the ability of *S. cerevisiae* to acquire resistance to fluconazole [95]. Another pioneering work employed the term persistence in *S. cerevisiae* to designate rare cells that grow very slowly and harbor increased expression of trehalose synthesis-related genes [96]. These cells were found to be probabilistically more resistant to heat killing. Thus, growth rate heterogeneity seems to serve as a bet-hedging mechanism, providing a benefit to the population across changing environments, especially in yeast. It was recently argued that while in bacteria, persistence relies on phenotypic switches, characterizing a persister state in yeast could be more difficult because of the more continuous distribution of states in the population between the majority of fast-growing cells with no expression of stress response genes and the slow-dividing cells with extreme levels of stress response proteins [97].

Persistent cells exist in *S. cerevisiae* both in biofilm and planktonic growth modes. Especially, treating a *S. cerevisiae* planktonic culture with AmB at 10-times the MIC in minimal medium, allows characterizing a persister subpopulation at the phenotypic and genotypic levels. The pathways responsible for *S. cerevisiae* persister cell survival in both

cultures were both the target of rapamycin C (TORC) and Ras pathways [98]. Finally, histone deacetylase inhibitors (HDACi) are known to enhance anti-fungal efficiency[99] and interfere with the phenotypic plasticity of *Candida* spp. by inhibiting biofilm formation and the generation of drug tolerant persister cells[100]. It remains unclear how these HDACi are able to kill persister cells, but they are also known to modulate gene expression noise[101]. It is therefore possible that the HDACi treatment modulates expression variability of these genes and thus modify the emergence of persistence as observed among cancer cells (see the section below)[102].

Cancer cell persisters

Genetic origins of drug-resistance

Genetic, epigenetic and gene expression variability and their phenotypic impact are commonly studied independently, while their multilayered interplay complexifies the way phenotypic variations appear. This interplay is especially interesting in the context of cancer drug resistance, considered as a model to study the complex mechanisms linking genotype and phenotype through adaptive variability and phenotypic plasticity. Historically, understanding resistance to therapy in cancer has been focused on genetic drivers of drug resistance, including genetic alterations that allow bypassing target inhibition (impaired drug binding), activation of downstream effectors in the same signaling pathway or activation of alternative pathways[103,104]. Evolutionary studies on this topic in the last decade helped to decipher if these mutations are pre-existing or acquired, and it appeared that clinically relevant drug-resistant cancer cells can both preexist and evolve from drug-tolerant cells in which resistance is then genetically assimilated (see[105]). Thus, the emergence of resistance results either from the selection of rare pre-existing genetic alterations upon drug treatment or the acquisition of *de novo* mutations during treatment[106].

Non-genetic origins of drug-resistance – drug-induced resistance

However, accumulating evidence shows that the ability to survive upon drug treatment cannot be reduced to simple genetic causes. The ability of a single cancer genome to produce multiple phenotypic states is now recognized and it is acknowledged that cancer cells can switch between these states without genetic alterations[107]. Importantly, such non-genetic reprogramming events are observed on therapy exposure, and these adaptive responses are associated with increased resistance to the treatment[108]. Thus a second model of therapeutic resistance acquisition, denominated as Lamarckian induction, proposes that resistant phenotypes are transiently acquired by a small subpopulation of cancer cells through epigenetic modifications[106]. A pioneering work in this conception showed the rapid generation of Multi-Drug Resistance (MDR) phenotype in HL60 leukemic cells and the increase in MDR1 expression following chemotherapy and suggested the early drug resistance phenotype is independent of selection and due to Lamarckian mechanisms[109]. Thus, the multidrug resistance in vincristine-treated cells would not be explained by Darwinian selection but rather by ‘Lamarckian induction’. Other examples in melanoma followed and all suggested that drug-tolerant cells in melanoma does not emerge through passive selection but rather appear transiently through active cell state transition[110,111]. Thus ‘Lamarckian induction’ now refers to such somatic evolution towards a better ‘adapted’ inheritable and advantageous phenotype induced by the environmental change [108]. The main difference between a classical Darwinian selection and such induced non-genetic cell response is that the adapted response is induced in cells that subsequently survive while not being more resistant *a priori*[112].

Non-genetic origins of drug-resistance – pre-existing resistance

A third pathway to drug resistance is now under intense investigations since the first description of a subpopulation of cancer cells called DTPs that transiently survives lethal drug exposures in non-small-cell lung cancer (NSCLC)[102]. In this pioneering study, the authors showed that a majority of cultured NSCLC cells exposed to therapy were killed but also that ‘drug-tolerant’ cells transiently and rapidly accumulated. Importantly, the kinetics and frequency of appearance of these viable cells cannot be explained by genetic modification and can be modulated by exposure to HDACi[102]. It had been earlier thought that drug tolerance corresponds to a state of transient survival with no proliferation upon treatment[108]. On the contrary, recent data showed that cycling cancer persister cells can arise due to metabolic and expression adaptations allowing cell cycle re-entry for a rare subset of persister cells[113]. In that specific case, persister proliferative capacity was associated with upregulation of antioxidant genes and a metabolic shift to fatty acid oxidation with in multiple cancer types. Impeding oxidative stress or metabolic reprogramming allowed altering the fraction of cycling persisters[113]. Colorectal cancer persisters generated upon targeted therapy are also able to slowly replicate and importantly they show an increased mutation rate under treatment[114].

The different ways in which persistent cancer cells can evade treatment is becoming a major focus in the field of cancer drug tolerance (see [115] for more details), with increasing attention on non-mutational mechanisms - phenotypic plasticity, metabolic switching and remodeling the tumor micro-environment. Moreover, DTPs have a high antioxidant stress response that protect themselves against the oxidative stress-induced cytotoxicity. For instance, DTPs are vulnerable to inhibition of glutathione peroxidase 4 (GPX4), leading to disabled antioxidant capacity and increased ROS levels[116]. Thus, redox signaling is of particular importance in DTP cells by allowing a robust antioxidant process[117] (Fig. 2D). Also, the DNA repair is compromised in persister cells from colorectal cancers and resulted in elevated DNA damage, most likely caused by the formation of ROS as a source of DNA damage[118].

From pre-existing to drug-induced non-genetic resistance

Despite extensive research in the past decade, whether DTP cancer cells harbor a specific ‘primed’ drug-tolerance or occur through non-genetic reprogramming by therapy remains unclear[108]. Nevertheless, patient-derived glioblastoma stem cells for instance can evade RTK inhibition and other therapies by acquiring a slow-cycling persister state through widespread remodeling of repressive chromatin[119]. In that case, cells reversibly transit between proliferative and slow-cycling states on an epigenetic basis, thus allowing tumors to propagate, adapt, and persist upon therapeutic pressure. Recent data on HER2+ breast cancer cells revealed rare cells of drug-tolerant cells that exist even before treatment. These cells that stochastically acquire a ‘pre-DTP state’ preferentially yield DTPs upon HER2 Tyrosine kinase inhibitors exposure[120]. Interestingly, lapatinib DTPs activate mTORC1 via a PI3K-dependent and AKT-independent pathway. The mTOR pathway plays a crucial role in the formation and maintenance of DTPs in many cases[121]. DTPs were also identified in colon cancer and melanoma, especially in a pioneering work where very rare melanoma cells were shown to transiently express very high levels of resistance genes prior to drug exposure (to vemurafenib) that allowed them to more likely survive to treatment compared to parental cells[122]. When the drug was removed, DTPs gave rise to sensitive cells, suggesting that the drug-tolerant state is reversible and transient rather than genetically heritable. Furthermore, although pre-resistant cells express only a small fraction of resistance genes (72 of 1456), the percentage of resistance genes expressed strongly increased after adding drug (600 of 1456 after one week and 966 of 1456 after 4 weeks), showing a progressive modification of the transcriptome as cells became stably resistant[122]. Thus, expression of these markers fluctuates in cancer cells and the rare

cells that express 'by chance' high levels of a sufficient number of resistance genes at the time of treatment survive and then modify their transcriptome to stably express a resistance gene signature[106]. Thus, DTPs seem to not be a pre-existing, well-defined subpopulation but on the contrary they stochastically arise from a dynamically fluctuating cell population. Further, similar to observations in bacteria (see section above), a large number of phenotypically distinct fates is seen within a seemingly homogenous cell population[123]. This single-cell variability occurs despite the clonal origin of the population and it homogeneous cell culture conditions. This rare sets of cells spontaneously emerge due to transient fluctuations in cell-state and can persist for 5–6 generations. Interestingly, this duration represents an intermediate timescale between gene expression changes driving differences between cell types in different tissues that are retained over a large number of cell divisions, and highly transient fluctuations in transcription referred to as gene expression noise that hardly associate with physiological distinctions between single cells[124]. Cell states that persist for several divisions are thus partly, but not indefinitely heritable. Importantly, they result from coordinated fluctuations in the expression of many genes in single cells. The timescale of maintenance of these cell-states was enabled by Memory-Seq to identify groups of genes co-fluctuating in rare-cell expression "programs" maintained over 5–10 cell divisions[124]. Applied to data on HER2+ breast cancer cells, this analysis revealed that the transient heritability of the DTP state significantly differed between cell lines ranging from 2 to 6 generations[125].

Pre-treatments to reduced drug-tolerant persister

Recently, analyses of human melanoma cells allowed quantifying long-lived fluctuations in gene expression that underlie resistance to targeted therapy and identified the PI3K and TGF- β pathways as modulators of gene expression memory and thus of the DTP state [126]. The authors proposed a strategy of 'pretreatment' of the primed state through PI3K inhibition before applying BRAF inhibitor in combination with a MEK inhibitor (BRAFi/MEKi). They succeeded in reducing the frequency of drug resistance by initially disrupting the DTP state and altering plastic cell states. Thus, these rare, heritable but ultimately reversible states are related to non-genetic mechanisms of therapy resistance in cancer[123]. Other examples in which short-term highly transient fluctuations can drive phenotypic changes have also been observed. Especially, a prominent role of gene expression noise was shown in the emergence of resistant cells among estrogen receptor-positive breast cancer cells[127]. Indeed, the reduction of gene expression stochasticity and of associated transcriptomic and phenotypic heterogeneity of non-genetic origin through inhibition of members of the KDM5 demethylase family decreased the number of cells acquiring resistance to endocrine therapies. Thus, reducing transcriptomic heterogeneity by acting on epigenetic mechanisms generates a lower risk of therapeutic resistance. A screening for single-cell variability modulators also identified genetic factors that changed the frequency of melanoma cells primed to survive BRAF^{V600E} inhibition and thus the degree of resistance of population[128]. These genetic factors modify cancer cell resistance to targeted therapies by altering their cellular plasticity. Finally, it appears that these non-genetic mechanisms could be tuned so as to modify the level of diversity and the number of DTPs that they generate, and to impact the evolution of drug resistance in mammalian cells. Expressing specific resistance proteins with high or low noise in a specific network circuit showed that only cells with the low noise gene circuit mutated to stably adapt stable resistance by acquisition of mutations within the circuits[129]. This shows an important interplay between non-genetic and genetic phenomena in the acquisition of cancer cell resistance to therapies[91] as mentioned above for bacterial cell populations.

Mathematical modeling of intracellular and population dynamics of persister cells

Along with experimental breakthroughs, mathematical models have also contributed to the efforts of understanding the emergence of persistence and resistance cell population. The mathematical modeling efforts can be broadly classified into two classes: 1) models that help understand regulatory and stochastic molecular-level processes enabling persisters and resistant cells in the population; 2) population dynamics models of sensitive cells, resistant cells and persisters.

Pre-existing drug-resistance and its heritability

The variability in protein levels in stress response pathways can lead to bet-hedging strategies where certain subpopulations survive the drug treatment (Fig. 2). For instance, using two synthetic dox-inducible acetyl-transferase (PuroR or pac) gene circuits with positive and negative feedback controls, it was shown that the positive feedback control circuit gave rise to more variability in PuroR levels than negative feedback control while keeping the mean PuroR levels similar, and therefore, conferred adaption and resistance to much higher concentration of the antibiotic Puromycin[129]. The authors further showed via population-level dynamical modeling that multiple cell-states – sensitive, non-genetic resistant and genetically resistant states – are required to explain extended periods of growth inhibition post-treatment with increasing concentration of drug in the experimental setting. This concept of decoupling mean and variability of expression levels was alluded to in another experimental study demonstrating that while the transcriptional burst frequency governs the variability in gene expression, the burst duration determines the mean expression levels. Importantly, the frequency and duration of transcriptional burst was found to be independent (orthogonal) to each other across genomic locations[130]. Not just variability in gene expression but also its extent of inheritance in successive generations plays important role for a population to survive drug treatment. In a mathematical model for the AcrAB-TolC efflux pump regulatory network (EPRN), epigenetic inheritance of transcriptional rates of the EPRN operon was shown to be necessary for bacterium to adapt rapidly to lethal drug treatment, while adaption on a long-term basis occurred through genetic inheritance (efflux pump efficiency)[131]. Further, because the efflux pumps also move out the essential metabolites for bacterial growth apart from flushing the drug out of the cell, it was shown that the high cost of efflux pump to population fitness post-treatment made the drug sensitive state (with lower efflux pump levels) to regain a majority in the population. Together, these population-level models highlight an interplay between genetic and non-genetic drug-tolerant subpopulations in determining the population-level fitness.

At an intracellular level, the determination of a persistence or resistance cell-fate is often enabled jointly by proteins belonging to several different pathways. Using mathematical modeling of TRAIL-induced apoptosis, Spencer et al. 2009 found that the cell-fate – survival or death – of HeLa and MCF10A cells on TRAIL-induced apoptosis was determined based on the combined levels of DR4/5 receptors, DISK components, CASP8 and BID in the apoptosis pathway[132]. Having large similarity in their protein distribution, the sister cells had similar time-to-death or survival probability than any two cells chosen at random. Recently, cell-fate determination in cancer cells by protein-levels distribution are more precisely reported using combination of lineage-tracing using DNA barcodes and mRNA single molecule fluorescent in-situ hybridization (sm-FISH). By following a pair of sister cells where one is drug-naïve and the other is exposed to vemurafenib drug treatment in BRAF^{V600E}-mutated melanoma cells, the drug resistant cells were shown to have coordinated high expression of more than three out of seven genes: *AXL*, *EGFR*, *NGFR*, *WNT5A*, *ITGA3*, *MMP1*, and *FN1* genes[122,123]. Using mathematical modeling of gene regulatory

networks, Schuh et al. 2020 showed that transcriptional bursting, where gene flips between transcriptionally active and inactive states, is necessary to produce such rare coordinated high states[133]. Further, the frequency of rare high states decreases with increasing connectivity of nodes, and is independent of number of nodes in the network. Moreover, while the entrance in the rare coordinated high state starts with a long transcriptional burst of one gene in the network, the exit from the cell-state happens through independent inactivation of individual genes expression.

Evolution of non-genetic resistance

With the emergence of drug persistent/resistant cell population in a population, it becomes critical to quantify the timescales at which the sensitivity of the population to the drug changes with time (Fig. 1). Mathematical modeling of the population growth of three breast cancer cell lines (MCF7, BT474, and MDA-MB-231) treated with doxorubicin for – 1) increasing concentration, 2) varying inter-treatment interval, and 3) varying number of doses – led to identification and quantification of both delayed apoptosis of sensitive cells and delayed proliferation of resistant cells on drug exposure[134–136]. Specifically, it was shown that cellular sensitivity to drug dynamically evolves during drug treatment and optimal inter-treatment interval is crucial for complete eradication of cancer cells. Significant efforts are being made to identify optimal treatment strategies with single and/or combination of drugs with the benefit of synergistic responses[137–139]. Thus, population-level models can be used to understand the emergence of persisters and resistant cells in a population at varying time-scales as well as suggest optimal therapy designs to maximize the therapeutic effect on a population.

Considering overall population growth as a fitness metric, multiple mathematical studies show cancer or bacterial cells optimally switch their cellular states to maximize their growth in variable environmental conditions. Initial theoretical endeavours in early 2000s showed that the switch between normal and persister bacterial cell states must be synchronised with the environmental fluctuation rate for the optimal growth of population[140]. Further, for optimal growth, stochastic cell-state switching was shown to be more feasible than sensing and responding to the microenvironment when the fluctuations were infrequent[141]. The bacterial cells population was shown to have two distinct cellular memories – phenotypic memory and response memory – to respond to the environment fluctuation occurring at different time-scales (>1 and <1 cell generations, respectively)[142]. Similarly, George et al. considered cells to switch their phenotype to fluctuating (favourable and unfavorable) environment while keeping track of past patterns in environmental fluctuations[143]. The authors showed that the extent of environmental state memory (number of past events recorded) determined the adaption time, and therefore suggested a dynamic memory size that maximizes the expected growth in all environmental conditions.

Switch from non-genetic to genetic drug-resistance

How do persister cells contribute to long-term emergence of resistant cells in a population has been investigated both experimentally and mathematically. For instance, in lung cancer cells exposed to erlotinib, persisters were shown to serve as a reservoir of heterogeneous drug-resistance mechanisms[144]. Similarly, *E. coli* strains showed a positive correlation between persistence and likelihood to gain genetic drug resistance. A theoretical model predicted that high mutation rates in bacterial persister cell state can enable them to act as a reservoir for emergence of resistant cells in the population. Thus, both a higher cell-state switching rate from sensitive to persister, along with high mutational rate in persisters, is required for emergence of stable resistance population that surpass drug intervention[54]. While cells adapt to their environment, interventions (drug exposure) can be made at

critical time to maximize treatment outcomes. For instance, non-genetic drug resistance can increase survival of the cell population while limiting the emergence of genetic drug resistance through competition between non genetically and genetically resistant sub-population. This depends multiple factors such as the carrying capacity of the environment, the treatment regimens and the fitness of the resistant sub-populations[145].

Discussion and perspectives

As recently highlighted, there is a need for a well-defined and consistent consensus on definitions among the different communities that would help comparison and progress toward understanding the mechanisms, either shared or unique, involved in the different organisms[68]. Such cross-kingdoms understanding of persistence holds the potential to brought up innovative strategies[146]. However, when analyzing in parallel the transient resistance, tolerance and persistence phenomena in face of antibiotic, anti-fungal and anti-cancerous treatments, some similarities already appear. First, when time-dependent experiments were performed (pre- vs post-treatment), they revealed that the persister states can preexist before treatment and that they spontaneously appear in the population, even if environmentally-induced heterogeneity in gene expression can also lead to persistence. Second, shared cellular processes can give rise to rare/edge cells that show persistent behavior (Fig. 2, Table 2): asymmetric cell division, epigenetic differences (at histone and/or DNA level) among cells and stochastic biochemical reactions, even if those mechanisms are less documents in fungi than in other kingdoms. Third, common pathways can be involved, especially the oxidative stress response (bacteria, fungi and cancer cells), or the activation of general stress response pathways (TOR/mTORC1 pathway in fungi and cancer cells and RpoS response in bacteria) (Fig. 2 and Table 2). Activated DNA damage response pathways seem also common among the persisters in the three kingdoms, and associated with increased mutagenesis (increased mutagenesis precedes drug treatment in yeast whereas it occurs during treatment in cancer cells). Also, among fungi and cancer cells, persister cells harbor a more open chromatin structure. Interestingly, common debates are also found, especially regarding the proliferation state of the persister cells. Recent works on cancer cells tend to validate the fact that persister cells can indeed proliferate, at least in some cases, while they were thought to be rather in a quiescent state at the time of treatment.

Some specificities can also be noted, such as the importance of pump efflux in the persistence phenotypes regularly showed in bacteria but not in the other contexts. Concerning the investigation methods, modeling studies have been mainly performed on cancer cells (and to a lesser extent on bacteria), and they should inspire more researches on yeasts when regarding the importance of being able to anticipate the behavior of persister cells under therapeutic pressure. Also, no study was published on the role of gene expression stochasticity in the generation of persisters in fungi while such fluctuations were shown to be of particular

Table 2

Shared molecular features of persister cells in bacteria, yeasts and cancer cells.

	Bacteria	Yeasts	Cancer cells
Oxydative stress response	56. V. Lagage <i>et al</i>	79. A. Bink <i>et al.</i> 80. T. Truong <i>et al</i>	115. M. J. Hangauer <i>et al.</i> 116. Z. Zhang <i>et al</i>
General stress response	33. O. Pacios <i>et al.</i> 42. O. Patange <i>et al</i>	82. P. Li <i>et al.</i> 97. R. Bojsen <i>et al</i>	119. C. A. Chang <i>et al</i>
Increased DNA damage	35. T. Dörr <i>et al</i>	83. G. Yaakov <i>et al.</i> 84. D. van Dijk <i>et al</i>	117. M. Russo <i>et al</i>

importance in bacterial and cancer persisters. It is entirely possible that a common origin resides in the random expression of specific sets of genes allowing the transient appearance of the persister state. Thus modulating phenotypic plasticity, especially by acting on gene expression stochasticity, seems to be a viable therapeutic option that should limit therapeutic escape as already partially shown on breast cancer cells [127]. Finally, after genetic mutations/mechanisms having been the focus of drug resistance studies and laboratory diagnosis for bacteria, fungi and cancer, researchers are now finding that alternative/non-genetic forms of resistance likely underly misdiagnosis and treatment failure, which might eventually lead to genetic forms of resistance, and be the target of new therapeutic strategies [147].

CRedit authorship contribution statement

Imane El Meouche: Writing – original draft, Writing – review & editing. **Paras Jain:** Writing – original draft, Writing – review & editing. **Mohit Kumar Jolly:** Writing – review & editing. **Jean-Pascal Capp:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Not applicable as this is a review article.

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