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Clinical trial designs for evaluating and exploiting cancer evolution

Alvaro H. Ingles Garces [a], Nuria Porta [b], Trevor A. Graham [c], Udai Banerji [a,*]

[a] Drug Development Unit, The Institute of Cancer Research and The Royal Marsden NHS Foundation Trust, UK
[b] Clinical Trials and Statistical Unit, The Institute of Cancer Research, UK
[c] Centre for Evolution and Cancer, The Institute of Cancer Research, UK

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ABSTRACT

The evolution of drug-resistant cell subpopulations causes cancer treatment failure. Current preclinical evidence shows that it is possible to model herding of clonal evolution and collateral sensitivity where an initial treatment could favourably influence the response to a subsequent one. Novel therapy strategies exploiting this understanding are being considered, and clinical trial designs for steering cancer evolution are needed. Furthermore, preclinical evidence suggests that different subsets of drug-sensitive and resistant clones could compete between themselves for nutrients/blood supply, and clones that populate a tumour do so at the expense of other clones. Treatment paradigms based on this clinical application of exploiting cell–cell competition include intermittent dosing regimens or cycling different treatments before progression. This will require clinical trial designs different from the conventional practice of evaluating responses to individual therapy regimens. Next-generation sequencing to assess clonal dynamics longitudinally will improve current radiological assessment of clinical response/resistance and be incorporated into trials exploiting evolution. Furthermore, if understood, clonal evolution can be used to therapeutic advantage, improving patient outcomes based on a new generation of clinical trials.

Introduction

Cancer is a genetic disease[1], with cells constantly turning over and acquiring changes in gene function over time. It can also be defined as a disease of evolution, governed by environmental selection of phenotypic features. Interestingly, while some of the genetic changes can be transient, such as modification in gene expression or post-translational function of encoded proteins, a proportion of genetic changes are irreversible and inherited by daughter cells leading to tumour evolution.[2–6] Some of these genetic changes are deleterious and result in cell death, others are neutral, and a few are advantageous and drive future generations of cancer cells.

The irreversible genetic changes over time can lead to temporal heterogeneity, i.e., differences in the genetic composition of cancer cells within a primary tumour or metastasis over time and, eventually, to spatial heterogeneity, i.e., differences in the genetic composition of cancer cells between different parts of a tumour or between different sites of metastases within the same patient.[7,8] The staggering degrees of heterogeneity within a single tumour or between different metastasis of the same tumour have been described in surgical samples of tumours removed at surgery, e.g., nephrectomy[9–12] or multiple samples at autopsies.[11,12] Moreover, the rapid role of next-generation sequencing (NGS) combined with the availability of curated sequential biopsies and circulating free-DNA samples collected longitudinally over time has allowed a glimpse into temporal heterogeneity.[13–17].

Both spatial and temporal heterogeneity of cancers are associated with resistance to anticancer therapy. Current methods of controlling or eliminating cancer cells such as chemotherapy, radiotherapy, targeted therapy, or immunotherapy rarely lead to cure once the tumour cannot be completely surgically excised. This is predominantly due to the presence of resistant clones within the population being treated (spatial heterogeneity), the future occurrence of new mutations, or re-population of tumours with resistant clones that were present in very small numbers in the initial population (temporal heterogeneity). It is also important to consider that tumour heterogeneity is not only the result of mutational events, but it is also related to changes in the tumour microenvironment (e.g., blood flow variation, distinct variation between tumour edge versus tumour core etc). Those mechanisms that do not rely on the presence of new mutations and cause cellular plasticity include epigenetic changes/rewiring of signal transduction/changes in

* Corresponding author at: Drug Development Unit, Sycamore House, The Royal Marsden NHS Foundation Trust, Downs Road, Sutton SM2 5PT, UK.
E-mail address: Udai.Banerji@icr.ac.uk (U. Banerji).

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gene expression, or lack of drug penetration due to stroma and the expression of drug efflux pumps. Further, the microenvironment where the tumour is placed is associated with fluctuation of immune cells, nutrients, growth factors and hormones, and alter spatial and temporal evolution of cancer cells within a tumour cell population.[18,19] Regardless of the method or combination of methods, resistance to therapy and eventually metastatic disease are the causes of morbidity and mortality in a wide range of cancers.

Thus, evolutionary forces will drive cancer cells to resistant states and populate tumours with resistant clones in a spatial and temporal fashion. Maximum-tolerated dose of drugs is one of our most common and current approaches where the same drug can tackle multiple grades of sensitivity in cancer cell populations, dealing, to an extent, with spatial heterogeneity. Also, the use of combinatorial therapy when the addition of individual drugs can affect clones resistant to the other drug (s) within the combination.[20,21] This rationale of combining mechanistically different drugs trying to overcome the tumour heterogeneity and promote more sustained remission/disease control[22,23] has also been found to fail as resistant phenotype(s) eventually emerge[24] even if the tumour adaptation in this situation requires a higher fitness cost [25].

Other methods of treating resistant clones identified over time include using new drugs of different classes like different chemotherapy regimens, targeted therapy, immunotherapy, or radiotherapy in a sequential fashion ("sequential therapy"). These approaches have led to considerable success in treating cancer, but cure or long-term responses (decades rather than months or a few years) in metastatic disease in a vast majority of solid tumours remains elusive.

A significant majority of randomised trials in metastatic cancer therapy are designed to evaluate interventions such as chemotherapy, targeted therapy, immunotherapy, or radiotherapy, used as a single agent or in combination, randomised against a control group aimed to demonstrate the superiority of the experimental arm. Following this model, there is a very high likelihood of the treatment eventually failing with the emergence of drug resistance due to cancer evolution. With our improving understanding of tumour evolution and the increasing availability of tools to track temporal (e.g., cell-free DNA) and spatial heterogeneity (widespread use of single-cell sequencing or functional imaging using antibody labelled PET tracers), the time is ripe to harness this understanding and use those tools to design the next generation of clinical trials (Table 1). Ultimately, this will result in improvement of the outcomes for patients with metastatic disease.

Table 1
Tools to design the next generation of clinical trials.

Tissue	Procedure	Techniques	Use of tissue
Tumour	Serial biopsies	Next generation sequencing, CpG methylation, gene expression and protein expression	Temporal changes in mutations, gene expression, protein expression and clonal dynamics
Tumour	Multi-regional sequencing	Next generation sequencing, CpG methylation, gene expression and protein expression	Spatial distribution of mutations, gene and protein expression, and clonal dynamics within different parts of the tumour or between metastasis
Tumour	Serial functional imaging	Antibody labelled Zr/Ga probes e.g. Zr^{89} labelled trastuzumab	Temporal changes in tissue expression of cell surface biomarkers and spatial heterogeneity of expression
Tumour Markers	Serial blood sampling	e.g. CA125, PSA ELISA	Dynamic study of tumour burden
Circulating tumour cells	Serial blood sampling	Cell counting, single-cell sequencing	Temporal changes in mutations, gene and protein expression, and clonal dynamics
Circulating tumour DNA	Serial blood sampling	Next generation sequencing and CpG methylation	Temporal changes in mutations, gene and clonal dynamics

Here we present fundamental concepts related to cancer evolution, i.e., herding of clonal evolution/collateral sensitivity and evolutionary game theory, and discuss clinical trial designs that could be used to evaluate our attempts to apply those concepts to therapeutic advantage.

Herding clonal evolution and collateral sensitivity

Cancer cells are often deficient in maintaining genome integrity[26], making them susceptible to rapid acquisition of additional genetic modifications such as mutations, rearrangements, amplifications or deletion. This process will lead to progressive intra-tumoral heterogeneity (ITH). Tumour cells also display differences in epigenetic and post-translational modifications[26], a key feature for drug tolerance/persistence and cells' ability to acquire resistance through stable non-genetic changes in gene expression.[27,28] Therefore, different strategies to reduce ITH need to be explored to improve long-term therapeutic responses, prolonged remission, and maybe tumour eradication.

"New" genetic mutations (not detected previously) are identified in tumour specimens in a clinically drug-resistant state following cancer treatment. These mutations could occur as *de-novo* mutations within tumours[29] or be pre-existent in very low numbers (therefore, not new mutations in fact, but not previously identified).[30] Either way, cells with genetic mutations that confer drug resistance increasingly populate tumours during therapy or relapse following an initial response, resulting in a clinical drug-'refractory' or -'resistant' status.[30,31] If it is possible to predict new mutations and/or new susceptibilities, we could hypothesise that we may eventually 'herd', 'steer', or direct cancer evolution for the tumours to acquire dominant populations of cells with known susceptibility to specific anticancer drugs. In addition, a few biological processes are known to be associated with an increased number of mutations. For instance, APOBEC expression in cancer cells has been shown to be associated with mutagenic showers or *kataegis.* [32–34] The importance of APOBEC, particularly later in tumour evolution, is highlighted by the observation that this mutational process was found to be a major source of subclonal cancer gene mutations in bladder, breast, head/neck squamous cancers, lung adenocarcinoma, and lung squamous cell carcinoma [33,35]. Therefore, developing preclinical models that track populations of cancer cells under the selection pressure of anticancer drugs could smartly predict the mutations of the subsequent resistant tumours. This information could potentially be exploited when using adjuvant/maintenance therapy following an initial tumour response to treatment to herd clonal evolution, reducing ITH, and enriching the tumour population of clones with known sensitivity to a given treatment (Fig. 1 A-B).

In addition to tracking and predicting mutations in drug-resistant populations and herding clonal evolution to enrich tumours with clones that are known to be sensitive to a specific treatment, it may be possible to exploit the concepts of evolutionary biology further. Studies of bacterial drug resistance have revealed evolutionary trade-offs related to key drivers/facilitators of cell growth that occur during changes in environment or antibiotic treatment.[36,37] Interestingly, there is now increasing new evidence that cancer cells may follow similar evolutionary trajectories, i.e., gaining an evolutionary advantage by acquiring mutations to become resistant to the selection pressure caused by an anticancer drug. This may result in dependence on other biological processes that make the cells more susceptible to other therapies. [30,38–41] Thus, in the future, it could be envisaged that one could artificially generate populations of cells within a tumour that would make it more sensitive to a treatment before the intervention. For example, if we could predict a population of cells with an increased expression of a unique surface protein or acquire a microsatellite instability, they would be vulnerable to treatments including antibody-drug conjugates/CAR-T therapy targeting the surface protein.[42,43]

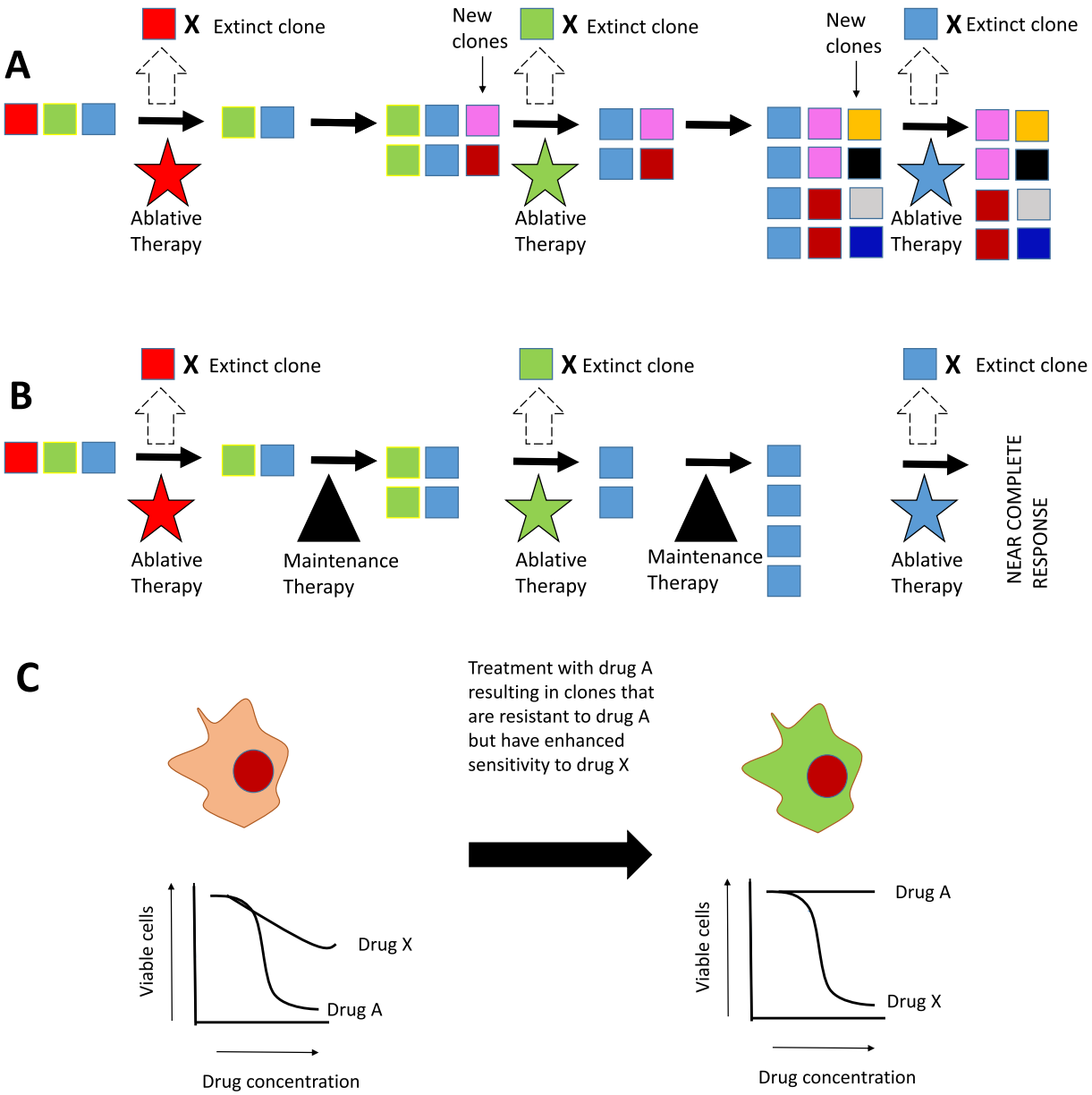

Fig. 1. Herding of clonal evolution and collateral sensitivity. A) Lines of therapy lead to tumour shrinkage and reduction in populations of sensitive clones but also to selection of resistant/tolerant clones and emergence of new clones that populate the tumours. Multiple lines of therapy lead to the accumulation of different drug-resistant clones and tumour heterogeneity that eventually lead to a lack of response and progression. B) Use of maintenance with a drug following a clinical response to the initial treatment. The role of the maintenance therapy is not to prolong the duration of response of the initial treatment but to reduce the number of clones that are resistant to subsequent therapies and to reduce heterogeneity. C) Collateral sensitivity. A cancer cell is initially sensitive to drug A and resistant to a range of drugs, including drug X. Upon becoming resistant to drug A, the cancer cell becomes dependent on other biological processes, e.g. a secondary mutation or, alternatively, acquires a phenotype that is not necessarily to survival advantage, e.g. overexpression of a surface protein. Either of these factors can make it sensitive to a drug that it was not before, e.g. drug X.

(Fig. 1 C). Mathematical models derived from preclinical experiments now offer multiple hypothesis as the most advantageous next line of therapy. While preclinical data offers us a glimpse of ways of applying the concepts of cancer evolution to gain an advantage in cancer therapy, it is key to consider the clinical settings and methodology to evaluate these hypotheses.

Herding design

There are a number of disease subtypes where the response to treatment is rapid but transient, where no maintenance therapy has been approved or when it is, the benefits are short-lived[44]. For example, small-cell lung cancer first-line maintenance therapy with immune checkpoint inhibitors has marginal clinical benefits.[44–47].

An example where there is no approved maintenance therapy, includes patients with metastatic KRAS mutated colon cancer treated with a chemotherapy regimen such as FOLFOX. This combination of chemotherapeutic agents often needs to be stopped after six months due to toxicity, such as neuropathy or neutropenia, despite a significant number of patients responding to treatment but unable to tolerate any further immediate subsequent systemic intervention (i.e. a maintenance therapy).

In the "herding" trial design, patients diagnosed with tumour subtypes where no maintenance therapy has been approved would receive the same standard of care first-line systemic therapy, according to their disease. Patients where an objective response measured by RECIST or its

variations/modifications is demonstrated, would be randomised to a control group (no maintenance therapy, as the current standard of care) or different maintenance treatments (investigational/experimental arms). Upon radiological progression/relapse, all patients receive the same second line of treatment. The primary outcomes could include time-to-progression to the second line of treatment (time on trial), measured from the randomisation point (experimental maintenance therapy versus no maintenance therapy), response rate to the second-line therapy, and other co-primary or secondary endpoints would be progression-free interval from the start of the second-line treatment (Fig. 2 A). This design essentially tests how different investigational maintenance treatments "herd evolution", influencing the response to the same second-line therapy and for how long the initial residual disease (molecular residual disease or radiologically measurable disease) would be controlled by the maintenance therapy. Important to consider that in our examples, we have listed the line of therapy as first and second lines, but this could be adjusted to "n" and "n + 1" lines of treatment, as long as all patients were chronologically at the same line of therapy.

It is important to distinguish between the herding trial designs proposed in this manuscript from trials of different maintenance therapies that have been attempted before. For example, the IFCT-GFPC 0502, a phase 3 randomised trial, compared maintenance therapy with gemcitabine or erlotinib or no intervention after first-line therapy with cisplatin-gemcitabine in patients with advanced non-small cell lung cancer (NSCLC) [48,49] The endpoint in the study was progression-free survival from the start of the maintenance therapy till progression. We would propose having a fixed next line of treatment and studying the objective response rate and progression-free interval of this fixed next line of treatment. Besides this, we also propose to study time on trial from randomisation to a selected maintenance therapy through to the fixed next line of treatment, and till progression to the following line of therapy (Fig. 2A).

There are multiple clinical trials that use different DNA damage/DNA repair inhibitors as a mechanism to increase the tumour mutational burden and prime cancer cells to improve outcomes with immune-checkpoint inhibitor therapy. For example, patients have been treated with olaparib or temozolomide, followed by the association of immune checkpoint inhibitors in trials such as MEDIOLA and MAYA studies, respectively[50,51]. The next step for these trials, in relevant tumour types, could include randomising patients to either placebo or different priming treatments, such as DNA damaging agents like temozolomide or DNA damage repair inhibitors like PARP or ATR inhibitors, followed by the combination of immune checkpoint inhibitor therapy. The primary endpoints would be the time to progression from the randomisation to the "priming agent" until the progression of disease on immunotherapy and the objective response rate of the immunotherapy phase. The secondary or co-primary endpoints would include progression-free survival while on immunotherapy.

Herding designs have limitations and confounding factors. Firstly, the "herding" trial design will only be applicable when there is a significant response to the first line or "nth" line of treatment – where a maintenance therapy is appropriate. Other potential confounding variables will be differences in the timing of starting the second line of therapy, as different patients will have progression of disease/relapse at different moments. It is fundamental that the timepoint when patients are randomised to start the maintenance therapy is within a predefined window of time after completing the initial line of therapy to evaluate the effect of this investigational maintenance treatment appropriately. The duration of maintenance therapy could be another confounding factor, as patients could have progression/relapse at different times. This could be overcome by limiting or fixing the duration of the

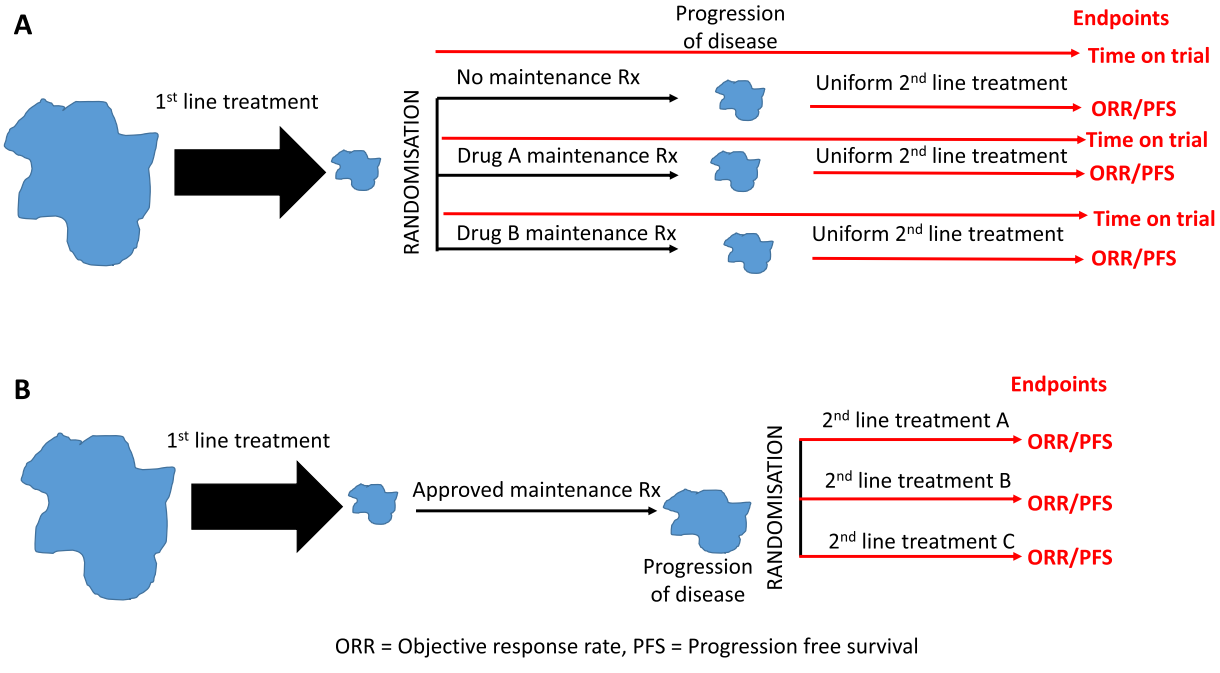

Fig. 2. Clinical trial designs to evaluate herding of clonal evolution. A) Herding design: following first line treatment of a tumour type where the standard of care does not include maintenance therapy, patients are randomised to a control arm with no maintenance therapy or a number of different maintenance therapies. After a fixed period of time or upon progression, patients receive the same approved second line treatment. The primary outcomes are lenght of time on trial from randomisation and objective response rate/progression-free survival of the approved second-line therapy. This trial evaluates the role of the experimental therapies to herd evolution that will be advantageous to standard of care second line therapy. The trial does not necessarily have to be first line; it could be nth line of therapy as long as a uniform n + 1 line of therapy is used. B) Collateral sensitivity or herded design: Cancers where maintenance therapy is standard of care or cancers where adjuvant therapy has been given in very high-risk cohorts could be considered. Upon progression, patients are randomised to receive a control arm that is justifiable by current standard treatment paradigms and 1–2 different experimental arms. The trial evaluates the role of the standard of care maintenance and/or adjuvant therapy to cause collateral sensitivity to multiple second line treatments. The trial does not necessarily have to be second line treatment, it could be nth line of therapy as long as a uniform previous n lines of treatment, and a uniform maintenance/adjuvant therapy is used.

maintenance or priming treatment schedule, as was done in examples related to the MEDIOLA and MAYA trials mentioned previously. These need to be worked out into inclusion criteria and/or accounted for in the statistical analysis and sample size calculation.

Translational research during trials with herding designs will enhance our understanding of cancer evolution and drug resistance. Liquid biopsies evaluating circulating-free DNA (cfDNA), circulating-tumour DNA (ctDNA) or circulating tumour cells (CTCs) could be collected before starting maintenance treatment, and then at progression - before starting second-line treatment, reflecting the 'herding process' (changes in clonal populations that have been herded by the different control/experimental arms).

In rare instances when sequential tumour biopsies could be taken at these time points (multisampling would be ideal, but it is not feasible), NGS sequencing of tumour tissue will provide insights into the development of new mutations following different experimental maintenance therapies. An anticipated outcome of herding is that clonal diversity is reduced as tumour cells are shepherded down a particular evolution path. Clonal diversity can be quantified in a straightforward fashion using the Shannon diversity index[52,53] or the "Evo and Eco-Indexes" – a tumour classification based on evolutionary and ecological features proposed by Maley et al. could also be used.[54].

Collateral sensitivity (herded) design

There are instances in cancer treatment where maintenance therapy is the standard of care, for example, PARP inhibitors following chemotherapy in a patient with advanced high-grade serous ovarian cancer who has responded to platinum-based chemotherapy and this "herded design" could be easily applied. Moreover, this model could be extended to adjuvant therapy in cases where the risk of relapse is high, such as surgery and chemotherapy followed by aromatase inhibitors in oestrogen-positive breast cancer with the involvement of axillary lymph nodes.

Patients in a herded clinical trial design will be treated with a known regimen that includes a primary treatment e.g., surgery, radiotherapy, or chemotherapy (or any line of therapy), followed by an adjuvant/maintenance therapy (which could be hypothesised to cause clonal herding) and established as standard of care. Upon progression following the adjuvant/ maintenance therapy, these patients will be randomised to multiple options of investigational treatment regimens. The endpoints will include objective response rates of the treatment post-randomisation and progression-free survival of the treatment offered post-randomisation (Fig. 2 B). This design tests the sensitivity of herded cancer cell clones within the tumour or collateral sensitivity caused by the adjuvant/maintenance therapy. Unintentionally, we have already done such clinical trial models in many instances. For example, randomised studies of different investigational regimens after relapse following adjuvant tamoxifen or aromatase inhibitor therapy in breast cancer could be considered, at some extent, a collateral sensitivity or herded design where patients treated with selective oestrogen receptor degraders or combinations of oestrogen receptor degraders and PI3K alpha inhibitors [55–59] The herded trial design could potentially be applied to any treatment regimens that are administered in a fixed sequence with the caveat that the first therapy (adjuvant or "n + 1") followed by maintenance is used for a uniform period and have the same outcome (disease control – partial or complete response).

Collateral sensitivity or herded designs also have limitations and confounding variables. Firstly, it can only be applied to a clinical scenario where adjuvant/maintenance therapy (or the n + 1 line) is standard of care. Secondly, in instances where adjuvant treatment (or the n + 1 line) contains multiple drugs, e.g., FEC (5-FU, epirubicin, cyclophosphamide) in node-positive breast cancer, it will be difficult to mechanistically attribute herding to one drug. On the other hand, it could be argued that this would not be problematic, as ITH is being addressed by the combination of the mechanistically different drugs. Finally, other confounding features may include length of time of adjuvant/n + 1 treatment and time between completion of adjuvant/n + 1 treatment and randomisation, maintenance therapy length, which all will need to be factored into the inclusion criteria and statistical analysis.

Translational research outputs in the herded design would be of particular interest to understanding collateral sensitivity although difficult to conduct. An option would be to include multiple sampling biopsies at surgery or a tumour biopsy immediately after response to the initial therapy and a second biopsy after randomisation and prior to treatment. Surface expression of a number of proteins actionable to ADCs/BITEs/CAR-T therapy in the pre- and post-adjuvant therapy could shed light on collateral sensitivity. Largely theoretical but technically possible would be the establishment of organoids of patient-derived xenografts (PDXs) from pre- and post-adjuvant/n + 1 treatment tissue. This could open out the possibility of testing drug sensitivity and thus the possibility of quantifying collateral sensitivity in these tissues and molecularly guided choices for further lines of therapy. [31,39,60–63].

Evolutionary game theory

Evolution game theory has been variously used in biological systems and economic decision-making models for many decades.[64–70] It is a mathematical toolkit to study how populations evolve when individuals in the population are competing with each other (playing a competitive "game"). In the context of cancer, it could be defined as the study of different strategic interactions of tumoural cells and their environment when cancer cell fitness depends on the environment, and different cancer cell phenotypes are present in the original tumour population (called "competitors" and "co-operators").[71] More specifically, applying this concept of cancer evolution, we could say that cancer cells are "players" and the "game strategies" are the different cell phenotypes that result from different mutations in a tumour population (clones/subclones).[71] Therefore, similar to a chess game, the oncologist, based on the current status of the disease, can plan and decide which therapeutic strategy will be used, and the cancer cells, in response, will try to adapt to the treatment that was applied. This is called Stackelberg game [72] (leader–follower), where the oncologist has the advantage of making the first move, leaving to the cancer cells the only option of "choosing" adaptive strategies followed by the selection pressure exerted by the treatment imposed by the oncologist.[24].

This model is not without downsides, as in reality, tumours are rarely constrained by a fixed anatomical boundary where they have to compete for resources. Furthermore, growing hypoxic tumours often create an environment of enhanced angiogenesis, which increases the supply of oxygen and nutrients, facilitating the growth of multiple clones simultaneously.

There are clinical trial designs that can be used to better understand and exploit the competitive interactions between cells; for instance, using intermittent schedules of the same treatment or, more speculatively, cycling different drugs/classes of drugs at regular intervals (Fig. 3).

Intermittent dosing to allow competition between sensitive and resistant clones.

Preclinical studies evaluating ovarian cancer xenograft models randomised animals to a) receive carboplatin at fixed intervals or b) an adaptive therapy based on adjusted doses, not allowing the tumour to grow, or c) no treatment at all (control group). Mice treated as "standard" recommendations (fixed doses and intervals) had the tumour initially reduced in size, but it started to grow again a few weeks later. The adaptive therapy group had stable disease throughout the experiment, allowing the mice to survive longer with a small tumour burden. [73] The same research group performed other experiments using breast cancer cells and mice, which showed that treatment with adaptive

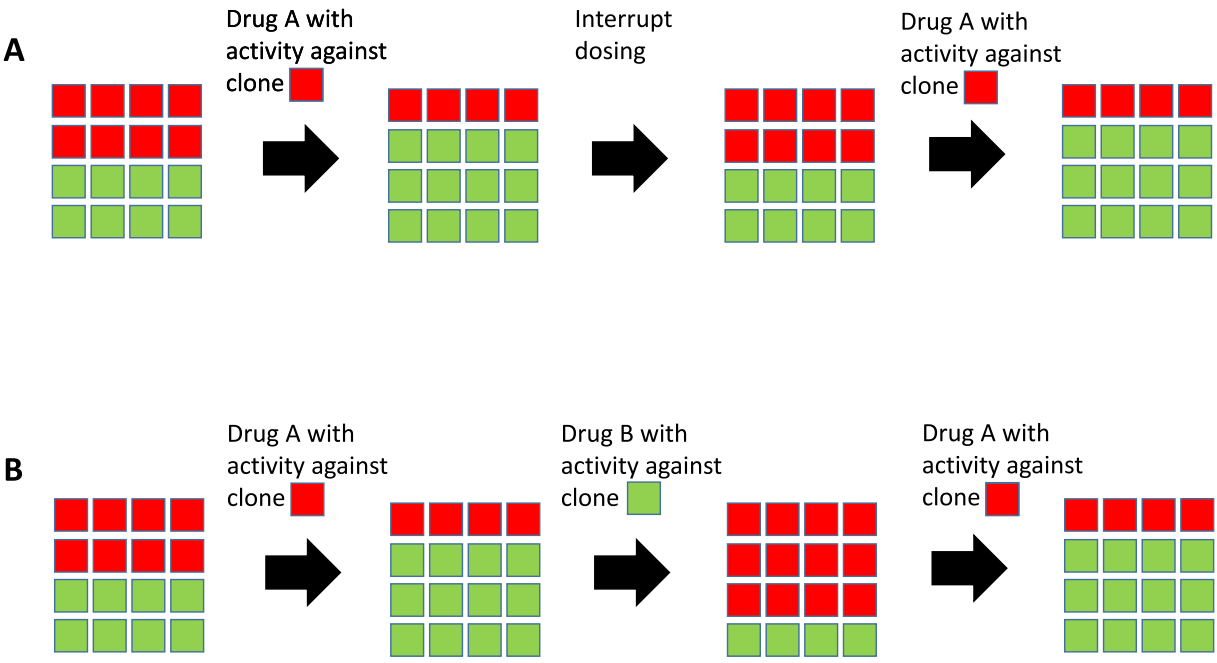

Fig. 3. Game theory and cancer therapeutics, A) Drug A is highly effective against a set of clones in a tumour type. Upon response, therapy is stopped to allow regrowth of the sensitive drug clones that will compete for resources (e.g., nutrients, blood supply) with the drug-insensitive clones. These trials will require frequent monitoring of tumour size to re-institute treatment and not let the tumour grow past its size at baseline. As it is not possible to image tumours so frequently, surrogate biomarkers of tumour size could be used. Examples of biomarkers could include PSA or CA125 in prostate or ovarian cancer. B) Drug A and Drug B have mutually exclusive subsets of clones within a tumour. Alternating between Drug A and Drug B in alternative cycles could lead to regrowth of clones sensitive to the drug that is not being used in every cycle resulting in competition for resources between two sets of clones within a tumour.

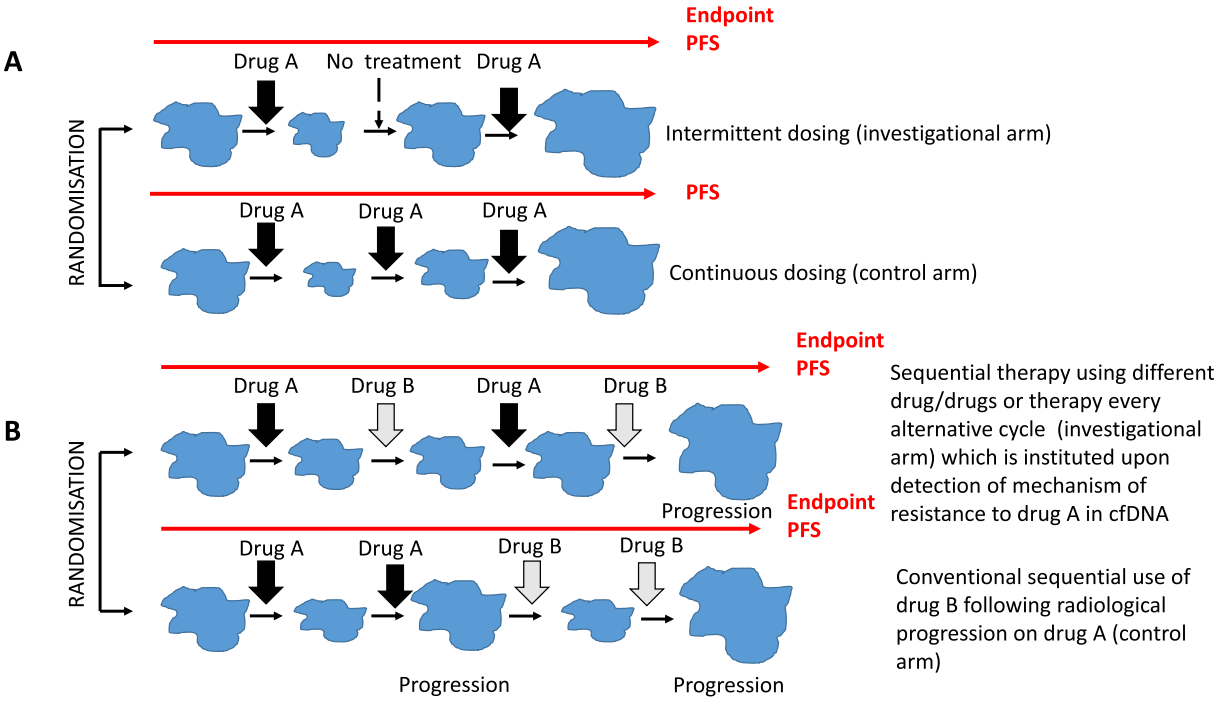

Fig. 4. Trial designs for evaluating game theory. A) Patients are randomised to continuous treatment or interrupted treatment. The endpoints include progression-free survival from the point of randomisation for the continuous treatment (control) and intermittent treatment (experimental arm). B) Patients are randomised to receive a sequence of drug A followed by drug B on alternate cycles following detection of mechanisms of resistance in ctDNA detected prior to radiological progression on drug A (experimental arm) followed till radiological progression. The standard of care (control arm) includes patients treated on drug A till progression, followed by drug B till progression. The endpoints would be progression-free survival, defined as time since randomised till the radiological progression in the experimental arm and the second radiological progression in the control arm.

therapy generated tumours with less necrosis and a more stable blood supply, suggesting that this approach could stabilise the resources in the "ecological" environment.[74].

Interestingly, such intermittent adaptive dosing has been evaluated in the clinical setting, for example, with prostate cancer. Patients with metastatic castration-resistant prostate cancer were enrolled in a non-randomised pilot study using abiraterone.[75] In this trial, treatment with abiraterone was interrupted when the PSA result was below 50% of the starting treatment level. Abiraterone was restarted if/when PSA levels were greater than 100% of the baseline when patients were enrolled on the trial (or radiological progression). Strikingly, 10 of 11 patients maintained stable oscillations of tumour burden; the median time to progression was at least 27 months compared to 16.5 months in the continuous use setting (non-head-to-head comparison).[75] Given the initial success, this study progressed, and the updated data showed that in a total of 16 evaluable patients following this adaptive strategy, the median radiographic time-to-progression was 30.4 months versus 14.3 months reported in the literature for the standard of care. Moreover, patients in the adaptive therapy group received an average abiraterone dosing rate (mg drug/patient/unit time) of 41% compared to standard of care.[76,77].

This clinical trial design would entail patients in the metastatic setting being randomised upfront to receive either continuous therapy until progression of disease, or intermittent therapy. In the intermittent scenario, treatment is to be stopped at a partial response or defined surrogate such as drop of tumour marker to a prespecified level or, if validated in the future, allele frequency of a specific driver mutation. The patients are then restarted when tumour reaches a predefined endpoint of regrowth (radiological, biochemical, or genetic), ensuring a degree of selection pressure to sensitive clones (Fig. 4 A). The clinical trial endpoints would be overall survival and progression-free interval from randomisation to progression. Ideally, we would define success as the intermittent schedule having a statistically significant longer progression-free survival and, ultimately, prolonged overall survival. Alternatively, the trial could be powered to show non-inferiority of the intermittent schedule compared to the continuous schedule as patients with the intermittent one are being spared the toxicity of treatment such as continuous chemotherapy. An important co-primary or secondary endpoint in the latter case would be a statistically better quality of life in the intermittent schedule. Tertiary and translational aims of these trials would be to demonstrate if maintaining different subpopulations of cells with different sensitivity to the same drug measured by multiple biopsies or circulating free DNA would compete with each other to therapeutic advantage.

Such trials are not without risks, including the *de-novo* development of drug-resistant clones (to the current drug and multiple future treatment options) during each interruption of therapy. The timing of stopping and re-starting the therapy that was put on hold is also crucial and would likely have to involve multiple scans (which might not be practical). Some cancers where tumour markers are a good surrogate for tumour growth, e.g., PSA for prostate cancer or CA125 in ovarian cancer (as being currently tested in the ACTOv trial, NCT05080556), may be better placed to be evaluated using this trial design. It is critical to genetically profile the balance between the drug-sensitive and less sensitive clones, and performing multiple biopsies every time a treatment is restarted is not feasible, leaving the use of circulating-free DNA as an alternative.

Alternate cycles of different anticancer agents to maintain a balance between populations of cells

Ideally, designing adaptive trials should be carried out after extensive *in vitro* and *in vivo* preclinical testing. This "alternate cycles of different anticancer agents" trial model design can be applied to a hypothetical situation where tumours consist of two or more sets of predominant clones, each clone exclusively sensitive to one drug and not to others used in the treatment regimen. Drugs would be used alternatively in sequence to promote a balance of the populations of clones within a tumour. This situation is hypothetical as it is uncommon for drugs to have mutually exclusive activity on different sets of clones. An example could include EGFR mutated NSCLC treated with EGFR inhibitors such as osimertinib. Mechanisms of resistance include MET amplifications [78–82] or C797X mutations [83–86]. A logical "alternating-cycles" clinical trial exploiting evolutionary principles could include a study where a patient with EGFR mutated NSCLC on osimertinib treatment is monitored for the appearance of MET amplifications and C797X mutations on ctDNA while still in radiological response or stable disease on osimertinib. Upon detection of these mechanisms of resistance in ctDNA, they will be randomised to continue on osimertinib till radiological progression, followed by treatment with a MET inhibitor or EGFR inhibitor that is effective against an EGFR C797X mutation till new progression (whichever is the detected mechanism of resistance) as the control arm. The experimental arm at randomisation could include alternating 28-day cycles of osimertinib and a MET or EGFR inhibitor active against EGFR C797X mutations (depending on the mechanism of resistance detected) till progression. This design will interrogate the ability to keep clones of cells sensitive to different drugs within a tumour to achieve control over a longer period rather than using drugs for shorter periods where tumours become rapidly and completely resistant to individual drugs used sequentially.

Therefore, this clinical trial design would include upfront randomisation to a control arm that sequentially uses two targeted regimens, one after another, following radiological progression after the first treatment. The experimental arm would include starting the first drug and, upon detection of a mechanism of resistance on ctDNA (while still radiologically not progressing on the first drug) and using alternating cycles of the first drug and a second one that will target the mechanism of resistance to be continued until progression. (Fig. 4 B). Theoretically, in the alternating schedule, we would aim to make two populations of clones in competition with each other, each with exclusive sensitivity to one of the two drugs. Translational studies tracking allele frequency of mutations that confer sensitivity to individual drugs in these novel drug schedules will shed light on the real-time biology of such patients. Clinical trial endpoints would include progression-free intervals of both drugs given in alternating cycles versus progression-free intervals measured from the start of the first treatment to the progression of the second drug in the conventional treatment arm. The critical challenge in these studies is monitoring the different clones to know when to initiate the changes in treatment. Clearly, to biopsy tumours after each cycle or a few cycles of treatment is not practical; however, assessing allele frequencies of mutations in ctDNA is feasible[87,88].

Challenges in implementing clinical trials to study cancer evolution

The foremost challenge would be negotiating funding for these trial designs. As some of these studies are paradigm-changing and include following up patients across two lines of treatment (herding design and herded design), those trials will be longer than the conventional ones evaluating one line of therapy. Further, some of the drugs in the herding and herded designs may involve standard-of-care treatment, and funding and monitoring of these will require negotiations with governmental and insurance companies. In designs such as intermittent dosing, a reduction in the amount of drug used compared to continuous dosing may not be in the interest of pharmaceutical manufacturers. In models using alternating drug schedules, the drugs may belong to different pharmaceutical companies, which may require complex negotiations.

A further challenge for implementing these trial designs is the increased use of monitoring patients with radiological measures such as CT scans or analysis of blood tests such as circulating-free DNA. These come with technical challenges such as quality control of acquisition and analysis of data across multiple clinical sites and increased costs[40]. It

will be required an increased investment into the capacity of clinical trials infrastructure, which is unlikely to be recovered from per-patient costs for clinical studies.

Another challenge is the training of the appropriate number of staff, including clinical, methodological and basic scientists, who will be required to interact regularly or, in many instances, in real-time to make and analyse treatment decisions in multidisciplinary meetings. [89,90].

Final considerations

Clinical data continues to emerge linking evolutionary forces to intra-tumoural and inter-patient heterogeneity. In some ways, the evolution of cancer cells within a tumour in the face of selection pressure should not be a surprise as it is a biological process seen widely across the animal and plant kingdoms. While evolutionary changes in nature occur over decades or generations (e.g., natural selection), it is accelerated to years or months in cancer due to the rapid and deregulated division of cancer cells and the selection pressure caused by anticancer drugs.

There are now many systemic treatments available, including chemotherapy, targeted therapy, complex molecules utilising localised mechanisms such as antibody-drug conjugates or bispecific antibodies, immunotherapy, and cellular therapies. Current clinical trials of new anticancer drugs focus on how to improve survival and, importantly, how to show the benefit of an individual intervention such as the use of a new drug as a single agent or in combination. There is also often a push to try to move all effective new therapies earlier on the treatment setting to maximize the chance of clinical success and drug tolerance, before patients develop extensive cancer-related morbidity. This generates intensive first line therapies often with poor quality of life for patients. For instance, FOLFIRINOX[91] or chemo-immunotherapy/chemo-immuno-antiangiogenic therapy [92,93] in pancreatic cancer and NSCLC respectively, may leave very few treatment options following progression to first-line treatment.

Another important point is the insertion of mathematical modelling in clinical trial design. It can be a powerful tool to understand the evolutionary process undertaken by the tumour in response to the different selective pressures and to try to predict the best approach as the next step for cancer treatment. Mathematical models can help to predict how different treatments may affect the tumour behaviour – growth/progression/response to different therapies, the timing for initiating and stopping drug interventions and even identification of predictive biomarkers. Mathematical modelling can, therefore, improve the design of trials and the decision for sequential lines of therapy. Considering the complex dynamics of cancer evolution and the development of cancer resistance, mathematical modelling can be used to simulate the tumour evolution and to predict how they will respond to different treatment strategies of sequential therapy.[94]

Based on factors such as tumour size, location of disease/metastatic sites, mutation rates, drug effectiveness, and retrospective data collected from other trials and clinical practice, models could be used to predict the optimal timing and sequencing of different therapies. Moreover, they can also be used to evaluate the potential risks and benefits of different treatment sequences and estimate the most likely to provide benefits and safety for patients.

The time is ripe for new generations of trials that need to be designed to show a sequential and better use of multiple effective drugs used as single agents or less intensive combinations in the patient journey. This is enabled by experimental models (wet biology and mathematical modelling) and technological advances allowing tracking of evolution, e.g., cell-free DNA monitoring, multi-site biopsies and functional imaging. Radically re-thinking the way we use existing and new treatments, and by exploiting the inevitability of cancer evolution rather than cataloguing and accepting its consequences, will result in patient improved patient outcomes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

All authors contributed to the conception and design of the work, review of the literature, draft, review, and edition of the manuscript. All authors have approved the final version and agreed to be accountable for all aspects of the work.

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