TITLE: Circulating tumor DNA in advanced prostate cancer: transitioning from discovery to a clinically implemented test.

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Summary:

The genomic landscape of metastatic castration-resistant prostate cancer (mCRPC) differs from that of the primary tumor and is dynamic during tumor progression. The real-time and repeated characterization of this process via conventional solid tumor biopsies is challenging. Alternatively, circulating cell-free DNA (cfDNA) containing circulating tumor DNA (ctDNA) can be obtained from patient plasma using minimally disruptive blood draws and is amenable to sequential analysis. ctDNA has high overlap with the genomic sequences of biopsies from metastases and has the advantage of being representative of multiple metastases. The availability of techniques with high sensitivity and specificity, such as next-generation sequencing (NGS) and digital PCR, has greatly contributed to the development of the cfDNA field and enabled the detection of genomic alterations at low ctDNA fractions. In mCRPC, a number of clinically relevant genomic alterations have been tracked in ctDNA, including androgen receptor (*AR)* aberrations, which have been shown to be associated with an adverse outcome to novel antiandrogen therapies, and alterations in homologous recombination repair (HRR) genes, which have been associated with a response to PARP inhibitors. Several clinical applications have been proposed for cfDNA analysis, including its use as a prognostic tool, as a predictive biomarker, to monitor tumor response and to identify novel mechanisms of resistance. To date, the cfDNA analysis has provided interesting results, but there is an urgent need for these findings to be confirmed in prospective clinical trials.

Introduction

Prostate cancer is one of the leading causes of cancer death in males in the western world[1], which occurs following the development of castration resistance. However, metastatic prostate cancers show heterogeneity in their clinical behavior, ranging from slowly progressing disease with long-lasting responses to antiandrogen therapy to rapidly lethal tumors characterized by resistance to available therapies. With the increasing number of options for the treatment of advanced prostate cancer[2,3], we hypothesize that further improvements in patient outcomes require molecularly directed treatments paired to clinically implementable biomarker tests. This approach requires a stepwise process from biomarker discovery and assay development to clinical qualification, first retrospectively and via the prospective analysis of retrospective collections and then, importantly, in prospective trials where the biomarker defines treatment randomization. The molecular landscape of metastatic castration-resistant prostate cancer has been characterized and includes several recurrent molecular pathways[4,5] that are putatively involved in tumor progression and resistance. These pathways include *AR* mutations and amplifications, *TP53* mutations and loss, DNA repair gene deletions and *PTEN* loss.

Intrapatient tumor heterogeneity has been associated with the reduced efficacy of systemic therapies in many forms of cancer[6-8]. Advanced prostate cancers are no exception; these cancers have been shown to be driven by multiple competing clones that display intra and intertumor heterogeneity[9]. Evolutionary diversity and clonal evolution might encounter several bottlenecks, including the development of metastasis and selective treatment pressure, that might limit the individual diversity of the main oncogenic drivers[10]. Treatment pressure induces the emergence of multiple clones either by selection or by divergent differentiation[9,11-13] (**Figure 1**).

Taking a biopsy of a metastasis is the gold standard approach for analyzing the genomic landscape of advanced disease. However, biopsy is invasive, carries a risk of morbidity, and can be technically challenging in bone or deep lymph nodes, which are the most frequent locations of prostate cancer metastases[3]. In addition, although the individual diversity of most oncogenic drivers seems to be limited in advanced metastatic disease, a single metastatic tumor might not capture the total burden of molecular alterations. Most likely, due to these limitations, unlike metastases of other advanced tumors that are biopsied for therapeutic decision-making, recurrent prostate cancer metastases are not routinely biopsied in most centers. An ideal method for personalized therapy needs to be able to, in real-time, identify the most relevant oncogenic drivers of the disease, accurately capture tumor heterogeneity, be minimally invasive and provide the possibility to periodically reassess the molecular phenotype of the disease[14].

Liquid biopsy

The term "liquid biopsy" describes the analysis of the molecular features of tumors in samples obtained from the blood and other body fluids such as urine and cerebrospinal fluid. Liquid biopsy includes the analysis of cfDNA[15], other nucleic acids[15], circulating tumor cells (CTCs) [16,17] and exosomes[18].

The presence of cell-free nucleic acids in human blood was first described by Mandel and Métais in 1948[19]. cfDNA can be found in varying quantities in blood, usually at low levels in healthy volunteers and at increased levels in patients after events such as exercise[20], myocardial infarction[21] or trauma[22]. To date, the most widespread translational research has been on the prenatal assessment of fetal DNA[23-25] and cancer[15,26,27].

The innovative approach of liquid biopsies for the analysis of components such as cfDNA enables the comprehensive characterization of the evolutionary processes that occur following

treatment response and subsequent progression. Being minimally invasive, cfDNA collection facilitates the repeated sampling in real-time needed to capture tumor evolution and the molecular dynamics of the disease[28].

The origin of tumor DNA in the circulation has been related to the properties of apoptosis and necrosis[29]. As a result of cell death, tumor DNA can be released from primary and metastatic tumors as well as circulating tumor cells. cfDNA is usually fragmented in small 70-200 base pair fragments with a peak of approximately 166 base pairs[30] corresponding roughly to the length of DNA wrapped around a single nucleosome[31]. Multiple studies have shown that DNA wrapped around nucleosomes is protected from the nuclease activity in blood and that even the tissue of origin can be estimated due to tissue-specific nucleosome positioning[32,33].

The presence of cfDNA in plasma is usually higher in cancer patients than in noncancer patients[34,35], with concentrations that range from 0 to more than 1000 ng/ml of blood, with an average of 19 ng/ml in first-line metastatic CRPC patients[36]. This concentration depends on several factors, including tumor metastatic volume, metastasis sites, and tumor progression. There is still a need for a better understanding of the differential contribution of tumor location and tumor microenvironment to the release of DNA. For example, for patients with tumors of the central nervous system, the molecular profile might be more accurately represented in the cerebrospinal fluid than in the bloodstream[37]. Likewise, for urological cancers, urine might be an attractive source for cfDNA. However, for cfDNA analyses in urine, the expected size of cfDNA is smaller, as cfDNA has to pass through the glomerular filtration system; downstream assays thus needs to be designed accordingly[38]. The concentration of cfDNA is also influenced by the clearance and degradation of DNA in the liver and the kidney and by nuclease activity in the blood. cfDNA has a relatively short half-life that ranges from minutes to several hours[39]. The short half-life gives cfDNA an advantage over protein-based biomarkers usually used in the clinic to monitor tumor progression, such as serum PSA, which have longer half-lives and

need several weeks to undergo changes representative of tumor dynamics[40]. In addition, cfDNA is pathway-agnostic, since it covers a broad range of molecular alterations and, unlike PSA, can be used in tumors that are not dependent on the AR pathway.

The proportion of tumor DNA in the pool of cfDNA is called the tumor fraction or tumor content (TC) and varies greatly from almost undetectable to more than 90%[41]. The presence of low cfDNA levels and a low tumor fraction have been limitations for cfDNA analyses for solid tumors. However, the introduction of new genomic technologies with high sensitivity and specificity, including next-generation sequencing[42] and digital PCR[43], has greatly contributed to the study of ctDNA[44,45]. While digital PCR is a relatively inexpensive and quick method of measuring the allelic frequency of rare mutations in a mixture of tumoral and nontumoral DNA and has increased the sensitivity for detecting allelic frequencies to as low as 0.01%[36], it does not provide a comprehensive profile of the molecular events that can be detected in cfDNA. Another limitation of digital PCR is that the sensitivity, particularly for identifying gene copy number variations, can be limited if the TC is low[41,46]. Next-generation sequencing (NGS), on the other hand, is capable of providing such information, and whole-genome, exome and targeted sequencing have all been successfully applied to cfDNA[41,46,47]. While NGS-based studies are still significantly more expensive than digital PCR, the number of questions that can be asked is higher for the same cfDNA input, but the tradeoff is a lower sensitivity than that of digital PCR for detecting specific alterations[48]. One important limitation for the identification of deletions using NGS is that NGS is limited to plasma with a high TC. Analysis of heterozygous single nucleotide polymorphisms (SNPs) provides an opportunity to overcome this limitation. If several gene aberrations are found to demonstrate predictive value, as seems very likely, NGS will be preferred. Indeed, NGS has already been implemented as a discovery tool in many clinical studies of novel agents[49-51].

Molecular alterations in cfDNA from advanced prostate cancer patients

The molecular landscape of advanced lethal prostate cancer has been defined based on tumor material obtained from tumor biopsies and warm autopsies[5,10,52,53] and possesses features absent or rarely found in primary tumors[54,55]. The accumulation of these aberrations follows a highly dynamic process directly related to the selective pressure imposed by the treatment of a genetically unstable disease. In advanced prostate cancer, the most frequently observed genomic alterations are the amplification or mutation of the *AR*, mutations of DNA repair genes and the loss or mutation of the tumor suppressor genes *TP53* and *PTEN* (**Figure 2**).

-*AR* aberrations.

The androgen receptor (AR) is the master regulator of prostate cancer [56,57,58] and castration is the backbone treatment upon which other treatments are supplemented. Intriguingly, molecular aberrations in the *AR* are seldom observed before castration[55,59], whereas lethal prostate cancers are frequently associated with AR aberrations, including *AR* gain, *AR* mutations and *AR* genomic structural rearrangements; furthermore, the detection of these aberrations increases during tumor progression, being observed in 10-15% of samples at castration resistance, 30-40% of samples from patients in second and later lines of advanced CRPC therapy[36] and up to 60%[10] of samples harvested at warm autopsy.

Analyses of cfDNA in sequential liquid biopsy samples have allowed the interrogation of the *AR* genomic landscape in CRPC and have greatly contributed to the understanding of the complex dynamics observed in the *AR* under the selective pressure of androgen deprivation and antiandrogen therapy during tumor progression and evolution.

Progression to bicalutamide therapy is associated with the emergence of a point mutation in the *AR*, c.2226G>T (p.W742C). This mutation has been associated with promiscuous AR activation and has been proposed to be involved in the observed PSA response to antiandrogen withdrawal[12]. Interestingly, the treatment of these patients with a new generation

antiandrogen, enzalutamide, is able to induce tumor responses and the regression of this clone in preclinical models[60]. In addition, there is evidence of the clinical efficacy of enzalutamide and abiraterone in patients harboring this mutation. Annala et al.[61] randomized 202 treatment-naïve CRPC patients to either abiraterone or enzalutamide. This mutation was detected before treatment in three patients, specifically, one on abiraterone and two on enzalutamide. A PSA decline was observed in all patients, and in two patients, the decline was greater than 50%, thus meeting the criteria for a PSA response. The *AR* mutation c.2623C>T (p.T875Y) has been associated with a broadened specificity *in vitro* to ligands such as estrogens, progesterone and adrenal androgens[62,63] and has been observed in patients receiving abiraterone[41,64]. This mutation was observed before treatment in nine patients in the study by Annala et al., specifically, three patients on abiraterone and six patients on enzalutamide. All patients on abiraterone had a PSA decline, and in two of the patients, the decline was greater than 50% and met the PSA response criteria. Five out of six patients on enzalutamide had a PSA response. These results suggest that the novel antiandrogens abiraterone and enzalutamide retain clinical activity in patients with these *AR* mutations detectable in plasma DNA.

The sequential analysis of cfDNA using NGS has identified two point mutations in the AR associated with resistance to abiraterone and prednisone: c.2105T>A (p.L702H) and c.2632A>G (p.T878A), which were previously associated with AR activation by prednisone and progesterone, respectively[36,41,46,62,65]. Another point mutation, c.2629T>C (p.F877L), has been associated with resistance to enzalutamide [66,67], although this mutation seems to be very uncommon[68].

The detection of *AR* gain in cfDNA has been associated with resistance to enzalutamide and abiraterone in chemotherapy-naïve and post-docetaxel CRPC[36,41,46,64,68-70] patients and has prognostic significance independent of other prognostic factors[71-73]. On the other hand, preliminary data suggest that tumors with AR gain are not resistant to taxane treatment[74],

which if prospectively confirmed could poise plasma AR as one of the first predictive biomarkers in prostate cancer. *AR* gain was previously defined based on the method used for detection: healthy volunteer data was used to estimate noise when analyzing cfDNA in plasma using NGS[46], and for ddPCR modeling, studies were used that estimated the likelihood of the *AR* CN cutoff value that best predicted the association with outcome[36]. A recent study that used a different method for *AR* gain estimation adjusted by the TC showed a worse outcome in univariate analysis [61], but the independent prognostic value of *AR* gain was unable to be confirmed. Differences in the method used for *AR* gain estimation preclude direct comparison with previous studies [75]. The analytical validation of new methodologies used to define *AR* gain, as well as a meta-analysis of *AR* CN data acquired from multiple trials at different institutions, is needed to identify the best cutoff value for considering *AR* CN gain clinically relevant.

AR genomic structural rearrangements (*AR*-GSRs) have been identified in one-third of CRPC patients and have been proposed to be potential drivers of resistance to antiandrogen therapies. These molecular alterations are highly heterogeneous in breakpoint location, rearrangement class and subclonal enrichment. However, most *AR*-GSRs are associated with the expression of splice variants lacking the ligand-binding domain[76]. These alterations can also be detected in cfDNA using whole-genome sequencing (WGS) and have been associated with the expression of AR splice variants in CTCs[47]. Although heterogeneous, *AR*-GSRs appear to be associated with a poorer outcome to antiandrogen therapy and an adverse prognosis. The correlation between both *AR*-GSRs and other *AR* aberrations and the clinical outcome needs to be further studied. One of the potential advantages of the study of *AR*-GSRs via cfDNA is that cfDNA analysis can be used in most patients, whereas other methods, such as tumor biopsy or CTC analyses, are limited by accessibility to the material.

cfDNA analyses can identify tumor heterogeneity and monitor clonal evolution during treatment. If clinically validated in advanced prostate cancer, cfDNA analysis could provide biomarkers to select patients more likely to benefit from antiandrogen therapies and those who should be switched to alternative therapies such as taxanes.

-Homologous recombination repair genes (HRR)

Prostate cancer patients with germline mutations are at higher risk of developing metastases, and germline mutations are associated with poor survival[77]. In lethal prostate cancer, tumor biopsies demonstrate increased germline HRR gene mutations with a frequent loss of heterozygosity (LOH)[5]. The loss of function of HRR genes predicts a response to PARP-inhibitors such as olaparib[78,79] and to platinum therapies[80]. The sequencing of cfDNA enables the detection of germline as well as somatic genomic alterations[81]. Moreover, next-generation cfDNA sequencing has shown a near-perfect correlation with biopsy samples for the detection of somatic mutations in HRR genes [82,83].

cfDNA analysis enables the detection and monitoring of HRR gene alterations during treatment[50,51]. A response to olaparib in prostate cancer patients with HRR germline mutations was associated with a decrease in the allelic frequency in cfDNA to approximately 50%, suggesting an abrogation of the LOH. For patients with somatic mutations in HRR genes, a response to PARP inhibitors was associated with a significant decrease in the allelic frequency of the mutations to less than 5%, confirming the presence of a molecular response[50].

BRCA1/2-deficient ovarian and breast carcinomas, which are initially sensitive to platinum and PARP inhibitors, develop treatment resistance associated with secondary mutations that restores the DNA repair function of BRCA1/2[84,85]. Interestingly, in prostate cancer, cfDNA sequencing has been shown to be a powerful tool to study the mechanisms of resistance to PARP inhibitors[50,51]. For example, in patients with *BRCA2* germline mutations who responded

to PARP inhibitors, additional somatic *BRCA2* frameshift mutations were observed at progression. These additional mutations were associated with the restoration of the open reading frame. Similar findings were observed in patients with *BRCA2* somatic mutations and patients with *PALB2* mutations, in whom additional alternative somatic deletions were associated with the restoration of the open-reading frame. These findings have great implications for the understanding of the mechanisms underlying the sensitivity and resistance to PARP inhibitors and likely to platinum compounds. PARP inhibitors have been demonstrated to exert a profound selective pressure associated with divergent subclonal evolution that restores HRR function, both in patients with germline mutations and in those with somatic mutations. It remains to be seen whether these clones behave as dominant clones after PARP inhibitor treatment is stopped. This knowledge could open the door to dynamic personalized therapies that include smart sequencing or a combination of anticancer therapies and treatment rechallenge depending on the molecular drivers controlling progression.

The efficacy of novel antiandrogens in HRR-deficient tumors is a matter of controversy. Whereas some authors suggest that enzalutamide or abiraterone treatment is associated with a poorer outcome [61,81,86], others either do not find a difference [87] or show a better outcome[88,89]. These studies are limited by the relatively low frequency of the mutations. Further studies are needed to clarify whether BRCA2, ATM and other mutations influence the treatment response differently; how LOH modifies this effect; in what ways frequently associated mutations in genes such as *TP53* contribute; and how prior therapies influence the development of resistance. The comprehensive and dynamic analysis of cfDNA might be helpful in solving these conundrums.

-*TP53* aberrations

Aberrations in *TP53* are observed in half of all lethal prostate cancers[5,10,52] and have been associated with castration resistance and the activation of androgen-independent pathways[90-

[92]. These aberrations are also found in plasma DNA[61,68,93]. Annala et al. identified mutations, rearrangements and deletions in more than fifty percent of patients with ctDNA above the threshold defined for detection in treatment-naïve CRPC (TC >30%)[61]. The presence of *TP53* inactivation was associated with worse outcome to abiraterone or enzalutamide in multivariate analysis independent of other clinical and genetic factors. Interestingly, patients who harbored two or more *TP53* defects had an even earlier time to progression than those who had only one TP53 defect. Although some patients harboring TP53 aberrations benefited from the treatment, most patients with primary resistance to abiraterone or enzalutamide and ctDNA above the threshold for detection (TC>30%) harbored mutations resulting in *TP53* inactivation. Although further studies are needed to use *TP53* as a predictive biomarker, a closer evaluation of these patients is recommended.

- PTEN and PI3K pathway defects

The PI3K pathway is altered in almost half of all advanced CRPC patients [5]. PI3K pathway alterations involve the loss or mutation of *PTEN* and aberrations in *PIK3CA/B* and *AKT*. These alterations have also been detected with a high concordance in ctDNA[68,82]. The loss of PTEN, which leads to PI3K activation, is known to mediate resistance to antiandrogen therapy[94] and predicts a response to AKT inhibitors[95]. PI3K pathway defects have been associated with a poor response to the novel antiandrogens enzalutamide and abiraterone[61] and suggests the possibility for potential treatment selection.

-Other genomic alterations

A number of other alterations have been described to have a role in metastatic prostate cancer and treatment response. The loss of *RB1* is frequently observed in lethal prostate cancers and has been correlated with lineage plasticity and the activation of *AR*-independent pathways when associated with *P53* loss. ctDNA analysis was able to detect losses of *RB1* associated

with a poorer response to enzalutamide and abiraterone[61,68,82]. Many other genomic alterations, including gains of *MYC, MET* and *CCND1*, have also been detected in cfDNA [64,68,93,96]. In addition, cfDNA has the potential to indicate the mutational load, a sentinel for mismatch repair (MMR)-deficient tumors, and to identify patients who could benefit from immunotherapy[97]. The possibility to characterize, in real time and serially, all these molecular alterations could expand the possibilities for the design and conduct of clinical trials, including umbrella and basket trials [92,98,99].

cfDNA analyses need to follow a qualification process

All biomarkers, including cfDNA analyses, need to follow a predefined analytical and clinical qualification process before being approved by the regulatory agencies and being implemented in the clinic. As an example, a plasma AR study is in the process of following a predefined roadmap for biomarker development and qualification (**Figure 3**). This roadmap includes the development of an accurate and reproducible assay to measure plasma *AR*[46] and a retrospective evaluation of the association of plasma *AR* with outcome in a prospectively collected set of patients[41]. After the analytical qualification was conducted, it was clinically validated in two homogeneous sets of patients with mCPRC treated with novel antiandrogenic agents[36]. The next step to clearly demonstrate the clinical utility of plasma AR will be to confirm these results in a clinical trial randomized by plasma AR status. Via a method analogous to that used in drug development, patients with the desired biomarker, in this case AR gain, would be randomized to the available treatments. Alternatively, randomized prospective trials including investigational therapies would need to be stratified and adequately powered to assess the predictive value of predefined biomarkers. These strategies would allow us to assess the predictive rather than the prognostic value of the biomarkers. All other cfDNA analyses need to follow a similar pathway of qualification before they can be routinely used in the clinic.

The association among different molecular alterations in mCPRC is not fully understood, and the relative contribution of each mutation to the clinical outcome needs to be carefully dissected and prospectively validated[96].

Potential clinical applications of cfDNA in advanced prostate cancer

Along with the improvement in molecular techniques and the increase in the knowledge about genetics and drug development, several clinical applications of cfDNA analysis can be foreseen in advanced prostate cancer (Table 1):

1.- As a prognostic tool. Circulating-free DNA, tumor fraction DNA and several molecular alterations, including the most relevant clinical prognostic factors in mCRPC, are associated with independent prognostic significance in multivariable analyses[36,41,71-73,96]. It will be interesting to design new prognostic nomograms that include both clinical and molecular features. cfDNA can be easily obtained at different time points and is a perfect candidate to improve currently available prognostic tools. It is tempting to study the clinical significance of cfDNA in localized disease and the association of cfDNA with tumor relapse and distant metastases[100].

2.- As a predictive marker. It is very likely that some molecular alterations, including HRR and *AR* aberrations, are associated with the differential efficacy of anticancer therapies. The detection of *AR* gain is associated with an adverse outcome to novel antiandrogen therapies. However, the predictive value of *AR* gain needs to be qualified in prospective studies before it can be used to make treatment decisions. In addition, some *AR* mutations that emerge during antiandrogen therapy are associated with bicalutamide or abiraterone-prednisone treatment and are known to be sensitive to other therapies. The identification of these aberrations can be helpful to guide treatment therapy. HRR gene mutations are associated with increased sensitivity to PARP inhibitors and to platinum therapies. The detection of HRR gene mutations

in plasma is highly correlated with the detection of these mutations in molecular analyses of biopsy tissues from metastases. If clinically qualified, cfDNA analysis could potentially become a new standard method to assess this feature.

3.- To monitor tumor response.

The sequential analysis of cfDNA allows a dynamic measure of its quantity and molecular alterations. Changes in the quantity of cfDNA during treatment closely correlate with the tumor response[50]. cfDNA has a relatively short half-life and can be more sensitive and specific than other protein markers such as PSA, particularly in challenging cases where the PSA level is very low or there is early rising PSA. As genomic analysis becomes more readily available for clinicians and methods are standardized, it can become part of the laboratory information available for the clinician. In addition, the molecular characterization of cfDNA allows the allelic frequency of mutations in key driver genes to be measured. The sequential analysis of cfDNA allows clinicians to closely follow the dynamics of these mutations. The clinical response to anticancer therapy in tumors that harbor somatic mutations is associated with molecular responses that include significant decreases in the allelic frequency. In contrast, the appearance of resistant clones can precede clinical or radiographic progression by several months and could provide the opportunity to introduce early changes to the anticancer treatment regimen in order to improve patient outcome.

4.- To identify novel mechanisms of resistance.

The analysis of circulating cfDNA provides an unprecedented opportunity for the identification of new mechanisms of resistance. Since the collection of circulating cfDNA is minimally invasive and allows us to serially obtain a comprehensive molecular analysis, cfDNA analysis gives us the opportunity to identify novel gene alterations associated with tumor progression. Moreover, cfDNA analysis allows us to better understand the mechanisms underlying tumor

progression and provides us with the opportunity to design novel drugs and treatment strategies, as it has allowed us to identify the methods underlying the acquisition of resistance to PARP inhibitors via the selection of mutations that revert the open reading frame of *BRCA2* [50,51].

Limitations of plasma DNA

However, cfDNA analysis is subject to relevant limitations: A) The sensitivity of the sequencing techniques, although improving along with the development of technology, is limited by the availability of an adequate amount of ctDNA. This limitation is particularly evident at earlier stages of disease and for the identification of gene copy losses, although it might also be relevant at later stages of disease and for the identification of other genomic aberrations. The restriction of the genomic analysis to the TC has partially alleviated this limitation. The estimation of the TC is usually defined by the presence of known tumor genomic aberrations. A restriction or adjustment for the TC, although appreciably improving the sensitivity, enriches the population with a cohort of patients with a poorer outcome and might neglect genomic events occurring in tumor clones lacking the predefined aberrations. This type of analysis might be relevant for the interpretation of the results and should be taken into consideration when comparing different studies; B) The specificity of plasma DNA analysis is limited by the quality of the DNA and by the sequencing technique. The identification of genomic aberrations in plasma DNA needs to be cautiously interpreted, since not all mutations or copy number variations are biologically meaningful. A comprehensive biological and clinical approach is required to validate new biomarkers; and C) The analysis of plasma DNA does not capture additional layers of biological complexity, such as differences in RNA expression or protein levels that might be clinically relevant. A study restricted to plasma DNA might be overlooking important information regarding the expression of AR splice variants, which might have prognostic and/or predictive value[101,102].

In summary, cfDNA analysis is very promising in advanced prostate cancer. cfDNA analysis is minimally invasive, is broadly applicable and provides a comprehensive molecular analysis of the tumor that would be difficult to obtain by other means. Precision medicine needs to integrate all this information in order to personalize the treatment of metastatic prostate cancer. The qualification of cfDNA-derived biomarkers is urgently needed before such biomarkers can be implemented in the clinic.

Figure 1. Prostate cancer is heterogeneous. A. Interpatient heterogeneity. Every patient has different genomic drivers of prostate cancer progression and resistance. The drivers are represented separately for simplicity, but multiple drivers can be present simultaneously in the same patient. **B.** Intrapatient heterogeneity. Distant metastases are composed of different metastatic clones. Circulating cell-free DNA has an advantage over tumor biopsy to capture genomic events from distant clones that are driving tumor progression. mCRPC= metastatic castration-resistant prostate cancer; HR = homologous recombination; MMR = mismatch repair.

Figure 2. The dynamic genomic landscape of advanced metastatic prostate cancer. The most relevant genomic events become more frequent during tumor progression and castration resistance. ctDNA can be studied in sequential liquid biopsies during tumor progression to capture the changing genomic landscape. Several additional molecular alterations are usually observed during progression. Many molecular events can be observed simultaneously in the same patient. The graph represents the genomic landscape of prostate cancer at different stages of the disease. A patient does not necessarily need to develop all the stages; some patients never develop metastasis, and some are diagnosed with *de novo* metastases. The relative frequencies of the aberrations are based on publicly available data[5,54,55]. Doce = docetaxel; Abi = abiraterone; HR = homologous recombination, MMR mismatch repair.

Figure 3. Roadmap for the qualification of plasma *AR* as a biomarker.

All biomarkers need to follow an analytical and clinical qualification process before they can be approved and implemented in the clinic. As an example, this figure shows the roadmap followed for plasma *AR* qualification and the future directions for the development of plasma *AR* as a biomarker.

Conflict of Interest Disclosures

The ICR developed abiraterone and therefore has a commercial interest in this agent. G.A. is on the ICR list of rewards to inventors for abiraterone. G.A. has received honoraria, consulting fees, or travel support from Astellas, Medivation, Janssen, Millennium Pharmaceuticals, Ipsen, Ventana, ESSA Pharmaceuticals, and Sanofi-Aventis and grant support from Janssen, AstraZeneca, and Arno. E.G.B. has received speaker honoraria or travel support from Astellas, Janssen-Cilag and Sanofi- Aventis. The other authors have no conflicts to declare.

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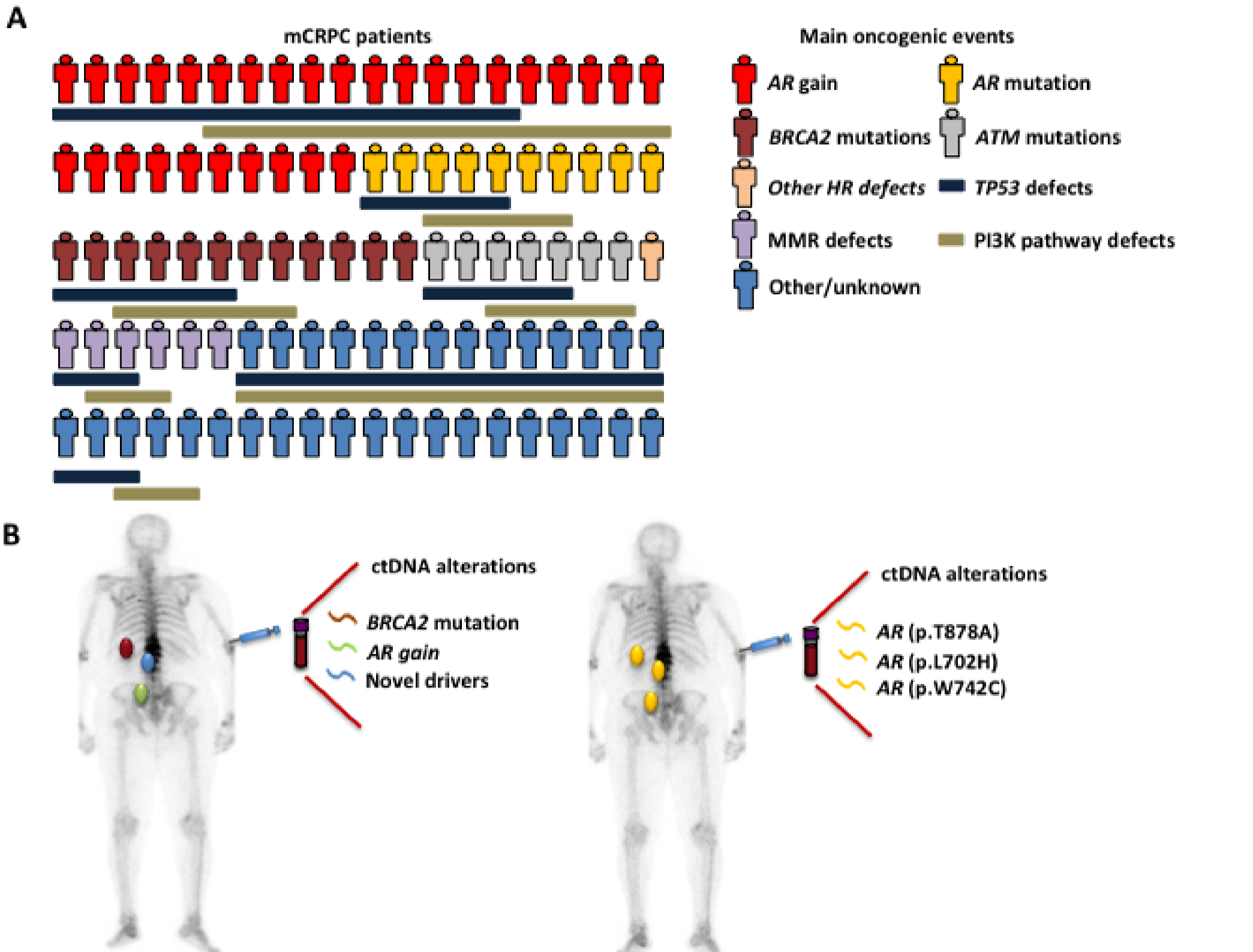
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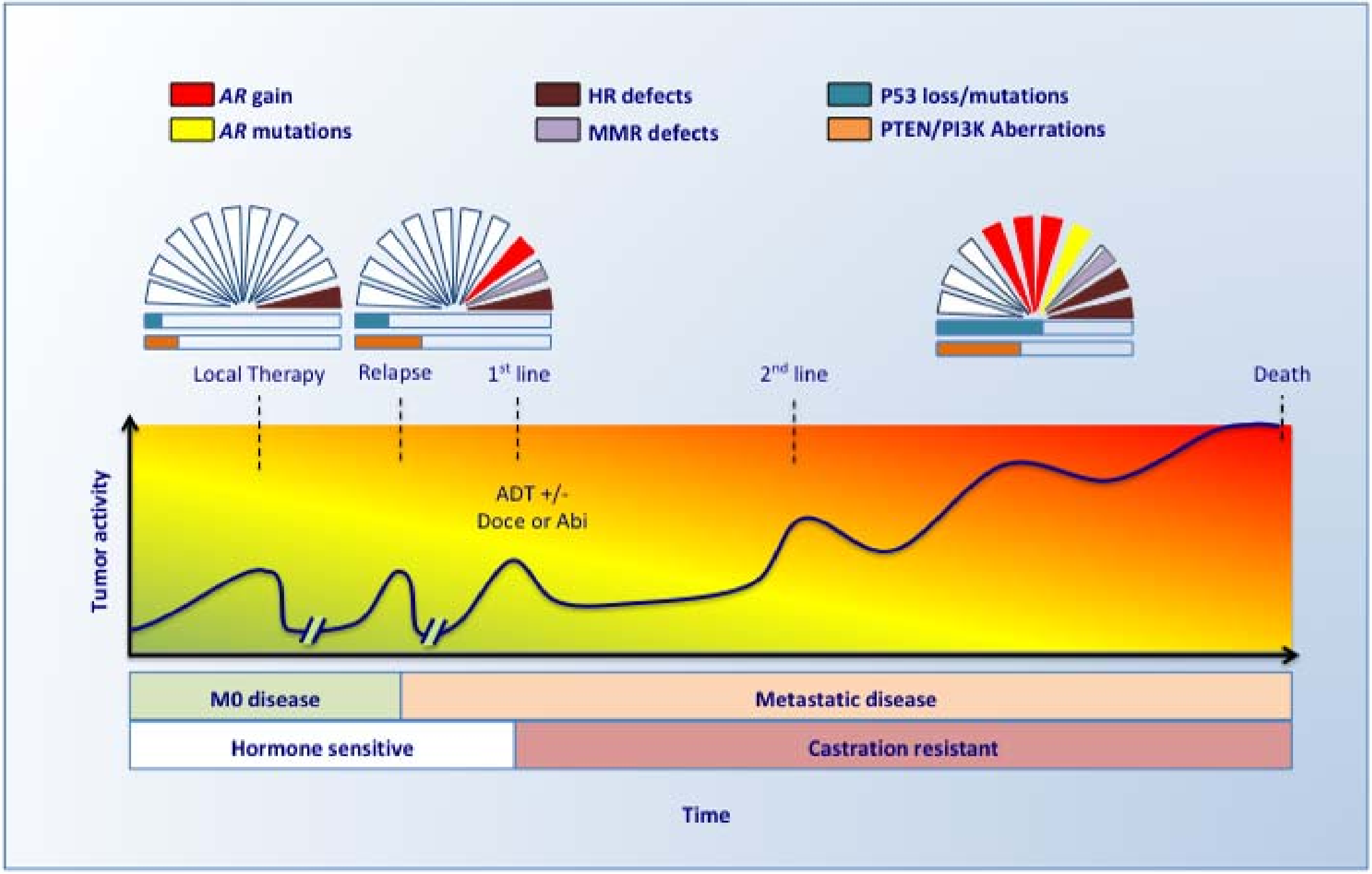
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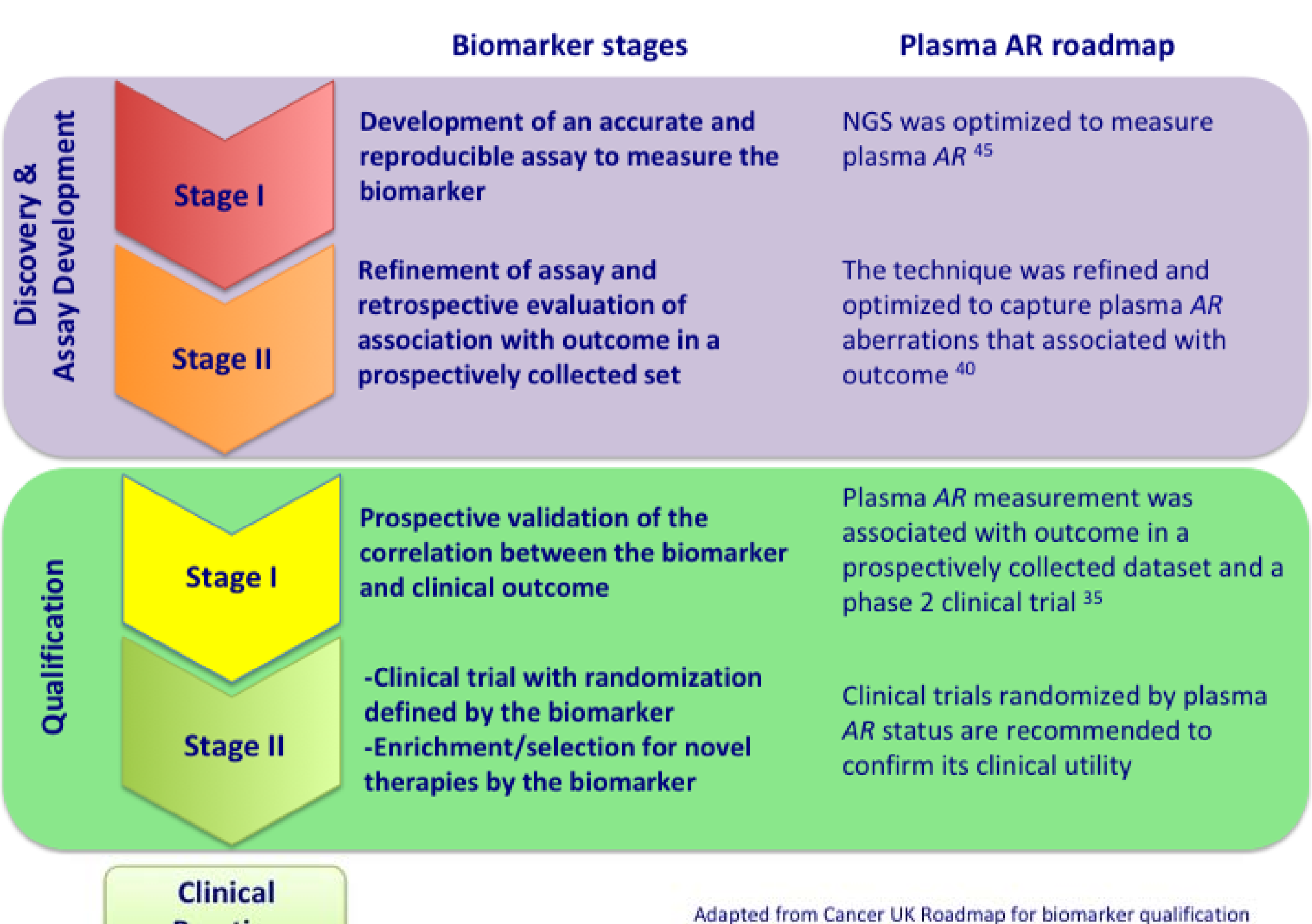
Biomarker stages
Plasma AR roadmap
Discovery & Assay Development
Stage I
Development of an accurate and reproducible assay to measure the biomarker
NGS was optimized to measure plasma AR [45]
Stage II
Refinement of assay and retrospective evaluation of association with outcome in a prospectively collected set
The technique was refined and optimized to capture plasma AR aberrations that associated with outcome [40]
Qualification
Stage I
Prospective validation of the correlation between the biomarker and clinical outcome
Plasma AR measurement was associated with outcome in a prospectively collected dataset and a phase 2 clinical trial [35]
Stage II
-Clinical trial with randomization defined by the biomarker
-Enrichment/selection for novel therapies by the biomarker
Clinical trials randomized by plasma AR status are recommended to confirm its clinical utility
Clinical
Adapted from Cancer UK Roadmap for biomarker qualification