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Tumour Review

Comprehensive intra-individual genomic and transcriptional heterogeneity: Evidence-based Colorectal Cancer Precision Medicine

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ABSTRACT

Despite advances in translating conventional research into multi-modal treatment for colorectal cancer (CRC), therapeutic resistance and relapse remain unresolved in advanced resectable and, particularly, non-resectable disease. Genome and transcriptome sequencing and editing technologies, coupled with interaction mapping and machine learning, are transforming biomedical research, representing the most rational hope to overcome unmet research and clinical challenges. Rapid progress in both bulk and single-cell next-generation sequencing (NGS) analyses in the identification of primary and metastatic intratumor genomic and transcriptional heterogeneity (ITH) and the detection of circulating cell-free DNA (cfDNA) alterations is providing critical insight into the origins and spatiotemporal evolution of genomic clones responsible for early and late therapeutic resistance and relapse. Moreover, DNA and RNA editing pave new avenues towards the discovery of novel drug targets. Breakthrough combinations of sequencing and editing systems with technologies exploring dynamic interaction networks within pioneering studies could delineate how coding and non-coding mutations perturb regulatory networks and gene expression. This review discusses latest data on genomic and transcriptomic landscapes in time and space, as well as early-phase clinical trials on targeted drug combinations, highlighting the transition from research to clinical Colorectal Cancer Precision Medicine, through non-invasive screening, individualized drug response prediction and development of multiple novel drugs. Future studies exploring the potential to target key transcriptional drivers and regulators will contribute to the next-generation pharmaceutical controllability of multi-layered aberrant transcriptional biocircuits.

Introduction

Genome and transcriptome sequencing and editing technologies, complemented by machine learning, are revolutionizing biomedical research shaping a shift from inexact science to Precision Medicine [1–8]. Especially for cancer, progress has been so impressive that a transition from research on spatiotemporal tumor heterogeneity [9–11] to early phase clinical trials is now beginning [4,12–17]. Precise characterization of dynamic intra-individual genomic and transcriptomic landscapes could enhance prediction of drug response in individual patients and optimize combinatorial therapy towards clinical Cancer Precision Medicine [7,8,18,19].

Colorectal cancer (CRC) has gathered immense research and clinical interest, with the standardization of treatment on the basis of clinicopathologic and genetic characteristics (*KRAS/NRAS/BRAF* and MSI status), as well as tumor staging, reflecting an initial step towards personalized medicine [20]. Multi-modal treatment of resectable tumors, including liver metastases, has showcased rapid advancements while progress in non-resectable disease remains slow [20,21]. Indeed, targeted therapy, which represents a major hope for clinical oncology, has not met expectations in the adjuvant or neo-adjuvant settings [20], while anti-EGFR (for *KRAS/NRAS* wild-type disease) and anti-VEGF agents have only moderate efficacy in the advanced or metastatic settings [22]. These advances and limitations mirror the standards of modern oncology, including the static tumor homogeneity and single biopsy-guided diagnostic approach, as well as the linear transcription dogma-based drug development [20,23,24].

A most rational perspective to overcome current unmet needs through understanding genome- and transcriptome-wide molecular mechanisms underlying drug resistance and relapse in each individual patient is provided by accumulating evidence on genetic, genomic and transcriptional heterogeneity both at bulk [10] and single-cell [9,25]

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resolution. A recent explosion in static tumor next-generation sequencing (NGS) analysis has led to the identification of new cancer-driver genes, mutations and oncotargets [26]. However, considering dynamic clonal evolution [27], spatiotemporal detection of genomic clones with both multi-regional (MR) tumor NGS and matched circulating cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA) NGS to identify bulk intratumor (ITH) [28] and circulating [29] heterogeneity respectively enable the delineation and improvement of therapeutic failure and relapse [30]. Moreover, beyond bulk sequencing, breakthrough technological systems, such as single-cell transcriptomics, CRISPR-Cas9 and their combination have returned exciting data on cell-by-cell variability-dependent drug sensitivity [9,31–33]. This pre-clinical effort is strongly backed by cancer models, particularly patient-derived xenografts and organoids [34]. Nevertheless, the design of optimized therapy comprises a framework combining both standard linear transcription-based drug development [23,33] and breakthrough discovery of next-generation drugs disrupting aberrant linear and non-linear regulatory networks [8].

The structure of this review is delineated in Fig. 1. We discuss valid published data on basket trials, bulk and single-cell NGS coupled with cf/ctDNA-NGS, DNA and RNA editing, as well as early-phase clinical trials, aiming for a transition from research to clinical Colorectal Cancer Precision Medicine. This shift sets the stage for overcoming three major clinical challenges: non-invasive screening for early diagnosis, individualized prediction of therapeutic response and discovery of multiple novel drug targets.

Clinical standards: state-of-the-art

The two pillars of Modern Oncology over the past decade have been the static tumor homogeneity concept and the single-biopsy approach for diagnosis, staging and treatment [20], as well as the single-gene transcription dogma [23], governing drug development. Regarding CRC, multi-modal treatment of resectable disease, not only for non-metastatic tumors, but for liver and/or lung metastasis as well in contrast to most other cancer types, has marked substantial progress [20]. Complete tumor resection (R0) has been the cornerstone of therapeutic modalities, complemented by adjuvant (AT) or neoadjuvant treatment (NAT). The over-abundance of prognostic factors used for patient subgrouping, as well as the lack of appropriate large-scale randomized clinical trials (RCTs) for specific subgroup analyses, represent a bottleneck for accurate decision-making among the multitude of therapeutic options available for several patient sub-populations [20,35]. Patient stratification is based on multiple prognostic factors including TNM stage, resection status (R0, R1, R2), high volume of surgery and high-risk factors such as poor differentiation, microsatellite instability status, lymphatic, vascular or perineural invasion, obstruction and perforation [20,35,36].

In colon cancer, surgery alone is indicated for stages I/II without high-risk features, while adjuvant chemotherapy is suggested for high-risk tumors and more advanced stages. Neoadjuvant chemotherapy may be considered in presence of clinical T4b stage disease and neoadjuvant radiation therapy with concurrent chemotherapy can be considered for initially unresectable non-metastatic colon cancer to aid resectability. For resectable liver and/or lung metastases R0 resection remains the standard of care with AT or NAT [20]. Perioperative chemotherapy has improved progression-free survival [37] but did not demonstrate any OS benefit [38]. On the other hand, standard treatment of resectable rectal cancer is centered around anterior resection or abdominoperineal resection with total mesorectal excision (TME). For clinically early stage rectal cancer (T1-2, N0) surgery or NAT is proposed. No adjuvant treatment is suggested for pT1-2, N0, while pT1-2, N1-2 requires adjuvant chemotherapy. In contrast to colon cancer, most other more advanced rectal tumor stages, resectable liver and/or lung metastases included, are treated with neoadjuvant chemo-radiotherapy, followed by surgery and AT [20].

Over the past two decades, laparoscopic resection has gained significant popularity versus open surgery. Randomized trials on minimally invasive surgery have already demonstrated improved short-term outcomes [39], while providing similar long-term oncological outcomes to open surgery in centers of excellence, initially for colon cancer [40], as well as for rectal cancer more recently [41]. Additionally, although high ligation of the inferior mesenteric artery has been the method of choice in many experienced institutions as opposed to low ligation [42], considering the low proportion of positive nodal disease at the inferior mesenteric root, no evidence from high quality RCTs currently exists supporting either approach [43].

Targeted therapy

Targeted therapy represents the major hope for improving disease-free (DFS) and overall survival (OS) rates, following a wide expansion of available targeted agents over the past decade. However, in contrast to great clinical success and dramatic reduction in relapse and cancer-related death rates for a few cancer types, such as breast, progress has been slow for many other major cancers, including colorectal [20]. Indeed, there is no evidence from RCTs that the addition of cetuximab or bevacizumab to adjuvant chemotherapeutic regimens improves oncological outcomes for resectable primary CRC without or with liver metastasis [20,35,44,45]. Although resection is preferable for oligometastatic disease, locally ablative techniques can be considered [20]. Moreover, the optimal first-line systemic treatment of unresectable advanced or metastatic disease remains under question. Recent RCTs suggest cetuximab or bevacizumab as standard additions to chemotherapy [46,47]. By contrast, modern recommendations include chemotherapy alone or combined with cetuximab or bevacizumab in the treatment alternatives, in the absence of strong evidence [20].

Basket trials

Recent evidence on genetic similarities between different cancer types, as well as the potential for further exploitation of available targeted drugs, approved only for specific cancers, have formed the basis of basket trials [48]. A prime paradigm is the *HER2* amplification, observed in breast and gastric cancer, successfully targeted by trastuzumab in both cancer types. A recent phase II basket clinical trial (NCT01953926) on 141 patients with 21 tumor types harboring HER2/3 mutations, including breast and colorectal, has demonstrated potential clinical utility of HER kinase inhibition with neratinib and trastuzumab across different malignancies [49]. Another ongoing, phase 2a, multiple basket study on 57 patients with treatment-refractory, HER2-amplified metastatic colorectal cancer (NCT02091141), which accounts for 2–6% of cases, demonstrated 32% objective response to dual HER2-targeted therapy with pertuzumab and trastuzumab [50]. Previously, the phase II HERACLES trial reported promising results for trastuzumab-lapatinib in patients with treatment-refractory HER2-positive metastatic CRC [51]. Similarly, a phase II basket study on 122 patients with *BRAF* V600 mutation-positive non-melanoma cancer (NCT01524978), including CRC, the response rates ranged from 42% for non-small-cell lung cancer to no response for CRC [52]. Additionally, initial results from the phase III BEACON trial suggest promising efficacy of the encorafenib-binimetinib combination added to standard cetuximab for *BRAF* V600E–mutant metastatic CRC after prior treatment failure [53]. Further phase III RCTs are thus required to validate the efficacy and survival benefit of targeted drug combinations in metastatic HER2-positive or *BRAF* V600E–mutated CRC. However, basket trials represent an initial step in the transition from inexact to Precision Medicine for CRC [8].

Unmet needs

Despite excellent prognosis of early-stage disease (T1-2, N0), 5-year

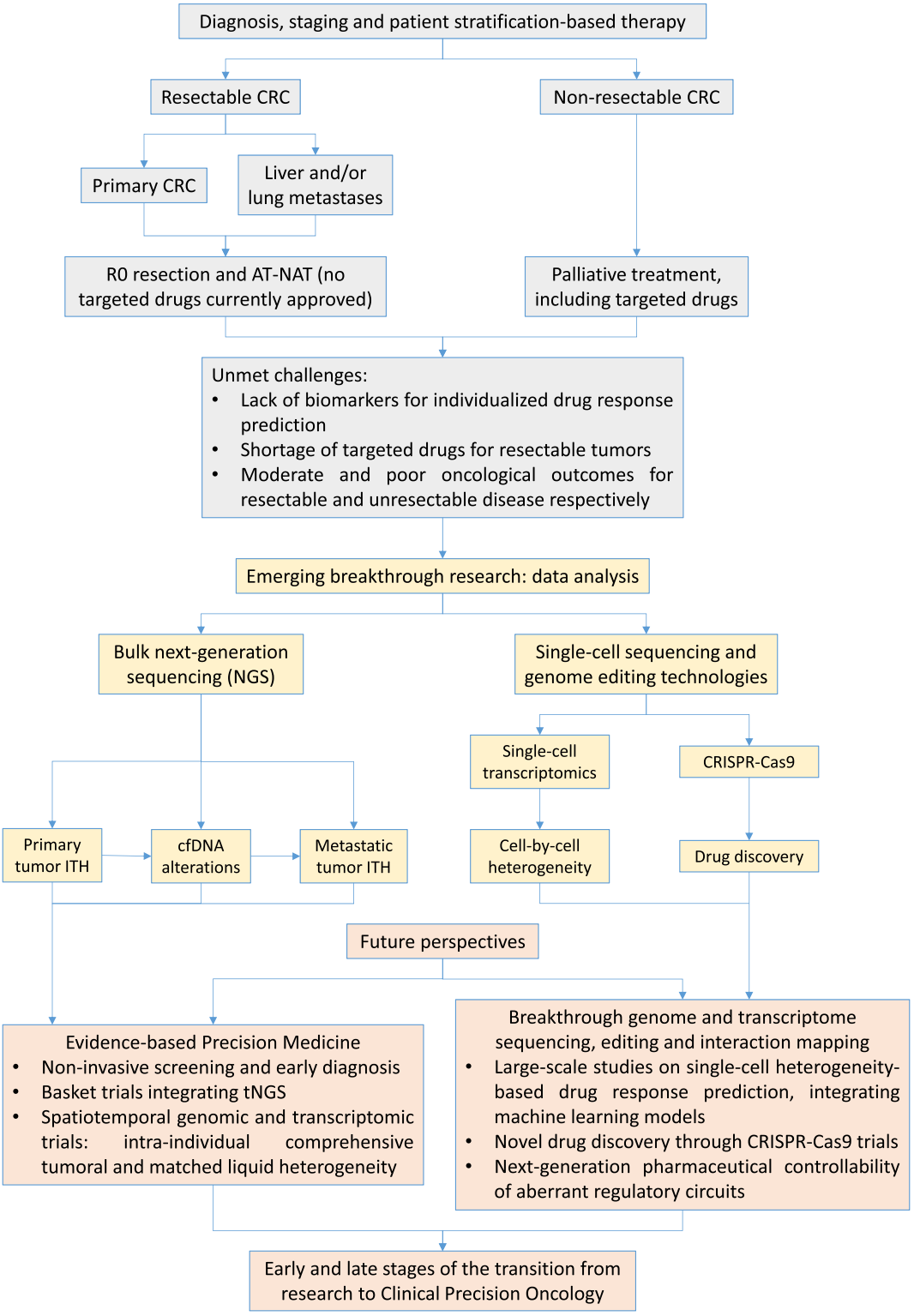

Fig. 1. Structure of the review. Colorectal cancer management state-of-the-art, unmet needs and potential solutions via the exploration of genome and transcriptome alterations implementing bulk and single-cell sequencing, editing and interaction mapping. **Abbreviations:** adjuvant treatment (AT), cell-free DNA (cfDNA), colorectal cancer (CRC), intratumor heterogeneity (ITH), neo-adjuvant treatment (NAT), next-generation sequencing (NGS), targeted NGS (tNGS).

DFS is approximately 70% for high-risk stage II and III CRC [35], while 4-year progression-free survival is approximately only 30% after resection of liver metastases [38,45]. Moreover, oncological outcomes remain dismal for non-resectable metastatic disease, with median overall survival ranging between 12 and 20 months [22]. These challenges, as well as the lack of high quality definite evidence on multimodal treatment [20], suggest that a valid innovative research strategy is badly needed. Breakthrough genome and transcriptome exploration in time and space, coupled with DNA and RNA editing, represent a highly exciting and promising perspective to address unmet needs.

Data analysis on genomic and transcriptomic landscapes

Over the past decade DNA and RNA sequencing and editing

technologies have transformed life sciences. Shortly after its market launch in 2005, NGS was introduced in the modENCODE [54] and ENCODE [55] projects, aiming to comprehensively characterize structural and functional, coding and non-coding genome elements. Since early integration of NGS into small studies, ease-of-application, continuously dropping cost and validity [56], coupled with recent recommendations on large sample sizes required for the valid identification of cancer driver genes and oncotargets [10], have led to an explosion in patient-derived sample NGS analyses [30]. Next-generation sequencing includes targeted (tNGS), whole-exome (WES), whole-genome (WGS), RNA (RNAseq) and chromatin immunoprecipitation sequencing (ChIP-seq). Sequencing of known gene panels through tNGS has rapidly provided clinical implications in daily practice, being the most appropriate method for basket trials. The protein-coding part of the genome (1.5%) is sequenced by WES, while WGS provides the unprecedented potential to identify all coding and non-coding alterations. This ability has changed the researchers' opinion on "junk" DNA, revealing the functionality of most of the non-coding genome [55]. Moreover, RNAseq represents a powerful tool for transcriptome exploration. Finally, the fifth technological capacity of NGS is chromatin immunoprecipitation sequencing (ChIP-seq), for genome-wide mapping of DNA-protein interactions via high-throughput DNA sequencing [1]. Rapid progress in the exploration of genomic and transcriptomic landscapes from static tumor homogeneity integrating a single biopsy [10,57] to modern spatiotemporal genomic clone evolution and tumor heterogeneity, requiring NGS of multiple tumoral and liquid biopsies, opens new diagnostic, predictive and therapeutic avenues [16,30]. Moving on from bulk NGS and ITH, to single-cell DNA and RNA sequencing has detected genetic, genomic and transcriptional heterogeneity, along with drug sensitivity variability, even among individual cells [9,25]. Moreover, genome and transcriptome sequencing and editing systems could uncover multiple therapeutic targets, required to overcome extensive inter- and intra-patient heterogeneity [33].

Translating static and dynamic tumor heterogeneity analysis into clinical implementation

Owing to the increasing affordability, accuracy and widespread availability of conventional NGS technologies and considering the magnitude of CRC as a public health threat, a multitude of studies have emerged, exploring the genomic and transcriptomic characteristics of CRC through static patient-derived tumoral sample analysis (Table 1) [10,26,57–66]. A major goal of single biopsy-based genomic studies is to expand the cancer type-specific catalog of driver genes and mutations towards completion, according to recent guidelines for large-scale analyses proposed by Lawrence et al. [10] for valid genomic discoveries. Indeed, numerous novel CRC driver genes have been identified by means of WES and WGS [10,57,65]. Additionally, apart from several putative new prognostic factors [62,63], researchers have proposed molecular classifications, aiming for clinical implications and accurate patient stratification [58,61,64]. Although the molecular features that could provide an optimal classification still remain unclear, the most robust stratification is currently the one documented by Guinney and colleagues [58], which classifies CRC into four consensus molecular subtypes (microsatellite instability immune, canonical, metabolic, mesenchymal) potentially informing future clinical trial designs and targeted drug development. Moreover, as many as 75% of CRCs have been reported to harbor genetic drug targets [65], including oncotargets of FDA-approved agents, such as *ERBB2* amplifications [57], suggesting the potential for biomarker-guided basket trials on combinational targeted therapy.

Over the past five years, more advanced NGS systems have emerged, in order to overcome the major limitation of single-biopsy analysis, namely the ability to investigate spatiotemporal clonal evolution, resulting in therapeutic resistance. These methods include both single- and multi-regional simultaneous analysis of matched primary and metastatic specimens, as well as NGS of plasma cf/ctDNA (Table 2) [17,28,29,67–80]. Comparative analysis of matched primary and metastatic tumors revealed high levels of mutational concordance, ranging between 80 and 100% for tNGS of known gene panels [68,70] and 65–70% for WES/WGS [67,73], while high heterogeneity was an adverse prognostic factor [67]. However, metastasis specific lesions, some of which were actionable, identified mainly by WES/WGS [67,73], suggest that genome analysis of the metastases, complementary to the primary lesion, could improve therapeutic decision making [73]. Furthermore, MR-NGS to identify primary and metastatic ITH uncovered variable degrees of intra- and inter-tumor heterogeneity [28,69,71,72], with de novo mutations in metastases indicating constant evolution in time and space [72]. Nevertheless, methodological flaws such as the small scale and heterogeneous protocols hinder the extraction of valid conclusions and warrant further investigation within prospective trials.

Forward from tumoral specimens, much interest has been directed towards patient-friendly blood-based methods to guide early diagnosis, as well as both primary and secondary decision-making through NGS of plasma cfDNA or ctDNA. Both terms have recently been utilized to describe circulating DNA alterations. Cell-free DNA with detectable cancer-specific mutations is usually termed as circulating tumor DNA (ctDNA). The mutational landscapes of plasma cf/ctDNA were found to reflect those of the primary tumor [74,75,78], including the detected resistant alterations [74,77]. Moreover, promising results regarding the capital hope of early non-invasive diagnosis via a blood test, such as the CancerSEEK [76], have already resulted in a very large-scale clinical trial titled "The Circulating Cell-free Genome Atlas" (NCT02889978). The study employs tNGS, WGS and whole-genome bisulfite sequencing of cfDNA to detect and localize multiple different tumor types, including CRC, and has already enrolled the vast majority of the 15,000 planned participants. In a recently reported preliminary sub-analysis on 1422 subjects the overall detection rates across all stages for 12 deadly cancer types were 76%, with a sensitivity of 74% for stage I-III CRC [17]. However, sensitivity heavily correlated to tumor stage, as rates of detection for stage I cancers were only 34% for the whole 12-cancer cohort, 77% for stage II and 84% for stage III, while localization rates were high for all stages [17]. Hence, sensitivity is currently insufficient to fulfill clinical criteria for screening and early diagnosis and waiting for the final results is mandatory before concluding on the test's cancer type-specific accuracy. By contrast, a very recent landmark study by Cristiano et al. [29] returned quite exciting data. The authors applied a machine learning model named "DELFI" to analyze cfDNA fragmentation patterns based on low-coverage WGS on seven cancer types, reporting detection sensitivity above 70% for CRC with very high specificity. However, the most impressive finding was a detection rate of 79% for stages I-II of all cancer types, while overall sensitivity increased to 91% when fragmentation profiles were complemented by mutation detection through tNGS [29]. These results for the first time suggest that, following validation within prospective clinical trials, this method could at last realize the long-term research and clinical dream of a simple blood test suitable for screening and early diagnosis.

Additionally to diagnosis and primary decision-making, serial cf/ctDNA-NGS before and during therapy has been used for the identification of emergent mutations responsible for therapeutic resistance and relapse as a result of dynamic clonal tumor evolution [79,80]. For instance, up to 32% of baseline *RAS* wild-type patients developed *RAS* mutations attributed to targeted treatment with panitumumab [80]. Thus, serial liquid biopsies is currently an attractive method for patient monitoring and prediction of therapeutic failure before the clinical diagnosis of relapse, but their use within appropriately designed studies is still very premature.

Early clinical trials

Following the explosive progress on NGS analyses, the first small patient-centric registered genetic trials are subsequently emerging, as a first step towards evidence-based precision (Table 3) [12–14,16]. For

Table 1
Identification of new cancer driver genes, molecular classifications and oncotargets through static next-generation sequencing analysis.

Patients	Technology	Findings	Implications	Ref
4151	RNAseq, Affymetrix and Agilent gene expression platforms	Four molecularly distinct consensus molecular subtypes were identified (MSI immune, canonical, metabolic, mesenchymal) with potential clinical utility	This is a robust CRC classification potentially enabling patient stratification within clinical trials	[58]
1439	WGS	• A strongly protective 0.3% frequency variant signal at *CHD1* was identified • 40 new independent association signals at $P < 5 \times 10^{-8}$ were discovered, including low frequency variants and lncRNAs, supporting a role for immune function • CRC risk was estimated as highly polygenic	Further large studies on rare variants are required to assist screening and drug development	[26]
999 (601 PTs, 533 MTs)	tNGS	• Right-sided microsatellite stable CRCs were associated with oncogenic alterations in *KRAS, BRAF, PIK3CA, AKT1, RNF43 and SMAD4* • Left-sided CRCs frequently had no genetic alteration in mitogenic signaling, but exhibited higher mitogenic ligand expression	Genomic variability could explain survival differences between right and left colon cancer	[59]
930 from 22 cancer types	WGS, RNAseq	• A global network of 193 non-coding loci was identified, mutations in which disrupt target gene expression • Many of the affected genes are not identified as drivers	Further studies are required to understand how non-coding mutations affect gene expression	[60]
921 (multiple GI cancers)	WES	• GI adenocarcinomas comprised five molecular subtypes: EBV, MSI, HM-SNV, CIN, and GS • Hypermutated tumors had variable immune characteristics • A genome-stable CRC subtype was identified with recurrent mutations in *SOX9* and *PCBP1*	Molecular subtyping could have prognostic significance	[61]
511 from QUASAR 2 trial	tNGS	• *TP53*, *KRAS*, *BRAF* and *GNAS* mutations were independently associated with shorter RFS ($p < 0.035$) • Total somatic mutation burden correlated with longer survival (HR 0.81; $p = 0.014$) • MSI was not independently associated with survival (HR 1.12; $p = 0.75$)	• Two novel prognostic factors were identified (*TP53* mutation and total mutation burden) • Gene panels can outperform MSI-based prognostic models	[62]
468	tNGS (1321 gene panel)	• A subset of 17 genes was identified as prognostic • Tumors without *APC* mutations have a worse prognosis	APC is a potential prognostic biomarker for the clinic	[63]
274 pts (tumor samples and mouse xenografts)	WES, WGS	• 3 clusters were identified based on CNAs, overlapping with consensus molecular subtypes • Intermediate-to-high CIN correlates to improved and low to worse outcomes respectively after bevacizumab combination therapy	Copy number load is a novel potential predictive biomarker of bevacizumab combination therapy	[64]
276 pts	224 WES, 97 WGS, 215 RNAseq	• 16% of CRCs were hypermutated, more likely from the right colon • Non-hypermutated tumors had remarkably similar genomic alterations • 24 genes were significantly mutated, some novel (*ARID1A, SOX9, FAM123B/WTX*) • Potential drug targets: WNT signaling, β-catenin, IGF2, IGFR, ERBB2, ERBB3, MEK, AKT and mTOR • Recurrent CNAs include potentially targetable *ERBB2* amplifications and a novel amplification of *IGF2* • A recurrent *NAV2-TCF7L1* fusion was identified • Increased activity of MYC was nearly ubiquitous	This study provides insight for future patient stratification for clinical trials on targeted therapies	[57]
233 (4742 from 21 cancer types)	WES	4 novel genes with clear connections to cancer were identified	Completion of cancer type-specific driver gene catalogues requires very large sample analyses and a P-value < 0.01	[10]
230 (9423 from 33 cancer types)	WES	• Almost 75% of CRC patients had druggable mutations • A total of 299 driver genes were identified, 59 novel		[65]
213 pts (tumor samples and cell lines)	WGS, ChIP-exo	• Non-coding point mutations at cohesin binding sites (CBSs) were frequent, as observed in other cancer types as well, and many are predicted to affect CTCF binding affinity • CBS mutations may drive tumorigenesis by causing aberrant gene expression, epigenetic changes or genetic instability	Large and appropriate studies are required to elucidate on the potential clinical role of non-coding mutations	[66]

Abbreviations: chromatin immunoprecipitation (ChIP), chromosomal instability (CIN), copy number alteration (CNA), colorectal cancer (CRC), gastrointestinal (GI), genome stable (GS), hazard ratio (HR),hypermutated (HM), long non-coding RNA (lncRNA), metastatic tumor (MT), microsatellite instability (MSI), patients (pts), primary tumor (PT), relapse-free survival (RFS), RNA sequencing (RNAseq), sungle nucleotide variant (SNV), targeted next-generation sequencing (tNGS), whole-exome sequencing (WES), whole-genome sequencing (WGS).

Table 2
Exploring static and spatiotemporal intratumor and circulating heterogeneity as preventive and predictive biomarkers to inform individualized therapy.

Patients (Samples)	Technology	Findings	Implications	Ref
Dynamic evolution of genomic clones and emergence of tumor heterogeneity				
88 pts (46 matched PT and MTs and 42 non-metastatic PTs)	WES	• Tumor heterogeneity was estimated through computational methods and was highly variable • In 70% the number of sub-clones was highly consistent between PT and LM • High heterogeneity correlated to worse outcomes	Validation is required for tumor heterogeneity as a predictor of liver metastasis	[67]
69 pts (matched PT and MT samples)	tNGS (WGS on 4)	Mutation profiles were 100% concordant for *KRAS*, *NRAS*, and *BRAF*, and highly concordant for recurrent alterations	NGS of either PT or MT could be used for diagnosis following validation	[68]
27 pts (97 samples from PT and MTs and 68 samples from a single PT)	tNGS (100 gene panel)	• Inter- and intra-tumor heterogeneity was attributed to gene copy-number alterations • Copy numbers are highly discordant between PT and MT	ITH could be used for future patient stratification	[69]
18 (matched PT and LM samples)	tNGS	79.3% of PT somatic SNVs are present in the LM and 81.7% of all LM alterations are present in the PT	This study supports linear progression of liver-limited metastatic CRC	[70]
17 (213 matched PT, LN and MT)	Polyguanine-repeat analysis	In 65% and 35% of cases, lymphatic and distant metastases arose from independent and single subclones in the PT respectively	Correlation between metastatic origin and clinical behavior should be further investigated	[71]
14 pts (70 MR samples from PT and matched liver and/or lung MTs)	tNGS	• PT *RAS* mutation status was maintained in MTs • De novo mutations in several genes were identified in MTs, correlating with site and time of metastasis	Dynamic NGS analysis is required to identify emerging targetable mutations	[72]
12 (matched PT and MT)	WGS	• 65% of somatic mutations shared a common progenitor, while 15% were tumor- and 19% metastasis-specific, supporting divergent evolution • Recurrently mutated non-coding elements: ncRNAs *RP11-594N15.3, AC010091, SNHG14*, 3′ UTRs of *FOXP2, DACH2, TRPM3, XKR4, ANO5, CBL, CBLB* • Actionable metastasis-specific lesions: *FAT1, FGF1, BRCA2, KDR*, and *AKT2-*, *AKT3-*, and *PDGFRA-3′* UTRs • Most patients featured a model of late metastasis	Metastasis-specific oncotargets could inform therapeutic decision-making in the metastatic setting	[73]
9 (75 MR PT and 2 LM samples)	MR-WES	• High ITH was identified in all tumors • Computational analysis attributed ITH to neutral evolution and therapeutic failure possibly to pre-existing low-frequency subclones	Further elucidation on cancer evolution could provide therapeutic insights	[28]
Next-generation sequencing of single liquid biopsies				
21,807 (> 50 late-stage cancer types)	tNGS	• cfDNA alterations in major driver genes matched those from tumor tissue sequencing (TCGA, COSMIC) • Differences in mutation prevalence were attributed to therapy and clonal evolution • Treatment-associated resistance was common and enriched in patients with targetable driver mutations (> 18.6%)	cfDNA reflects clonal evolution and could detect resistant driver alterations	[74]
1,422 (sub-study, from 21 tumor types, NCT02889978)	cfDNA-tNGS/WGS/WGBS	• Overall sensitivity was 55% and sensitivity for 12 deadly cancers (incl. CRC) was 76% at 99% specificity • Sensitivity for stage I-III CRC was 74%	Methylation profiling of cfDNA could aid non-invasive cancer diagnosis	[17]
1397 (advanced CRC patients)	tNGS	• Mutation frequencies in ctDNA were comparable to those of tissue in other independent compendia • A novel cluster of extracellular domain *EGFR* mutations was identified, with 91% of these patients harboring multiple distinct resistance alterations	ctDNA sequencing can effectively dissect CRC mutational landscapes with potential implications for treatment	[75]
1005 pts (8 cancer types)	CancerSEEK (multiplex-PCR and 8 protein biomarkers)	Sensitivity of the CancerSEEK test was approximately 65% for CRC, varying according to stage, with very high specificity	Technological refinements are required to achieve accurate non-invasive diagnosis	[76]
236 (7 cancer types)	DELFI (based on low-coverage cfDNA-WGS), tNGS	• The DELFI machine learning model had detection sensitivities of 57% to > 99% at 98% specificity • Overall sensitivity was approximately 79% for stages I-II • Sensitivity for CRC was 81% and 70% for 95% and 98% specificity respectively • Combination of DELFI with tNGS increased overall sensitivity to 91%	The small amount of WGS required, suggests that DELFI could be broadly used for cancer diagnosis or even screening	[29]
	BEAMing, tNGS			[77]

(*continued on next page*)

Table 2 (*continued*)

Patients (Samples)	Technology	Findings	Implications	Ref
100 pts (matched PT and plasma samples after anti-EGFR)		• Resistant alterations identified: *KRAS*, *NRAS*, *MET*, *ERBB2*, *FLT3*, *EGFR*, *MAP2K1* • Treatment cessation leads to re-emergence of drug sensitivity	Liquid biopsies could be a valuable resource to monitor treatment response and mechanisms of resistance	
80 pts	WGS	• Tumor-derived cfDNA was higher in plasma than serum • Common CNVs were identified in multiple chromosomal regions • CNVs were associated with disease stage and prognosis	Plasma cfDNA-NGS could be used as a diagnostic and prognostic tool in advanced CRC	[78]
Patient monitoring and prediction of acquired drug resistance and relapse through serial cf/ctDNA-NGS				
261 (ASPECCT study, plasma samples at baseline and after panitumumab)	tNGS	• Higher *RAS* mutant allele frequency at baseline correlated to worse outcomes • Baseline EGFR pathway mutations correlated to shorter survival • An increase in tumor mutational burden in time was observed	• Further studies are required to investigate mutation frequency as a prognostic marker • Liquid biopsies could be used for primary and secondary decision-making	[79]
238 (ASPECCT study, plasma samples at baseline and after panitumumab)	tNGS	• 79% of baselines samples were WT and 21% mutant *RAS* • 32% of baseline-WT had emergent *RAS* mutations • Patients with baseline mutant RAS had worse outcomes, while emergent ctDNA RAS mutations had no correlation	Further research is needed to investigate the clinical significance of baseline and emergent ctDNA *RAS* mutations	[80]

Abbreviations: beads-emulsion-amplification-magnetics (BEAMing), cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), colorectal cancer (CRC), DNA evaluation of fragments for early interception (DELFI), intratumor heterogeneity (ITH), liver metastasis (LM), lymph node (LN), metastatic tumor (MT), multi-regional (MR), next-generation sequencing (NGS), patients (pts), primary tumr (PT), single nucleotide variant (SNV), targeted NGS (tNGS), whole-exome sequencing (WES), whole-genome bisulfite sequencing (WGBS), whole-genome sequencing (WGS).

Table 3
Early-phase targeted next-generation sequencing-based clinical trials highlighting the beginning of evidence-based Precision Medicine.

Patients (samples)	Technology	Findings	Implications	**Ref**
107 diverse advanced cancers (34 CRC, Static PT samples)	tNGS and array-based transcriptomics	• This is a registered clinical trial (WINTHER trial: NCT01856296) assigning patients to genome- and transcriptome-based therapies • Rates of stable disease and PR or CR were 26.2% (arm A: 23.2%; arm B: 31.6%; P = 0.37)	This trial suggests that genomic and transcriptomic profiling could guide further studies on individualized therapy	[12]
100 diverse advanced cancers (23 CRC, Static ctDNA samples)	ctDNA-tNGS	• This is an early phase clinical trial (TARGET study) • Drug targets were identified in 41/100 pts, with 11/41 receiving a matched therapy • PR was achieved in 4/11 and stable disease 7/11 patients	This study supports the application of ctDNA-tNGS in early-phase clinical trials to enhance stratification for targeted therapy	[13]
83 diverse advanced cancers (14 CRC, Static PT and ctDNA samples)	tNGS, ctDNA-tNGS	• This is a registered clinical trial (I-PREDICT: NCT02534675) • 30% of pts evaluable for response achieved disease control (6-month stable disease, N = 4; CR, N = 1; PR, N = 16) • Targeting of more drug targets correlated with significantly improved disease control, PFS and OS rates	This trial supports a shift from the current clinical trial paradigm for precision oncology (one driver mutation, one drug) to individualized combinatorial therapy	[14]
47 (Archived PT, double MT samples at baseline, PR and progression (core biopsies) and serial plasma samples every 4 weeks)	tNGS, cfDNA-tNGS	• This is a prospective phase II trial (NCT02994888) • 50% of tumor *RAS* wild-type patients harbor *RAS* pathway mutations in pretreatment cfDNA • Primary and acquired cetuximab resistance are often of polyclonal nature and can be tracked by serial tissue and liquid biopsies	Frequent serial liquid biopsies could predict therapeutic failure and progression	[16]

Abbreviations: cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), colorectal cancer (CRC), complete response (CR), metastatic tumor (MT), partial response (PR), primary tumor (PT), targeted next-generation sequencing (tNGS).

instance, the WINTHER [12] and the I-PREDICT [14] clinical trials investigated whether tNGS of the tumor and matched tumor/ctDNA respectively could effectively guide combinatorial targeted therapy. Both basket studies included patients with diverse lethal refractory cancers, including CRC. A specialized committee reviewed the genetic results and suggested combinational targeted drug regimens, based on both on- and off-label matches. Off-label examples of drugs approved by FDA for other cancer types include pertuzumab and trastuzumab for *ERBB2* mutations, afatinib for *ERBB3* mutations, dabrafenib, trametinib and vemurafenib for BRAF V600E mutations and others (Supplementary Table 1). A "matching score" was calculated based on the number of mutation-drug matches for each patient, with several patients receiving a combination of two or three agents, such as afatinib, trametinib and sulindac for *KRAS*, *ERBB3* and *APC* mutations, as well as cetuximab plus trametinib for *KRAS* and *SMAD4* alterations. Both studies concluded that patients with a higher matching score achieved better disease control rates (> 6 months stable disease, partial or complete response) and more favorable oncological outcomes, including progression-free and overall survival [12,14]. In specific, the I-PREDICT study reported a significant median progression free survival increase from 3.1 to 6.5 months ($P = 0.001$) for high versus low matching score patients [14]. Similarly, the TARGET study [13] evaluated the potential to guide targeted therapy via ctDNA-NGS, concluding that tNGS-guided matched therapies resulted in better rates of disease control than non-matched treatments. These data support the integration of genetic profiling into clinical practice following large-scale confirmation, challenging the current clinical trial paradigm of Precision Oncology, matching one driver mutation to one agent and suggesting a shift to combinatorial optimized therapy irrespective of cancer type.

Despite all these intriguing early results, static analyses are incapable of identifying tumor evolution responsible for therapeutic failure and spatiotemporal studies within a clinical setting are still scarce. One such trial was lately published by Khan and colleagues [16], focusing on the emergence of resistance to cetuximab. For the first time within a prospective phase II study, the researchers analyzed over therapy longitudinally acquired multi-regional samples from matched primary, metastatic and progressive tumors, along with frequent cfDNA liquid biopsies by tNGS and reported several interesting findings. A significant percentage as high as 50% of supposedly *KRAS* wild-type patients harbored RAS pathway mutations at baseline cfDNA, conferring pre-existing primary cetuximab resistance. Regarding acquired resistance, tissue and liquid biopsies were highly concordant and both accurately captured tumor dynamics resulting in treatment failure, highlighting that resistance is polyclonal in nature and by one-third attributed to aberrations in genes outside the RAS pathway, such as *PIK3CA* and *ERBB2*, not routinely tested in clinical practice. Especially serial liquid biopsies at dense time intervals (< 2–3 months), coupled with mathematical modelling, were most successful to track dynamic tumor evolution and emerging heterogeneity and provided sufficient predictive power to readily detect therapeutic resistance and progression a few months before clinical diagnosis. Therefore, dynamic tissue and liquid sampling could effectively inform clinicians about the timing of clinical decisions and future treatment strategies for CRC [16]. Nevertheless, despite this study being the first spatiotemporal trial with a prospective phase II protocol, major limitations include the small sample size (47 patients), the limited focus on the RAS pathway and cetuximab, as well as the very small number of cancer-related genes within the utilized tNGS panel (77 genes). Further prospective confirmation dynamic studies, integrating more advanced technological systems exploring intratumor and circulating heterogeneity through WGS, are hence warranted to determine the true benefit of longitudinal sampling in a clinical setting.

Sequencing at single-cell resolution and genome editing

Recently, technical improvements of the single-cell technique in combination with NGS technologies have enabled the identification of molecular differences between individual cells. Progress over the past few years has been so impressive that single-cell RNA sequencing (RNAseq), in conjunction with machine learning models, has already successfully been applied to delineate the transcriptional dynamics of normal organogenesis in mice, analyzing a striking total of two million single cells [2]. Especially in cancer, bulk multi-regional NGS is conceptually incapable to enable full exploration of genetic, genomic and transcriptional ITH and the detection of rare subclones within the primary tumor, possibly driving progression and metastasis [81]. At present, several reports on CRC single-cell genomics and transcriptomics have been published, analyzing a few thousands of individual cells (Table 4) [9,25,82–92]. In an exemplary study, Roerink et al. [25] subjected single-cell-derived clonal organoids from three CRCs to RNAseq with groundbreaking results. All three tumors contained cells resistant to common drugs but most importantly differential drug response was identified even between closely related cells, showing a model of late emergence of resistance during tumorigenesis consistent with Darwinian dynamic evolution [27] and suggesting the need for early diagnosis and effective intervention [25]. A crucial implication of single-cell transcriptomics for the precise characterization of cell-to-cell heterogeneity-dependent drug sensitivity is the optimization of multi-drug treatment at the individual level [9,19]. Single-cell next-generation sequencing (scNGS) has further been utilized as a means of more detailed CRC sub-classification compared to bulk analysis [83,84], allowing for the detection of rare aggressive cell sub-populations, potentially associated with cancer progression and metastasis [85]. Moreover, scNGS of matched primary and metastatic tumor has been used to track spatiotemporal tumor evolution as well as the dynamic tumor immune microenvironment through single-cell multiomics and single T-cell RNAseq respectively [82,83], potentially informing future implications for cancer treatment and, particularly immunotherapy. Taking into consideration that integration of scNGS into translational research is only now beginning, future large patient-centric studies are highly anticipated and expected to revolutionize current approaches to cancer research.

In parallel to clinical and translational studies, CRC constitutes a critical object for fundamental research on cancer models, such as cell lines and organoids, integrating highly innovative technologies and primarily CRISPR-based genome engineering in conjunction with DNA/RNA NGS (Table 4) [9,25,82–92]. Targeted genome manipulation using engineered nucleases via CRISPR-Cas systems has surfaced as a novel approach with potentially crucial clinical implications [3]. The power of CRISPR-Cas9 to identify and prioritize potential drug targets has recently been demonstrated by CRISPR-based screening of more than 300 cancer cell lines from 30 different tumor types, including CRC, by Behan and colleagues [86]. For instance the Werner syndrome ATP-dependent helicase was verified as a synthetic lethal target in microsatellite instable tumors in a cancer type-agnostic manner, raising promises for adjunct therapy to immune checkpoint inhibition. This study underlines the capacity of genome editing to inform modern drug development by enriching and diversifying the portfolio of cancer therapeutic targets, in order to create a systematic framework for effective tailored treatment [86]. Several other studies have also exhibited the capabilities of CRISPR-Cas in cancer target discovery, especially for CRC [88,89,92]. Therefore, the revolutionary capacity for precision editing both ex-vivo and in-vivo has delivered a transformative tool in the discovery of novel oncotargets and drugs in pre-clinical studies, while the first early-phase clinical trials on various diseases, including leukemias and solid tumors, implementing genome editing have just begun to materialize [3,4,93]. Furthermore, innovative studies implementing various combinations of NGS technologies, and particularly WGS and RNA sequencing, with interaction mapping via

Table 4
Integration of breakthrough single-cell sequencing and editing technologies into fundamental and early translational research.

Sample type	Technology	Findings	Implications	Ref
Single-cell DNA and RNA next-generation sequencing				
12 pts (11,138 single T-cells)	Single-cell RNAseq, TCR tracking	20 distinct T-cell subsets were identified, correlated with distinct functions, as well as dynamic relationships among different groups	Single T-cell transcriptome analysis could provide immunotherapeutic implications, particularly for MSI-H tumors	[82]
12 pts (1,900 single cells and bulk multi-regional PT and MT)	Multiomics including single-cell Trio-seq and bulk MR-WGS	• Cancer cells were classified into several genetic subclones • PTs showed higher subclonality than MTs • DNA methylation profiles were stable	Single-cell multiomics sequencing can trace epigenomic and transcriptomic dynamics during progression and metastasis	[83]
11 pts and 7 cell lines (590 patient–derived and 561 cell line-derived single cells)	Single-cell RNAseq and RCA algorithm	Single-cell RNAseq generated further sub-classification of CRC subtypes found by bulk RNAseq, with prognostic significance	Single-cell RNAseq could enable clinically relevant patient stratification	[84]
3 pts (Single cell-derived clonal organoids)	tNGS, WGS, RNAseq	• Drug response differed even between closely related single cell-derived clones, suggesting late emergence of resistance during tumorigenesis • All three colorectal cancers contained cells resistant to common drugs • Diversification of methylation and transcriptome state was heritable, stable and independent • of the tumor microenvironment	Single cell-derived organoid clones could provide a powerful cancer model to study ITH through functional assays	[25]
2 pts (360 single-cells and bulk PT and LM samples)	Single-cell tNGS, bulk WES	• Single-cell and bulk analysis were 100% concordant • Monoclonal and polyclonal seeding was identified • Rare cell sub-populations were identified as associated with progression and metastasis • However, a late-dissemination model was identified, consistent with high concordance between PT and MT	The late dissemination model suggests that early surgical intervention could prevent metastasis	[85]
Genome engineering, cancer models and discovery of novel drug targets				
Multiple cancer types, including CRC (106 cell lines and 26,465 individual cells)	WGS, tNGS, single-cell RNAseq, CRISPR-Cas9	Extensive genetic and transcriptional heterogeneity arises both from pre-existing subclones and de novo, producing rapid diversification and differential drug sensitivity	• Clinical models and translational research is required • Transcriptional heterogeneity suggests the need for extensive drug development	[9]
30 cancer types, including CRC (324 human cancer cell lines)	CRISPR-Cas9	The WRN helicase, which physically interacts with MMR proteins, was identified as a synthetic lethal target in multiple cancer types with MSI	Clinical development of WRN antagonists is a potential therapeutic strategy, following confirmation	[86]
Cell-lines	Hi-C, ChIP-seq, CRISPR-Cas9, RNAseq	• A recurrently mutated *cis*-regulatory element was identified to interact with the *ETV1* promoter affecting gene expression and subsequently cell viability and patient survival • *RASL11A* was found a target of a previously identified enhancer amplification	• Identification of *cis*-regulatory elements affecting *ETV1* expression could be used as prognostic biomarkers • *ETV1* inhibitors should be further evaluated in clinical trials with patient stratification	[87]
Cell lines	CRISPR-Cas9	CRISPR-Cas9-mediated gene replacement of WT *KRAS* with a mutant allele to produce homozygous mutant clones sensitized HCT116 CRC cells to MEK inhibition	This study provides potential translational implications for targeted therapeutic strategies	[88]
Cell lines from multiple cancer types	ChIP-seq, 3C, CRISPR-Cas9, RNAseq	• 3 distinct cancer type-specific mechanisms of *KLF5* activation by somatic coding and noncoding mutations were identified • Cancer cells with *KLF5* overexpression are dependent on KLF5 for their proliferation	These data suggest *KLF5* as a potential novel oncotarget following validation	[89]
Multiple cancer types (19 CRC, FFPE samples)	ChIP-seq/FiT-seq	Chromatin immunoprecipitation sequencing identified tumor specific *cis*-regulatory elements correlating with known oncogenic drivers	Epigenomic analysis could potentially elucidate on gene regulation and uncover novel non-coding biomarkers	[90]
10 pts and cell lines (PT before and after cetuximab resistance and pt-derived cell lines)	RNAseq, WES, ChIP	• The lncRNA MIR100HG and two microRNAs, miR-100 and miR-125b, were overexpressed without cetuximab resistant mutations in both cetuximab-resistant cell lines and tumors • miR-100 and miR-125b repressed five Wnt/β-catenin negative regulators, resulting in increased Wnt signaling • Wnt inhibition restored cetuximab responsiveness • miR-125b upregulates MIR100HG by inhibiting GATA6 expression	MIR100HG could be a novel predictive biomarker guiding Wnt targeting in future trials investigating cetuximab resistance	[91]
PDA cell lines and xenografts, CRC organoid cultures	CRISPR-Cas9, RNAseq	Antibodies that selectively bind FZD5 and FZD8 robustly inhibited the growth of RNF43-mutant PDA cells and CRC organoid cultures in vitro and PDA xenografts in vivo	CRISPR-based genetic screens can be used to identify novel oncotargets	[92]

Abbreviations: chromosome conformation capture (3C), chromatin immunoprecipitation (ChIP), clustered regularly interspaced short palindromic repeats (CRISPR), colorectal cancer (CRC), fixed-tissue chromatin immunoprecipitation sequencing (FiT-seq), formalin-fixed paraffin-embedded (FFPE), high-throughput chromosome conformation capture (Hi-C), intratumor heterogeneity (ITH), liver metastasis (LM), long non-coding RNA (lncRNA), metastatis tumor (MT), micro RNA (miR), microsatellite instability high (MSI-H), multi-regional (MR), pancreatic ductal adenocarcinoma (PDA), patients (pts), primary tumor (PT), reference component analysis (RCA), sequencing (seq), T-cell receptor (TCR), targeted next-generation sequencing (tNGS), triple omics sequencing (trio-seq), wild-type (WT), whole-exome sequencing (WES), whole-genome sequencing.

Hi-C and ChIP-seq, as well as DNA editing have explored and dissected coding and non-coding, genomic and transcriptional heterogeneity at bulk and single-cell resolution, substantially advancing our understanding of gene expression [9,26,60,66,73,87,90,91]. In the following years, in-depth delineation of coding and non-coding cell-to-cell heterogeneity is expected to remodel the concepts of precise prediction and drug development.

Future outlook

Exploiting breakthrough technologies and appropriate methodologies, emerging research focuses on overcoming three major challenges: late diagnosis, lack of robust predictive biomarkers and limited efficacy of available targeted agents. Innovative approaches exploring genome- and transcriptome-wide aberrations in time and space encourage the realization of long-term research dreams, including non-invasive screening, individualized drug sensitivity prediction and development of a drug bank to successfully address extensive genetic, genomic and transcriptional heterogeneity. Patient-centric expectations comprise medium-term clinical implications through a new generation of clinical trials on the one hand [14], and a distant horizon of translational integration of breakthrough technologies into appropriately designed studies to understand genome and transcriptome regulation in health and disease on the other [94,95].

Evidence-based precision medicine

The explosive progress in NGS studies has highlighted the beginning of a new era of early-phase clinical trials of simplistic design evaluating the clinical utility of bulk ITH and cf/ctDNA-NGS to improve oncological outcomes [12–14,16]. The development of effective non-invasive screening could dramatically increase early-stage diagnosis, reducing cancer-related death rates and public health costs. Currently, NGS of cf/ctDNA represents the most rational and promising perspective. An underway large-scale clinical trial integrating cfDNA-tNGS/WGS/WGBS has already preliminarily reported high overall sensitivity rates for several cancer types, such as CRC, but the final results are necessary to determine the suitability of this platform as a screening tool, considering that sensitivity rates for stage I tumors was only 34% [17]. By contrast, much more promising data have more recently been reported through the combination of cfDNA-tNGS/WGS with machine learning models, with overall combined sensitivity reaching 91% on a patient population comprised in the majority by stage I and II disease [29]. Nevertheless, despite this method appearing to meet the criteria for screening, large-scale confirmation clinical trials are required. Furthermore, exploitation of both available drugs targeting specific gene mutations irrespective of cancer type and extensive panels for tNGS, has enabled the conduction of basket trials on individualized combinational therapies. The positive results returned by Sicklick et al. in a small scale combined analysis of tissue and matched ctDNA [14] are limited by the simplistic tNGS-based design disregarding genome- and transcriptome-wide alterations and warrant further larger and more sophisticated validation trials.

Although several studies have provided exciting results on dynamic genomic clone and tumor evolution, as of yet, there has been no comprehensive study or trial evaluating bulk intra-individual genomic and trancriptomic landscapes in time and space. We propose an innovative triple comparison between primary tumor ITH, serial cf/ctDNA-NGS and metastatic tumor ITH for each individual patient within a new generation of prospective patient-centric trials (Fig. 2). This protocol could generate a series of research discoveries and clinical implications. First, it provides the potential to assess the magnitude of genetic, genomic and transcriptional intratumor heterogeneity (Table 2). Second, the role of ITH as a prognostic and predictive biomarker guiding primary therapeutic decision-making could be validated and established. Third, the current grand challenge of detecting minute amounts of cfDNA, as in the case of minimal residual disease after surgery, could be overcome by combining DNA fragmentation patterns analyzed within machine learning models and mutation analysis [29]. Thus, dynamic cf/ctDNA-NGS could contribute to primary decision-making on adjuvant treatment through comparisons between NGS of cfDNA before and after R0 resection with ITH. In the neo-adjuvant setting, as is the case for most rectal cancers, comparison of multi-regional NGS before and after treatment could reveal the emergence of resistant clones and guide effective adjuvant therapy [96]. Moreover, serial NGS liquid biopsies could be used for patient monitoring to predict acquired resistance and relapse several months before imaging diagnosis and enhance secondary decision-making by early targeting of resistant mutations [19,29,97]. Fourth, ITH analysis of synchronous or metachronous metastasis can identify alterations responsible for resistance and hematogenous spread. And fifth, the precise characterization of the complete repertoire of intra-individual alterations in time and space promises to provide high-quality evidence on the origins and evolution of therapeutic resistance, highlighting the advent of Colorectal Cancer Precision Medicine [19].

Breakthrough genome, transcriptome and regulatory network exploration

Lately, integrated multiplex technological frameworks, including single-cell genomics/transcriptomics, CRISPR-Cas9 and interaction mapping, have opened new avenues towards individualized therapeutic response prediction, discovery of novel drug targets and understanding complex dynamic regulatory circuitry [6,94,98]. The recent evidence on cell-by-cell variability-dependent drug sensitivity in an analysis of thousands of cells from multiple cancer types [9] and the necessity of machine learning to analyze transcriptional landscapes of two million cells [2] suggest that high-precision dissection of drug resistance at single-cell resolution within the primary tumor containing hundreds of millions of cells will require sophisticated artificial intelligence systems [99]. In addition, single-cell RNAseq could also add to the understanding of intricate cell-to-cell interactions potentially informing therapeutic resistance [100]. Future large-scale single-cell NGS studies and trials will enable full and detailed exploration of ITH [81], significantly increasing the accuracy in the assessment of therapeutic response for each patient. Beyond precise prediction, genome and transcriptome editing technologies pose as a compelling opportunity, in addition to NGS, to address the necessity of developing multiple novel drugs to overcome extensive intra-individual heterogeneity. In addition to genome editing [4,86], latest technological advances allowing precise RNA editing can be used to modify transcripts containing coding and non-coding pathogenic mutations [101], as well as for transcriptome reprograming with breakthrough drug systems [102].

Beyond long-term conventional linear experimentation, DNA and RNA sequencing and editing technologies have provided critical insight into the complex regulation of gene expression in health and disease. However, the precise mechanisms through which coding and non-coding variation affects gene control remain poorly understood. A recent landmark paper integrating WGS, RNAseq, ChIP-seq, Hi-C and computational algorithms unveiled the intricacy with which genetic variation perturbs cis- and *trans*-regulatory coordination and gene expression unraveling the multiple network layers between genetic variants, regulatory elements and gene expression underlying disease phenotypes [94]. In cancer, future studies integrating a variety of techniques and methods, including detection of bulk and single-cell intratumor transcriptional heterogeneity [25,103] and transcription factor-binding site mutations [60], as well as transcriptome editing [101,102], could not only achieve high precision in drug response prediction but also discover next-generation drugs targeting key transcriptional elements and regulators of aberrant linear and non-linear transcriptional circuits [8,104–106]. In summary, the future development of an extensive drug bank, including both the continuing discovery of drugs based on single-gene linear transcription and regulatory

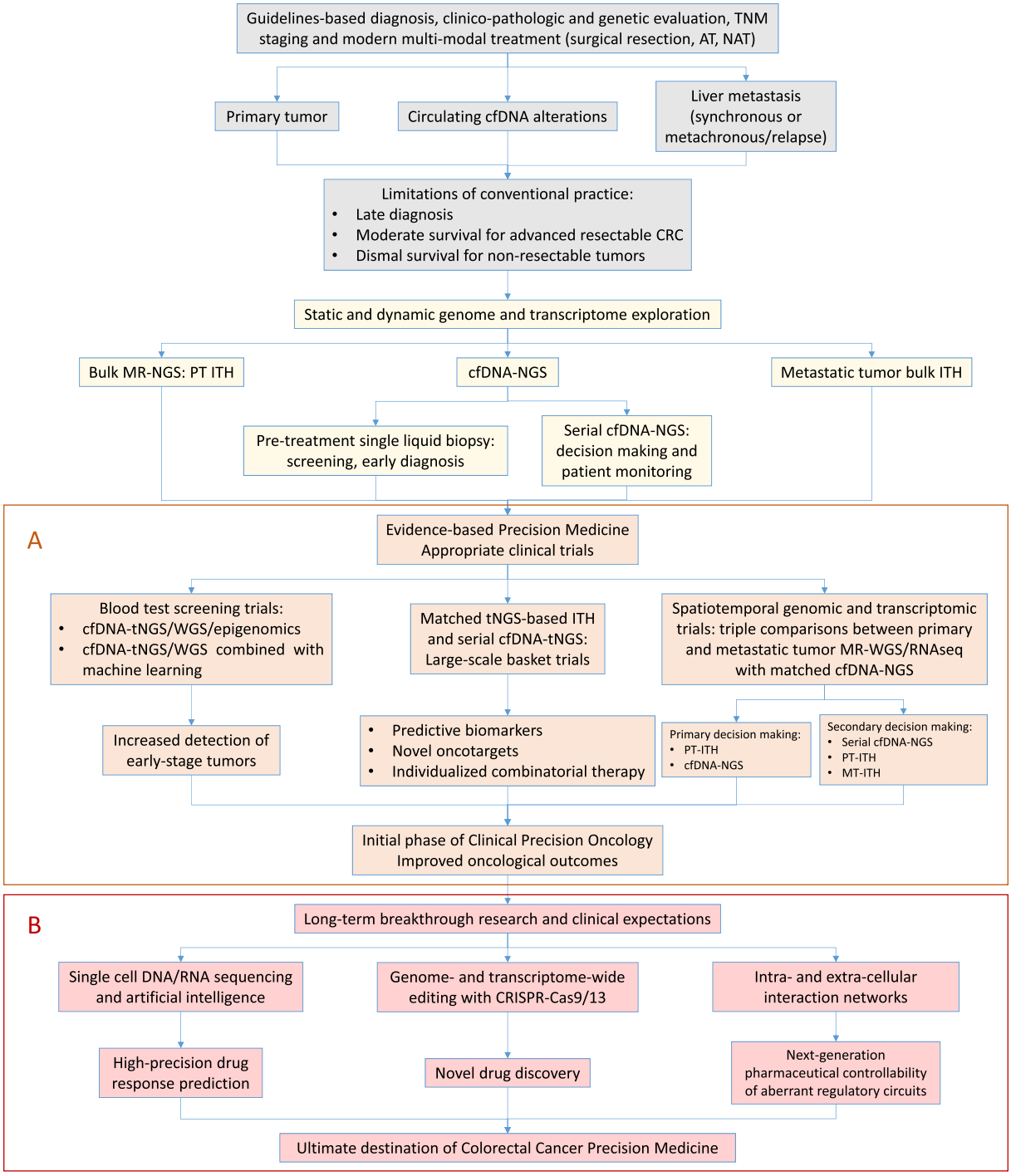

Fig. 2. Exploration of the comprehensive intra-individual genetic, genomic and transcriptional heterogeneity towards early and final stages of Colorectal Cancer Precision Medicine. Step-wise flowchart including primary tumor ITH with matched cfDNA alterations and metastatic liver tumor ITH, towards individualized precise drug response prediction and optimization of therapy. A. Medium-term evidence-based implications through bulk NGS and matched serial cfDNA-NGS trials: novel screening via cfDNA-NGS and machine learning, development of predictive biomarkers and discovery of new CRC-specific oncotargets and drugs towards optimization of primary and secondary therapeutic decision-making. B. Long-term expectations: accurate individualized prediction of cell-by-cell heterogeneity-dependent drug sensitivity, discovery of novel drug targets and development of next-generation therapies disrupting aberrant transcriptional biocircuits. **Abbreviations:** adjuvant treatment (AT), cell-free DNA (cfDNA), colorectal cancer (CRC), intratumor heterogeneity (ITH), metastatic tumor (MT), multi-regional (MR), neo-adjuvant treatment (NAT), next-generation sequencing (NGS), primary tumor (PT), RNA sequencing (RNAseq), targeted NGS (tNGS), whole-genome sequencing (WGS).

network-directed agents, could enable optimization of therapy on the basis of precise prediction and selection of the most effective drug combination.

Conclusions

Genome and transcriptome sequencing and editing technologies, coupled with interaction mapping and machine learning systems, as well as the static and dynamic exploration of genetic, genomic and transcriptional heterogeneity within early and underway clinical trials

highlight a shift from research to clinical evidence-based Cancer Precision Medicine. Future prospective CRC studies implementing a triple comparison between primary and metastatic tumor ITH with serial liquid biopsies could provide high-quality evidence on the origins and evolution of genomic subclones responsible for drug resistance and relapse. These advancements advocate the realization of three prime clinical goals, namely non-invasive screening for early diagnosis, development of multiple novel targeted drugs and individualized prediction of sensitivity to drug combinations. Deeper insight into coding and non-coding genome- and transcriptome-wide alterations deregulating gene expression through linear and non-linear cis and trans regulatory networks will unravel the next-generation therapeutic controllability of perturbed transcriptional circuitry.

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Declaration of Competing Interest

The authors have no competing interests to report.

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Appendix A. Supplementary material

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