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Chromatin factors: Ready to roll as biomarkers in metastatic colorectal cancer?

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A B S T R A C T

Colorectal cancer (CRC) ranks as the third most prevalent cancer globally and stands as the fourth leading cause of cancer-related fatalities in 2020. Survival rates for metastatic disease have slightly improved in recent decades, with clinical trials showing median overall survival of approximately 24–30 months. This progress can be attributed to the integration of chemotherapeutic treatments alongside targeted therapies and immunotherapy. Despite these modest improvements, the primary obstacle to successful treatment for advanced CRC lies in the development of chemoresistance, whether inherent or acquired, which remains the major cause of treatment failure. Epigenetics has emerged as a hallmark of cancer, contributing to master transcription regulation and genome stability maintenance. As a result, epigenetic factors are starting to appear as potential clinical biomarkers for diagnosis, prognosis, and prediction of treatment response in CRC.In recent years, numerous studies have investigated the influence of DNA methylation, histone modifications, and chromatin remodelers on responses to chemotherapeutic treatments. While there is accumulating evidence indicating their significant involvement in various types of cancers, the exact relationship between chromatin landscapes and treatment modulation in CRC remains elusive. This review aims to provide a comprehensive summary of the most pertinent and extensively researched epigenetic-associated mechanisms described between 2015 and 2022 and their potential usefulness as predictive biomarkers in the metastatic disease.

Abbreviations: 5-FU, 5-Fluorouracil; AZA, 5-aza-2'-deoxycytidine; bp, base pairs; CIMP, CpG island methylator phenotype; CIN, chromosomal instability; CMS, consensus molecular subtype; CRC, colorectal cancer; CT, chemotherapy; DFS, disease-free survival; DNMT, DNA methyltransferase; DSBs, double strand breaks; EMT, epithelial-to-mesenchymal transition; FFPE, formalin fixed paraffin embedded; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDACi, HDAC inhibitor; HDM, histone demethylase; HMT, histone methyltransferase; HR, homologous recombination; ICI, immune checkpoint inhibitor; IT, immunotherapy; MSI, microsatellite instability; OS, overall survival; PDX, patient-derived xenograft; RFS, relapse free survival; ROS, reactive oxygen species; TILs, tumor-infiltrating lymphocytes; TS, thymidylate synthase; TTP, time to progression; wt, wild-type.

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1. Introduction

1.1. Genetic basis of CRC

Colorectal cancer (CRC) ranks as the third most common cancer worldwide and stands as the second leading cause of cancer-related deaths, resulting in 1.9 million new cases and 935,173 deaths in 2020 [1].

The majority of CRC cases are sporadic and develop slowly over several decades through the adenoma-carcinoma sequence, while hereditary cases account for only 5–10%. Tumor progression is facilitated by the accumulation of chromosomal and genomic alterations, frequently initiated by mutations in the *APC* gene. Early changes lead to the activation of oncogenes like *KRAS* and the inactivation of tumor suppressor genes such as *TP53*. This characteristic sequence is often accompanied by chromosomal instability (CIN), including aneuploidy and chromosomal alterations. Additionally, sporadic CRC tumors can exhibit mutations in microsatellite repeat sequences due to deficiencies in the DNA mismatch repair system, leading to microsatellite instability (MSI). Moreover, they can be characterized by a severely hypermethylated status of gene promoter regions, resulting in a CpG island methylator phenotype (CIMP) [2,3].

Nevertheless, many tumors do not fit into this characterization (CIN, MSI and CIMP), complicating the establishment of clear therapeutic guidelines for this type of cancer. In 2015, Guinney and colleagues [4] established an international consortium and published an extensive study that integrated mutational, transcriptional and DNA methylation data. All these layers of "-omic" information led to the identification of four consensus molecular subtypes (CMSs): i) CMS1: is the MSI immune group, representing 14% of all CRC tumors characterized by a hypermutated status frequently associated with *BRAF* mutations and a strong activation of immune evasion pathways; CMS2: represents the canonical phenotype with epithelial differentiation and marked WNT and MYC signaling activation, accounting for 37% of CRC tumors; CMS3: denotes 13% of CRC tumors and comprises the metabolic phenotype accompanied with *KRAS* mutations; CMS4: corresponds to the mesenchymal phenotype, which accounts for 23% of CRC tumors, and is characterized by activation of TGF-β signaling, angiogenesis, and matrix remodeling.

However, it is important to note that 13% of CRC cases do not fit any of these four CMSs, highlighting the high heterogeneity and complexity of CRC disease.

1.2. Current treatment of metastatic colorectal cancer

Approximately 20–50% of patients initially diagnosed with localized CRC will eventually develop metastases, mainly in the liver, followed by the lung, peritoneum and distant lymph nodes. In addition, 15− 30% of cases are first diagnosed with disseminated disease. In these cases, the 5-year survival rate is about 10–15%. The current treatment approach for non-resectable metastatic disease heavily relies on systemic treatment with cytotoxic chemotherapy (CT) involving fluoropyrimidines, oxaliplatin, and/or irinotecan, which impact DNA biology, leading to cell death. FOLFOX (folinic acid, fluorouracil and oxaliplatin), FOLFIRI (folinic acid, fluorouracil and irinotecan) and their variants are the most frequently used schedules [5]. The chemotherapy backbone is often combined with targeted therapies such as anti-EGFR (cetuximab or panitumumab, only in RAS wild-type patients) or anti-VEGF drugs (bevacizumab) [6].

However, a significant challenge in achieving a cure for CRC lies in overcoming therapeutic resistance. Surprisingly, since the approval of cetuximab, panitumumab and bevacizumab in the early 2000's no other drugs have been successfully introduced in first-line treatment and indeed, most CRC patients have not benefited from immunotherapies (IT) based on revolutionary immune checkpoint blockers or PARP inhibitors, which have shown success in other types of tumors. Consequently, after undergoing two or three lines of treatment, most of the patients progress while still being in good physical condition, and unfortunately, no further therapeutic options are available for them. This underscores the urgency to identify biomarkers that can better predict response to existing treatments and to discover novel effective drugs targeting different aspects, aiming to eradicate metastatic disease and overcome resistance.

To comprehend the potential mechanisms of resistance in CRC treatments and the possible involvement of chromatin, it is essential to provide a concise overview of their modes of action. 5-Fluorouracil (5-FU) is an uracil analogue where a hydrogen atom in the carbon 5 of the pyrimidine ring has been replaced by a fluorine atom. Through a series of enzymatic reactions, 5-FU is converted into FdUMP, FdUTP, or FUTP, which can inhibit thymidylate synthase (TS) after being incorporated into DNA or RNA. Inhibition of TS results in reduced levels of dTMP, causing an imbalance in the deoxynucleotide pool, particularly in the ratio between dATP and dTTP. Consequently, this imbalance severely affects the synthesis and repair of DNA, leading to lethal damage [7]. Irinotecan, also known as CPT11, is a soluble derivative of camptothecin, a plant alkaloid. Once metabolized, it forms SN-38, which acts as an inhibitor of the enzyme Topoisomerase I (Topo I). More precisely, SN-38 binds to the DNA-Topo I complex, impeding cell replication and inducing double-stranded cleavage within the DNA molecule, ultimately leading to cell death. These actions take place during the DNA synthesis phase (S phase), making irinotecan a cycle-specific drug [8]. Oxaliplatin exerts its action by creating bonds within the same DNA strand or between the two strands, leading to the formation of covalent bonds between the platinum active complex and specific bases within the DNA sequence. Consequently, these drug-DNA bonds hinder DNA synthesis, disrupt transcription processes, and elicit DNA damage responses, ultimately initiating cell death. Notably, this mechanism operates independently of the cell cycle [9]. While 5-FU might be administered as a single agent in certain situations, such as in the adjuvant setting, oxaliplatin and irinotecan are commonly used in combination with 5-FU for enhanced therapeutic effects [6]. Besides cytotoxic and cytostatic drugs, inhibitors for tyrosine kinase pathways such as cetuximab and panitumumab could also impact chromatin since tyrosine kinases may directly phosphorylate and thus regulate DNA repair and cell cycle checkpoint proteins. In addition, they regulate DNA damage by phosphorylating core histones as well as chromatin modifiers at critical tyrosine residues; for instance, EGFR has been shown to regulate phosphorylation of H4 at Y72 and of *ATM* at Y370 affecting DNA synthesis and repair in both cases, or *PCNA* at Y211 deregulating mismatch repair [10]. In the context of metastatic cancer treatment, anti-EGFR drugs are typically combined with a cytotoxic drug backbone and are specifically administered to patients with RAS wild-type (wt) tumors. This combined approach aims to capitalize on their effects and improve therapeutic outcomes. Finally, immunotherapies (IT) utilizing monoclonal antibodies targeting co-inhibitory immune checkpoints, such as PD1, PD-L1, and CTLA-4, have demonstrated clinical efficacy in various malignancies [11]. However, their impact on CRC is limited to tumors with a defective mismatch repair system leading to MSI, which comprises less than 5% of total metastatic cases [12]. In a broader context, the ineffectiveness of IT in CRC can stem from several factors, including: 1) insufficient generation of anti-tumor T-cells; 2) inadequate function of tumor-specific T-cells; 3) impaired formation of T-cell memory [13], or 4) impaired antigen presentation. Furthermore, each of these dysfunctions may be attributed to an altered chromatin status of genes involved in mediating these critical functions.

This fact is exemplified by the efficacy of epigenetic modifying agents, such as methyltransferase inhibitors and histone deacetylase inhibitors, which can influence the expression of antigen-processing and -presentation machinery elements, novel tumor-associated antigens, and cytokines [14]. These agents have the potential to enhance and bolster the immune response within tumor cells. Additionally, it has been observed that chromatin-remodeling pathways play a role in immune checkpoint inhibitor (ICI) resistance in melanoma by repressing

interferon-stimulated genes [15]. These discoveries suggest two significant concepts: first, that targeting chromatin remodelers could potentially convert resistant "cold" tumors into responsive "hot" ones, and second, that alterations in specific chromatin remodelers may indicate a favorable immune environment that could be therapeutically exploited.

In conclusion, chromatin regulation plays a fundamental role in the pathogenesis of CRC and its response to therapy. By understanding and manipulating these epigenetic mechanisms, we may be able to enhance treatment responses and potentially overcome resistance in CRC.

1.3. The landscape of chromatin regulation

Eukaryotic genomes are organized into a hierarchical structure known as chromatin. The fundamental unit of chromatin is the nucleosome, which consists of a core of histone octamers (two copies each of histones H2A, H2B, H3, and H4) around which a 147 bp stretch of DNA is wrapped. Furthermore, histone H1 binds to nucleosomes, acting as a "gripper," and plays a role in additional packaging into higher-order structures.

Chromatin is intricately associated with a diverse array of enzymes, transcriptional factors, cofactors, and non-coding RNAs, collectively playing pivotal roles in DNA repair, transcription, and replication. These chromatin regulators interpret various signaling pathways to modulate chromatin activity, thus laying the molecular foundation for genetic regulation. Epigenetic mechanisms encompass this repertoire of chromatin actors and the biochemical modifications they induce, including:

1) Addition or removal of methyl-, phospho-, acetyl-, glycosyl-, or nitrosyl- groups, among the most extensively studied, to histone tails protruding from nucleosomes. Enzymes responsible for these modifications are often referred to as "writers," such as histone acetylases and methylases, or "erasers," like histone deacetylases or demethylases, to name a few.

2) Addition of a methyl group to a cytosine nucleotide when followed by a guanine, a process managed by DNA methyltransferases (DNMTs), or oxidation of the methylated cytosine by ten-eleven translocation enzymes (TETs): TET1, TET2 and TET3.

3) ATP-dependent sliding, eviction, or alteration of histone composition within nucleosomes through the action of chromatin remodeling complexes, such as SWI/SNF, ISWI, CHD, or INO80 complexes.

These modified sites and loci serve as anchor points for proteins and complexes that participate in DNA repair, initiation of replication, or activation of transcription. Consequently, chromatin identity, supported by epigenetic mechanisms, is a complex and dynamic process involving a vast array of combinatorial possibilities that are only just beginning to be unraveled (Fig. 1).

1.4. The role of epigenetics in cancer

More than three decades ago, a significant breakthrough in understanding the association between epigenetic aberrations and cancer emerged in the context of retinoblastoma tumors, a pediatric malignancy affecting the eye. It was well-established that mutations and deletions were responsible for inactivating the tumor suppressor gene RB1 [16]. However, a groundbreaking observation revealed that RB1 promoter DNA hypermethylation resulted in transcriptional repression [17]. This finding was revolutionary in linking epigenetic modifications to the development of cancer. The exact cause of hypermethylation is not fully understood, as it doesn't always correlate with mutations in DNMTs or TET enzymes, suggesting additional factors and mechanisms are likely at play; for instance, novel modifications or impaired recruitment of crucial proteins are some to be considered.

In the last decade, sequencing efforts have revealed mutations in chromatin regulators across various cancer types [18], suggesting their significant role in cancer development. Moreover, not only mutations but also expression of genes encoding chromatin regulators is also found frequently altered in many different cancer types [19]. However, the impact of such alterations on oncogenesis can vary depending on tumor type, disease phase and other concurrent changes. To address this contradiction, researchers are integrating multiple -omic data (genomic, epigenomic, transcriptomic, metabolomic, and proteomic) to categorize tumors more accurately. As explained above, in CRC, this integrative approach has led to the CMSs classification [4], which aims to device personalized treatments based on molecular characteristics to enhance treatment efficacy. Further validation is achieved through *in vitro* and animal models, confirming the contributive role of epigenetic alterations in oncogenesis and exploring potential vulnerabilities for targeted treatments.

1.4.1. DNA methylation

In humans, DNA methylation occurs predominantly in cytosines in

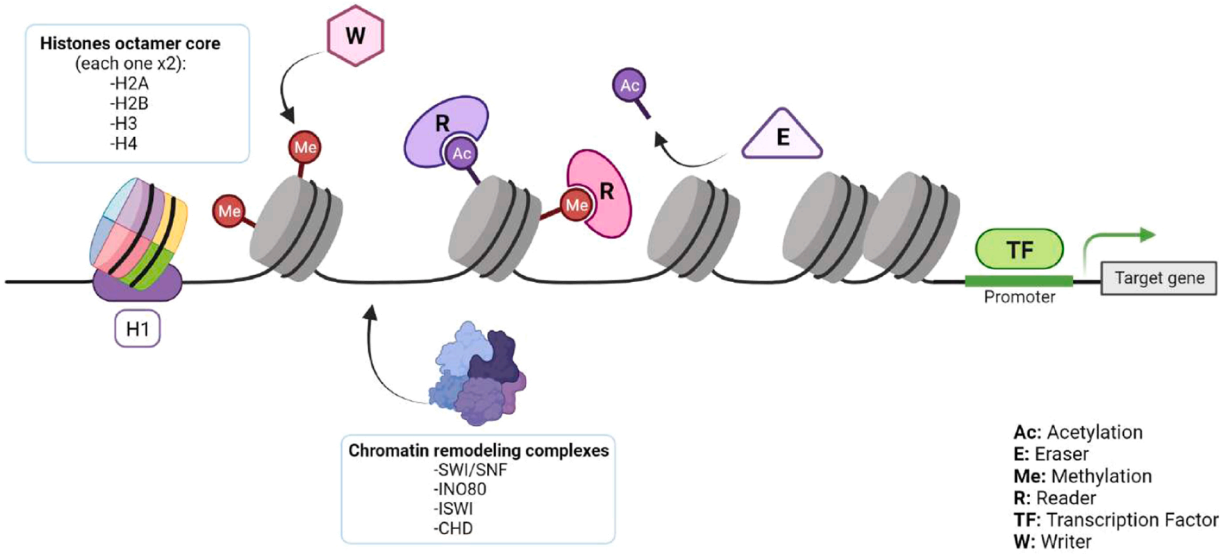

Fig. 1. Detailed chromatin structure of histone subunits and epigenetic modifiers. Chromatin is packed in octamer units, which are formed by the combination of four different histone subunits linked by histone 1. Writers (W), erasers (E) and readers (R) are key factors in charge of regulate chromatin structure by adding or removing epigenetic marks, such as methylation (Me) or acetylation (Ac). Moreover, changes in chromatin conformation may facilitate or impair the access of transcription factors (TF) or remodeler complexes, such as SWI/SNF, to promote gene expression. Created with BioRender.com.

the context of CpG dinucleotides, with some exceptions in embryonic stem cells and neural development [20,21]. Highly methylated CpG-containing regions are often found in the genome to repress transcription. However, CpG-rich regions, known as CpG islands, which have a CG content over 50% in a 200–2000 bp span, are usually demethylated in normal cells. As such, these CpG islands are indicative of active or potentially active promoters and enhancers [22–26].

In tumors, DNA methylation patterns undergo significant changes compared to normal tissues. Tumors are globally hypomethylated, particularly in repetitive DNA sequences, coding regions and introns [23,27], leading to the activation of oncogenes and altered splicing profiles [28]. In CRC, genome-wide hypomethylation was one of the first aberrant methylation events detected in all stages of the disease: hypomethylation of oncogenes, such as *MYC* or *HRAS*, and repetitive elements (LINE-1 and macrosatellites) promote genomic instability and tumor development [25,29,30].

Interestingly, malignant cells in CRC often exhibit hypermethylation of CpG islands, resulting in the transcriptional repression of tumor suppressor and DNA repair genes [22–26,31]. This hypermethylation is frequently observed in a specific subset of colorectal tumors known as CIMP tumors [30,32,33]. However, the specific genomic loci undergoing hypermethylation can vary among different CIMP tumors, leading to controversies in choosing markers for the CIMP phenotype. The mechanism driving aberrant hypermethylation in cancer remains complex and poorly understood.

1.4.2. Histone modifications

Another level of epigenetic regulation involves histone proteins and their dynamic post-translational modifications, which indicate different states and functionalities based on the cell's needs. These modifications directly impact DNA accessibility by altering the three-dimensional structures of nucleosomes. Consequently, they can either increase or decrease chromatin compaction, leading to transcriptional activation or repression, respectively.

Histone modifications occur in the tails of all histone proteins and their variants, providing docking sites for regulatory protein complexes. These protein complexes can possess enzymatic activities that further influence chromatin structure and function. The most well-known histone modifications occur at specific amino acid residues and include methyl, acetyl, phosphate, ubiquitin, and citrulline groups. These modifications play crucial roles regulating gene expression and other chromatin-dependent processes [24].

1.4.2.1. Histone acetylation. Histone acetylation is a critical epigenetic modification that occurs in their lysine residues and influences the interaction between histones and DNA. When histones are acetylated, lysine residues gain a negative charge, leading to a repulsion of the negatively charged DNA. This process results in less compacted chromatin and creates docking sites for various enzymatic activities. The global impact of histone acetylation is significant in cancer development and progression. Hyperacetylation of histones is linked to the abnormal activation of oncogenes, promoting tumor growth. On the other hand, hypoacetylation, promoted by histone deacetylases (HDACs), can silence tumor suppressor genes, contributing to tumor expansion and progression [25].

Histone acetyltransferases (HATs) are enzymes that promote gene transcription by exposing gene promoter sites to the transcriptional machinery, thereby facilitating the expression of specific genes essential for cell function and proliferation. The dysregulation of histone acetylation by HATs and HDACs has significant implications for cancer biology and treatment [34].

1.4.2.2. Histone methylation. Histone tails can undergo methylation at various residues such as arginines, lysines, and histidines. The enzymes responsible for catalyzing histone methylation and demethylation are called histone methyltransferases (HMTs) and histone demethylases (HDMs), respectively.

Unlike histone acetylation, histone methylation does not seem to directly impact DNA compaction. Instead, it creates sites that can be recognized by different protein complexes, leading to diverse biological outcomes. For instance, methylation of arginine residues promotes transcriptional activation, while methylation at lysine residues can result in either transcriptional activation or repression, depending on the specific site of methylation. Moreover, histone methylation can occur in different degrees, represented by mono-, di-, or tri-methylation, adding an additional layer of regulation and complexity to each methylation site.

The alterations in histone methylation processes can contribute to the activation of oncogenes and the silencing of tumor suppressor genes, thereby influencing the development and progression of cancer. These dynamic modifications of histone proteins play a pivotal role in regulating gene expression and have significant implications in carcinogenesis and tumor progression [25,31].

1.4.3. The SWI/SNF complex

The SWI/SNF complex has been extensively studied as a chromatin remodeler. It consists of an ATPase subunit, BRG1 or BRM (encoded by SMARCA4 and SMARCA2 genes, respectively), along with core subunits BAF155 (encoded by SMARCC1 gene), BAF170 (encoded by SMARCC2 gene), and INI1 (also known as SNF5 or BAF47, encoded by SMARCB1 gene) [35,36]. Additionally, it contains 6–11 accessory subunits (ARID1A, ARID1B, ARID2, BAF47, BAF60a, BAF60b, BAF60c). The various compositions of SWI/SNF accessory subunits confer distinct activities and cell/tissue specificities [37].

The main functions studied include altering chromatin organization by sliding nucleosomes or evicting histones, which impacts the accessibility of the transcriptional machinery and DNA repair mechanisms [38,39]. Altered chromatin remodelers were identified in cancer over twenty years ago, but their contribution to disease development remained unclear. While Brg1 + /- mice suggested a tumor suppressor role, upregulated SW/SNF subunits in breast tumors indicated a potential pro-oncogenic role [40]. Recent integrative bioinformatic analyses from different cancers tend to support a tumor suppressor role [41], involving impaired decatenation, which prevents Topoisomerase IIα access to DNA, rather than altered transcription [42].

Furthermore, mutations or loss of SWI/SNF subunits have been linked to sensitivity to DNA double-strand break-inducing agents in several cell types [43,44]. SWI/SNF complexes are rapidly recruited to DNA double-strand breaks, promoting two main DNA repair pathways: non-homologous end joining and homologous recombination (HR) [45–47]. HR impairment has been observed in cells deficient for SWI/SNF subunits, but the exact mechanism connecting both events remains poorly understood [38,46].

2. Methods

The objective of this study was to conduct a systematic literature review to investigate the significance of chromatin regulators and epigenetic marks as potential predictive and prognostic biomarkers in CRC and their associations with specific therapies. The sections of the study were categorized based on factors that have been reported to influence the effectiveness of fluoropyrimidines (used as a single agent), oxaliplatin-based, and irinotecan-based treatment regimens in CRC.

A systematic bibliographic search was performed using the "Entrez" option in PubMed database filtering by using "Publication date" as a main criterion and "Custom range" from 1st January 2015 until 31st December 2022. Extracted papers were grouped by keywords (colorectal cancer and drug/target and epigenetic regulator/mark). The "drug/target" keywords were the following: "panitumumab", "cetuximab", "fluoropyrimidines", "5-fluorouracil", "topoisomerase", "irinotecan", "oxaliplatin", "capecitabine", "bevacizumab", "immunotherapy", "PD-

1", "PD-L1", "CTLA-4" and "pembrolizumab". In the case of "epigenetic regulator/mark", the search included: "DNA methylation", "histone methylation", "histone acetylation", "chromatin helicase", "SWI/SNF", "ISWI" and "INO80". microRNAs and long non-coding RNAs, considered also as epigenetic regulators, were excluded from this review due to the extensive amount of recent bibliography available in several reviews.

Following the above-mentioned criteria, 356 papers were found. Duplicated entries were discarded before paper's eligibility process (n = 15). First paper selection was performed according to the concordance between used keywords and abstract-contained information (discarded n = 254). A second selection was based on the journal quartile: papers were prioritized and considered as candidate entries if they belonged to the first quartile, checked through "Academic accelerator" web page (n = 6). After the two-step screening process, additional papers were used from other sources to reinforce the information found in Pubmed, following the same criteria used before (n = 67). The search of suitable papers to be cited in i) "Introduction" section, ii) the introductory information from each subsection and iii) studies related with other types of cancers did not follow the criteria used for papers selected to be reviewed. In this case the most relevant authors related with the topic described were cited. As a result of the screening process, 148 papers were selected for deeply reading. The clinical relevance of some of the described epigenetic alterations related to the modulation of treatment efficacy were highlighted by the support of international clinical trials results found by using *ClinicalTrials.gov* web page (n = 8). The flow chart of final paper selection is represented in Fig. 2.

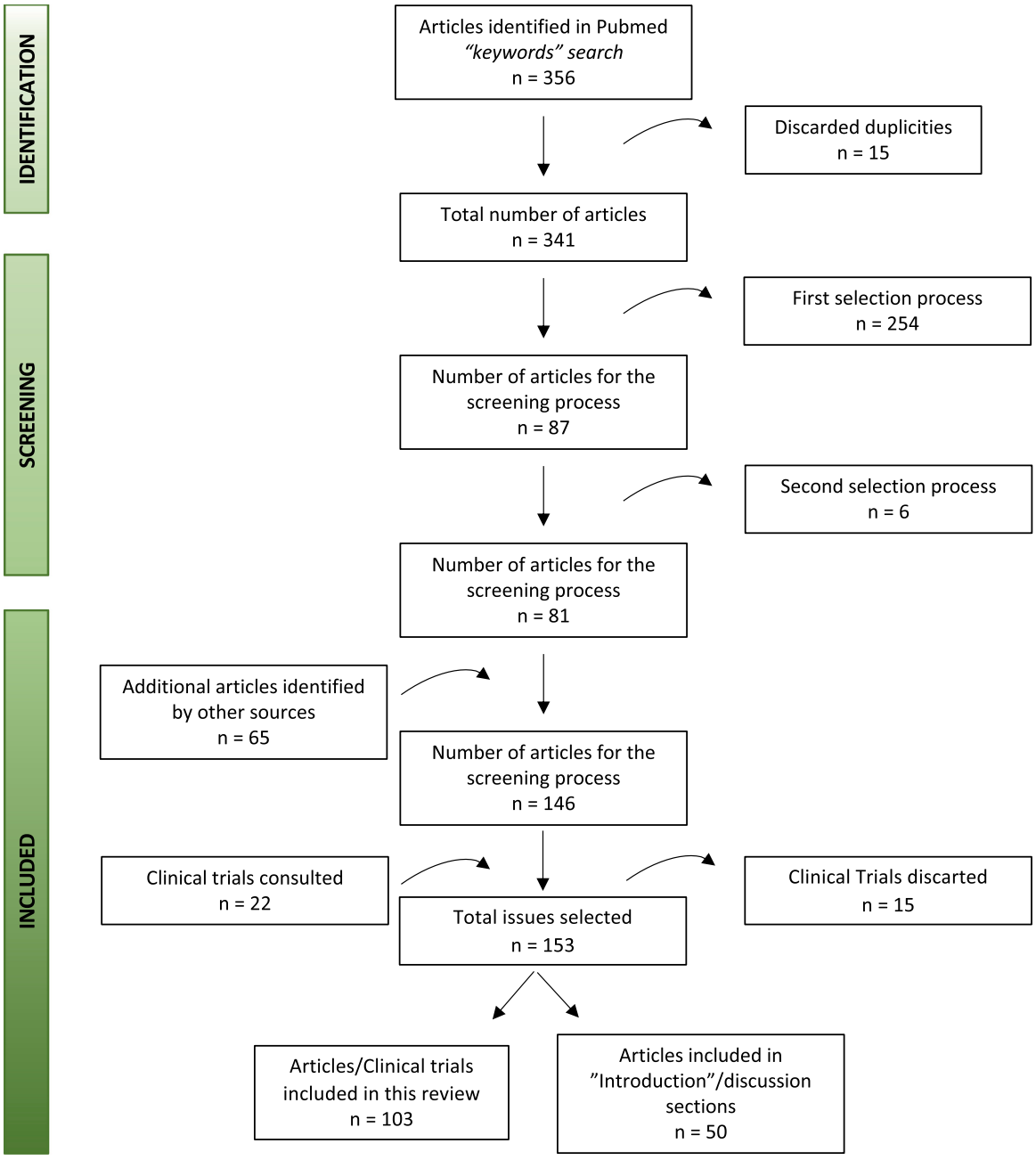

Fig. 2. Flowchart of the selection process. Flow diagram of selection procedure of studies assessing the implication of epigenetic and chromatin factors in the resistance to treatment in CRC. It specifies the inclusion and exclusion of articles considered for this systematic review.

3. Results

3.1. Epigenetic features affecting fluoropyrimidines efficacy

In CRC, changes in DNA methylation patterns have been associated with the promotion of metastasis and have also shown potential as prognostic biomarkers. Despite the critical role of methylation in CRC development, there is limited information available regarding the connection between aberrant methylation and drug response. Several studies have reported that epigenetic mechanisms can influence the response to 5-FU-based treatments by altering the expression of genes involved in its metabolism [48]. For example, Kunicka et al. investigated the methylation status of DPYS, an enzyme responsible for inactivating 5-FU during pyrimidine degradation. They found higher levels of DPYS methylation in CRC tumors compared to normal adjacent mucosa, however, they did not find conclusive associations with disease stage neither treatment reponses [49]. Nevertheless, the significance of DPYS in 5-FU resistance has been observed in triple-negative breast cancer. In this context, hypermethylation of DPYS may confer resistance to 5-FU treatment, particularly in HER2-positive cohorts of patients [50,51]. These findings highlight the potential importance of DPYS, with its enzymatic activity, in relation to 5-FU metabolism. However, further research is needed to better understand whether DPYS methylation status directly impacts 5-FU response or could be used as a predictive biomarker.

In recent years, the analysis of tumor methylomes available in public repositories has become a new approach to discover gene signatures that could serve as effective prognostic biomarkers [52]. Comparing the methylation profiles between normal and tumoral tissues can reveal gene families that are abnormally regulated by DNA methylation. In a study using data from the TCGA, Giri and colleagues identified the PEA3 transcription factor subfamily [53], which is associated with the MAPK signaling pathway [54], as potentially involved in 5-FU resistance.

Among the PEA3 members, the expression of ETV4 and ETV5 transcription factors is regulated by methylation. Interestingly, while upregulation of ETV4 is correlated with hypomethylation in the promoter region, ETV5 upregulation is associated with promoter hypermethylation. Strikingly, only ETV5 overexpression was found to be significantly linked to worse relapse-free survival (RFS), suggesting that ETV5 could serve as a potential predictive biomarker for 5-FU treatment response [53].

To further explore the clinical implication of methylation signatures, the group of Baharudin analyzed the epigenome of 48 CRC tumors with an Illumina methylation beadchip assay. They found a positive correlation between promoter hypermethylation of NKX6.1, TFAP2E-DKK4, and IGFBP3, and resistance to 5-FU chemotherapy [55]. However, the specific mechanism by which this effect is promoted was not further explored.

Nevertheless, *NKX6.1*, a member of the NKX family of transcription factors, has been extensively studied in various cancer types, including lung and cervical cancers [56,57]. *NKX6.1* has been implicated in epithelial-to-mesenchymal transition (EMT) and chemoresistance to 5-FU. In CRC, Chung et al. demonstrated the importance of NKX6.1 hypermethylation in promoting resistance to chemotherapeutic agents *in vitro*. They also showed that *NKX6.1* overexpression could enhance the sensitivity of CRC cells to 5-FU and oxaliplatin *in vitro* [58]. This association between aberrant *NKX6.1* expression and drug resistance was further supported by *in vivo* experiments conducted by Chang's group. The researchers analyzed the methylation status of *NKX6.1* in 151 stage II CRC patients and found a correlation with certain clinicopathological features. They also discovered that patients with methylated *NKX6.1* experienced reduced 5-year overall survival (OS) and disease-free survival (DFS) after receiving adjuvant 5-FU-based chemotherapy [59]. Considering the findings from both Baharudin's and Chung's studies, *NKX6.1* emerges as a promising predictive biomarker candidate not only in CRC but also in other tumor types.

In the study conducted by Luo et al., the focus was on capecitabine, another fluoropyrimidine agent used in the treatment of CRC. The researchers investigated the role of circFoxp1 (a circular RNA derived from the FOXP1 gene) in colon cancer cells' response to capecitabine treatment using *in vivo* models. The study found that downregulation of circFoxp1 sensitized colon cancer cells to capecitabine treatment. The mechanism underlying this effect involved circFoxp1's role in recruiting DNMT1 to the *FOXP1* gene promoter, leading to hypermethylation of *FOXP1* and subsequent transcriptional inhibition of the gene. Importantly, *FOXP1* is known to function as a tumor suppressor gene, so circFoxp1's regulatory role in DNA methylation could have significant implications for cell proliferation and response to chemotherapy [60].

The methylation status of promoter regions has been found to be critical for the function of *NME2*, a gene that promotes invasion and metastasis formation in cancer. Wen et al. conducted a study in which they observed lower *NME2* methylation levels in 5-FU-resistant CRC cell lines compared to parental cell lines. To investigate the role of *NME2* in chemoresistance, they downregulated *NME2* using siRNA. The results showed that NME2 knockdown restored 5-FU sensitivity, increased apoptosis, and reduced cell survival, indicating that *NME2* plays a role in mediating 5-FU responses. Conversely, when *NME2* was overexpressed, it led to the acquisition of 5-FU resistance [61]. This aberrant methylation pattern affecting *NME2* has also been observed in other types of tumors, such as lung cancer [62]. These findings suggest that epigenetic modifications, particularly methylation of the *NME2* promoter, can influence the sensitivity of cancer cells to 5-FU treatment and may have implications for the development of chemoresistance.

Aside from classical *MYC* or *HRAS* oncogenes, other oncogenes have been revealed due to their hypomethylated status in cancer. One such example is Nrf2, a transcription factor that plays a role in protecting cells from oxidative damage. Hypomethylation of Nrf2 has been linked to its upregulation, and this has been associated with chemoresistance in cancer. In particular, hypomethylation of Nrf2 was described as a consequence upregulation of the TET family. TETs are enzymes involved in the oxidation of methylated cytosines [63], leading cytosines to a non-methylated status thus contributing to upregulation of gene expression. When colon cancer cells are treated with 5-FU, reactive oxygen species (ROS) are released, which upregulate and activate TET1, leading to demethylation of *Nrf2* gene promoter [64] Additionally, the MLL/COMPASS-like complex is recruited to trimethylated H3K4 of *Nrf2*. These combined processes result in the transcriptional activation of Nrf2, which leads to chemoresistance [64]. This intricate regulatory network involving Nrf2 and epigenetic modifications highlights the complex interplay between oxidative stress, epigenetic regulation, and drug response in cancer cells. Understanding these mechanisms could open up new opportunities for developing targeted therapies to overcome chemoresistance and improve treatment outcomes for cancer patients.

A commonly altered histone acetyltransferase in cancer is *PCAF* (P300/CBP-associated factor), which has been related to cell growth and tumorigenesis. Somatic inactivating mutations of *PCAF* have been prevalent in various malignant tumors, including prostate and bladder cancers [65–67]. Several studies have specifically investigated the role of PCAF in resistance to 5-FU-based chemotherapy, particularly in CRC. Liu and colleagues demonstrated that a 5-FU resistant CRC cell line showed decreased PCAF levels that correlated with increased H3K27me3 in the promoter. The opposite situation was also supporting a role for PCAF in 5-FU response; *in vivo* models indicated that PCAF overexpression resulted in decreased tumor size upon 5-FU treatment. Importantly, patients with CRC tumors with lower PCAF expression showed poorer survival outcomes, as well as those with lower p21. *In vitro* data strongly indicates that the relationship between PCAF and sensitivity to 5-FU is reliant on p21. Unfortunately, data on 5-FU treatment in these patients was not available [68]. Du et al. also observed a similar trend in their study, where low levels of *PCAF* were associated with reduced long-term DFS and increased early progression

in stage II-III CRC patients [69]. While the current evidence suggests *PCAF* as a promising biomarker for 5-FU resistance in CRC, more research is needed to establish its clinical utility and fully elucidate its role in drug resistance mechanisms.

Another HAT involved in the modulation of 5-FU response is *NAA40*. The study conducted by Demetriadou et al. provides evidence that high expression levels of *NAA40* in CRC primary tumors might be associated with reduced sensitivity to 5-FU treatment. Indeed, downregulating *NAA40* in CRC cell lines, decreased proliferation and increased sensitivity to 5-FU [70]. Moreover, a recent study by the same group further supports the idea that *NAA40* could be involved in 5-FU resistance through its acetyltransferase activity, which would promote the expression of *TS*, a key enzyme in 5-FU metabolism. These findings agree with *in silico* analysis using a TCGA cohort, where non-responders to 5-FU based chemotherapy exhibited higher *NAA40* expression levels compared to those who responded [71]. The involvement of *NAA40* in other types of cancers, such as lung and liver cancers [72,73], suggests that its drug-modulating role may extend beyond CRC.

Conversely, acetylation reactions can be reversed by histone deacetylases (HDACs). Their activity has been linked to various cellular processes, including senescence, apoptosis or autophagy as well as EMT or DNA damage repair [74]. The correlation between the activity of these enzymes and drug resistance was already the focus of some studies in breast cancer several years ago [75,76]. The role of HDACs in modulating the activity of 5-FU and oxaliplatin in CRC is becoming increasingly evident. In this regard, Alzoubi et al. demonstrated that downregulation of *HDAC2* using techniques like shRNA or the HDAC inhibitor Vorinostat (SAHA) sensitizes CRC cell lines to 5-FU and oxaliplatin, whereas increased *HDAC2* expression is associated with 5-FU resistance [77]. The mechanism by which HDACs inhibition could enhance 5-FU cytotoxic activity involves the activation of caspase-3/7 and the p21-mediated apoptosis pathway [78]. Similarly, in the case of oxaliplatin, *FKBP3* downregulation, which attenuates HDAC2 activity, appears to increase sensitivity to this drug by activating the apoptosis pathway [79].

Another HDAC, HDAC7, has been implicated in 5-FU response, although not in CRC but in hepatocellular carcinoma (HCC). In HCC, inhibition of HDAC7 using SAHA leads to increased acetylation of the OAT2 promoter gene, which is a transporter for 5-FU, resulting in enhanced 5-FU sensitivity [80]. Given the importance of HDACs in drug response and their involvement in various cancer-related processes, selective HDAC inhibitors have been evaluated in clinical trials as potential therapies for different types of cancer. For instance, Vorinostat efficacy has been tested in CRC as a single agent, but adverse effects were high, which limited a reliable efficacy assessment [81].

Additional trials have tested the effectiveness of Vorinostat or Panobinostat when combined with 5-FU (NCT01238965 and NCT00336141). However, comprehensive findings pertaining to the outcomes of these combined therapeutic approaches have not yet been posted [81,82]. Vorinostat's potential has also been explored in conjunction with radiation therapy or Irinotecan in other types of cancer, such as gastrointestinal and pancreatic cancer [82–85].

Regarding histone methyltransferases and fluoropirimidines responses, overexpression of *SETDB1* is linked to 5-FU resistance. Chen and colleagues found SETDB1 to promote proliferation and migration of CRC cell lines, while inhibiting apoptosis induced by 5-FU treatment [86]. Moreover, they confirmed these findings using immunohistochemistry on FFPE tissue and in mice models where *SETDB1* downregulation suppressed cell growth. Regarding combinatorial 5-FU–based treatments with targeted therapies such as anti-EGFR, recent studies have explored the relationship between resistant outcomes and epigenetic mechanisms. In 2020, Hou et al. proposed that inhibiting *SETDB1*, which activates Akt through K64 methylation [87], could be a potential strategy to overcome resistance to cetuximab. They demonstrated that *KRAS*-mutated CRC cell lines and xenograft models became sensitive to anti-EGFR treatment when combined with mithramycin, a *SETDB1* inhibitor [88]. This data emphasizes how epigenetic inhibitors can impact on non-chromatin proteins such as the Akt pathway; arguing in favor of combinatorial treatments of epigenetic drugs with targeted therapies, that in principle, were not expected to synergize. Table 1 summarizes the genes and epigenetic marks involved in sensitivity or resistance to fluoropyrimidines treatment, while Box 1 highlights a potential biomarker role for an helicase and cetuximab responses; even if this is a less common therapeutic approach than combinatorial treatments with chemodrugs, distinct vulnerabilities at the chromatin level represent attractive approaches in cetuximab exclusive treatments.

3.2. Chromatin alterations affecting efficacy of oxaliplatin-based schedules

Oxaliplatin treatment, similar to 5-FU, induces the generation of ROS, and its accumulation has been found to have a synergistic effect in oxaliplatin-based combinations [90]. Unlike in *in vitro* experiments, in the clinic, oxaliplatin is always combined with a fluoropyrimidine and is never administered as a single agent. Therefore, it should be noted that in the following lines, many of the results described, and especially those referring to studies in patients, are often associated with combination treatments with 5-FU or capecitabine.

A gene associated with antioxidant regulation in this context is *GPX3*, a peroxidase whose abnormal methylation pattern has been observed in various tumors, including ovarian and prostate cancers [91,92]. Notably, hypermethylation of the *GPX3* promoter has been linked to increased sensitivity to oxaliplatin in CRC cell lines. This effect was further confirmed by observing tumor regression when *GPX3* knock-out xenografts were treated with platinum drugs [93], providing additional support for the predictive role of *GPX3* in oxaliplatin sensitivity.

Although *RASSF1A* is not directly involved in antioxidant metabolism, its methylation status has been found to influence the response of CRC patients to oxaliplatin treatment. When *RASSF1A* is not methylated, CRC patients tend to exhibit a better objective response, aligning with the well-established tumor suppressor role of this gene [94]. Moreover, the absence of methylation in the promoter region of *RASSF1A* has been significantly correlated with improved OS and independently associated with a better prognosis [95]. Interestingly, *RASSF1A* is frequently inactivated in various human tumors through promoter methylation, as seen in lung, ovarian, or esophageal squamous carcinoma [96–98]. However, the specific mechanism by which *RASSF1A* may modulate sensitivity to oxaliplatin in CRC remains unknown and warrants further investigation.

Through *in silico* analysis, Wang and colleagues directed their research towards investigating in CRC stem cells the methylation status of *MEIS2*, a gene involved in transcriptional regulation. Upon analyzing microarray data comparing CRC stem cells to non-stem cells, they observed higher levels of promoter methylation in stem cells. This hypermethylation of *MEIS2* was found to be associated with a worse response to oxaliplatin. To gain insight into the underlying mechanisms, the researchers conducted a pathway enrichment analysis (GSEA) and found that the Wnt-ß-catenin pathway emerged as the primary mediator of *MEIS2* [99]. The Wnt-ß-catenin pathway is one of the most crucial signaling pathways involved in various aspects of tumor biology, including proliferation, apoptosis, invasion, stemness, and resistance to chemotherapy [100]. Considering these findings, *MEIS2* holds promise as a potential biomarker candidate gene that merits further in-depth investigation.

In addition to hypermethylation, the loss of methylation is also implicated in oxaliplatin resistance, as demonstrated by the case of *PARPBP*, a gene involved in maintaining genomic stability and DNA damage repair. Hong and colleagues investigated the relationship between *PARPBP* promoter hypomethylation and oxaliplatin resistance in CRC cell lines and found that parental cells exhibited lower expression of *PARPBP* than resistant ones. Higher expression levels of *PARPBP* led to the activation of *PARP1* and a subsequent increase in the repair of DNA

Table 1
DNA and histone epigenetic modifications in fluoropyrimidines treatment.

Epigenetic mark	Status	Target gene	Model of study	Effect	Reference
DNA methylation	Hypermethylation	**ETV5**	TCGA data	Upregulation by hypermethylation correlates with lower RFS.	[53]
		NKX6.1, TFAP2E-DKK4, IGFBP3 (SIGNATURE)	SW48, HT-29 cell lines and 48 tumors (frozen and FFPE)	Correlation with 5-FU chemotherapy resistance.	[55]
		NKX6.1	HT29, HCT8, HCT116, SW480, SW620 cell lines and xenografts		[58]
			HT29, HCT116, SW480, SW620, TCGA data, 151 stage II patients (frozen)	Reduced 5-year OS and DFS after adjuvant 5-FU-based chemotherapy.	[59]
		Foxp1	LOVO, HCT116, SW840, SW620, and HCoEpiC cell lines, 78 patients (FFPE) with matched paracancerous tissues, xenograft models	circFoxp1 recruits DNMT1 to Foxp1 promoter. Downregulation of circFoxp1 sensitizes colon cancer cells to Capecitabine.	[60]
	Hypomethylation	**NME2**	HCT8 cell line	Correlation with 5-FU chemotherapy resistance.	[61]
		Nrf2	SNU-C5 cell line	5-FU resistance mediated by TET family upregulation.	[64]
Histone modification	Methylation	**MLL/COMPASS complex**	SNU-C5 cell line	Promotes H3K4me3 of Nrf2 promoter, leading to chemoresistance.	[64]
		SETDB1	SW48, SW480, LOVO cell lines, 60 patients (FFPE), and xenografts	Overexpression promotes proliferation, migration, and resistance.	[86]
	Acetylation	**PCAF**	SW620, SW48, SW480, HCT116 cell lines, xenografts, and TCGA data	Downregulation correlated with resistance to 5-FU-based chemotherapy (only *in vitro* and *in vivo*).	[68]
			HCT116, HCT8, SW480, SW620, and 262 stage II-III patients (FFPE)	Low levels correlated with reduced long-term Disease Free Survival and increased early-progression.	[69]
		NAA40	HCT116, HT29, SW480, SW620 cell lines, xenografts, 318 patients and healthy donors, and TCGA data	TS acetylation by NAA40 correlated with resistance to 5-FU.	[70]
			HCT116, HT29, SW480, SW620 cell lines, xenografts, and TCGA data	Downregulation results in an increase in sensitivity.	[71]
		HDAC2	HCT116, SW480, HT29 cell lines, and xenografts	Downregulation by shRNA or SAHA (Vorinostat) correlates with higher sensitivity to 5-FU and OXA, whereas increased expression associates with resistance, only to 5-FU.	[77]
			Cell culture of 12 patients, 58 patients (frozen)	HDAC2 downregulation by FKBP3 increases sensitivity.	[79]
SWI/SNF complex		**ARID1A**	SW480 and SW620 cell lines, 86 patients (FFPE)	Decreased ARID1A inhibits apoptosis induced by 5-FU.	[109]

Box 1
Chromatin helicase and cetuximab efficacy.

In the context of cetuximab-exclusive treatment in CRC, which is less common than combinatorial therapies but still could offer relevant predictive information for combinatorial treatments with chemotherapy, CDH6 has emerged as a significant modulator of this anti-EGFR drug. CDH6 belongs to the chromodomain helicase DNA binding protein family and plays a role in unwinding double-stranded DNA by catalyzing hydrogen bonding. Zhang and colleagues conducted a study showing that CDH6 is highly expressed in CRC tumor samples compared to normal tissue. In a patient-derived xenograft (PDX) model with wild-type KRAS, they observed a more significant reduction in tumor growth when CDH6 was highly expressed and treated with cetuximab. The mechanism underlying this effect involves the regulation of EGF in stabilizing CHD6. When cetuximab is present and inhibits the EGF pathway (in KRAS wild-type scenarios), it promotes the ubiquitination of CHD6, leading to the deregulation of mitochondrial activity via TMEM65. These changes collectively contribute to reducing cell proliferation, tumor growth, and the metastatic capacity of colon cancer cells [89]. *Nevertheless, additional research is needed to fully understand the potential role of CDH6 as a predictive biomarker in combination with cetuximab.*

damage caused by oxaliplatin. To validate this effect in CRC patients, the researchers analyzed 148 tumor samples using immunohistochemistry. The study revealed that patients with higher *PARPBP* expression had worse prognosis when treated with oxaliplatin-based therapies [101]. Since previous works showed the potential involvement of *PARP1* in oxaliplatin resistance it is reasonable to consider combinatorial drug therapies that promote DNA damage alongside the inhibition of several DNA damage repair proteins, such as PARPBP and PARP1, to enhance treatment effectiveness. By targeting both aspects of DNA damage response, it may be possible to overcome resistance and improve treatment outcomes for CRC patients receiving oxaliplatin-based therapies.

Currently, there is limited knowledge about the potential role of histone methylation in modulating oxaliplatin treatment. Recent research has only identified a correlation between oxaliplatin and the methylation pattern of H3K27me3, indicating that decreased levels of the H3K27me3 mark were more frequently observed in oxaliplatin-resistant patients. Building upon this observation, Wang and colleagues investigated the link between low H3K27me3 levels and increased oxaliplatin resistance, through the activation of NOTCH2 in a patient-derived xenograft (PDX) model. Their study revealed that the NOTCH signaling pathway promotes the activation of stemness-related genes, leading to an increase in oxaliplatin resistance [102]. While these findings show promise, further experiments are required to fully validate and substantiate these preliminary results. The information of this section is summarized in Table 2. .

The relationship between the SWI/SNF complex and resistance to 5-FU treatment in CRC has been previously reported [109], in particular, decreased ARID1A inhibited apoptosis induced by 5-FU. However, its role in oxaliplatin treatment response remains unclear. Nonetheless, various research groups have investigated the involvement of the SWI/SNF complex in oxaliplatin resistance in other types of tumors. For instance, in pancreatic cancer *SMARCA4* has been identified as a potential mediator of sensitivity to oxaliplatin. Depletion of *SMARCA4* in *in vitro* models led to a significant increase in sensitivity to oxaliplatin and irinotecan, with a moderate increase observed for 5-FU. This effect appears to be associated with impaired DNA repair pathways, leading to the activation of apoptosis to promote cell death upon oxaliplatin treatment [110].

On the contrary, mutations in SMARCA4 have been linked to oxaliplatin and taxane resistance. Given the crucial catalytic role of SMARCA4, its phosphorylation status has been investigated in ovarian cancer. Aberrant activation of *SMARCA4* was found to be linked to decreased sensitivity to oxaliplatin. A model based on SMARCA-4 phospho-mutant and phospho-mimic mutations revealed that reduced phosphorylation of SMARCA4 contributes to tumor malignancy and promotes chemoresistance by reducing chromatin accessibility mediated by SWI/SNF activity. This modulation of chromatin accessibility affects the capacity of DNA damaging agents, such as oxaliplatin, to access DNA, thereby impacting the response to the drug [111]. These findings shed light on the potential involvement of the SWI/SNF complex and *SMARCA4* in oxaliplatin resistance in certain tumor types, providing valuable insights into the mechanisms that influence treatment response and chemoresistance. However, further investigation is required to fully understand the complex interplay between SWI/SNF components and oxaliplatin sensitivity and resistance in CRC and other cancers.

Table 2
DNA and histone epigenetic modifications in oxaliplatin and FOLFOX treatments.

Epigenetic mark	Status	Target gene	Model of study	Effect	Reference
DNA methylation	Hypermethylation	**NKX6.1**	HT29, HCT8, HCT116, SW480, SW620 cell lines and xenografts	Attenuated sensitivity when downregulated by shRNA.	[58]
		GPX3	RKO, SW48, LOVO, HCT116, SW480, SW620, COLO205, CACO2, HT29 cell lines, xenografts, and TCGA data	Increased sensitivity and tumor regression.	[93]
		MEIS2	CACO, and HCT116 cell lines, 30 metastatic patients (frozen), and TCGA data	Worse response through Wnt-ßcatenin pathway.	[99]
	Hypomethylation	**RASSF1A**	108 stage II-III patients (blood)	Better objective response, overall survival, and prognosis.	[95]
		PARPBP	HCT116, and DLD1 cell lines, 148 stage II-III patients (FFPE)	Worst prognosis.	[101]
		LINE-1	SW480, HCT116, CACO2, RKO cell lines, and 40 FFPE primary tumors	Patients present worst OS and PFS. Prediction of non-response to FOLFOX.	[106]
			336 stage III and high-risk stage II patients (FFPE, frozen and blood)	Worst performance status in stage II and III patients, and shorter RFS in stage III patients.	[107]
			129 stage III patients (FFPE)	Early postoperative recurrence and lower DFS in CRC patients.	[108]
Histone modification	Methylation	**H3K27me3**	HCT116, SW620 cell lines, and patient-derived xenograft models	Low levels are related to increased resistance through NOTCH2 activation	[102]

Box 2
LINE-1 hypomethylation.

Hypomethylation of repetitive elements is a common DNA methylation alteration observed in cancer [103,104]. *In particular, LINE-1 hypomethylation has been found to be inversely correlated with MSI and CIMP status* [23,105]. *However, the exact consequences of LINE-1 hypomethylation in the oncogenic process are still unclear. It has been proposed that hypomethylation may activate LINE-1 elements, causing them to act as retrotransposons, potentially inserting themselves into genomic unstable regions and promoting genomic instability. Additionally, LINE-1 deregulation has been associated not only with cancer development but also with potential resistance to chemotherapy.*

In the context of CRC, Kaneko's group conducted a comprehensive study to explore the role of LINE-1 elements. They analyzed LINE-1 hypomethylation status in a cohort of 40 CRC FFPE primary tumors and observed that patients with hypomethylated LINE-1 had worse OS and PFS. Furthermore, in patients treated with FOLFOX chemotherapy, LINE-1 hypomethylation was predictive of non-response to this treatment combination. Importantly, in a multivariate analysis using the COX proportional-hazards model, LINE-1 hypomethylation was identified as an independent factor associated with poor prognosis [106]. *These encouraging findings prompted further investigations in larger patient cohorts. In these studies, a correlation between LINE-1 hypomethylation and worse performance status was observed in stage II and III CRC patients. Additionally, in stage III patients treated with FOLFOX, RFS was shorter in those with LINE-1 hypomethylation* [107]. *Similarly, a stratified study with a cohort of advanced CRC patients conducted by Lou* et al. *demonstrated that patients with LINE-1 hypomethylation experienced early postoperative recurrence and lower DFS when treated with FOLFOX* [108]. *Taken together, while additional studies are necessary to fully understand its potential clinical utility, LINE-1 hypomethylation shows promise as a predictive biomarker in CRC. Its assessment could aid in treatment decision-making and prognosis determination for CRC patients.*

3.3. Epigenetic features affecting efficacy of irinotecan-based schedules

Irinotecan, together with 5-FU and oxaliplatin, is one of the most commonly used drugs to treat advanced CRC patients. Table 3 represents the epigenetic modifications and genes involved in the modulation of irinotecan-based treatment described below. Recognizing the significance of irinotecan in clinical practice, Cha and colleagues conducted a study to explore the relationship between DNA methylation and irinotecan resistance. In their investigation, they focused on *CHFR*, an E3 ubiquitin ligase known for its involvement in the response and repair of DNA damage induced by irinotecan. The researchers found that *CHFR* promoter was hypermethylated in metastatic CRC, and its expression level appeared to modulate sensitivity to irinotecan in *in vitro* experiments. Specifically, hypermethylated *CHFR* was associated to irinotecan sensitivity probably by impairing DNA damage repair mechanisms. AZA-induced CHFR expression could reverse irinotecan sensitivity while knocking down CHFR restored it. Moreover, the study observed that the hypermethylation status of *CHFR* was associated with better treatment outcomes, specifically longer time to progression (TTP), in a cohort of 102 metastatic CRC patients. These findings suggest that the methylation status of *CHFR* could serve as a useful biomarker to aid in patient selection for irinotecan treatment [112].

Global demethylation has been recognized as a significant strategy to enhance sensitivity to chemotherapeutic drugs, using demethylating agents like 5-aza-2'-deoxycytidine (AZA). In the context of irinotecan-based treatments, *in vitro* models have demonstrated that 5-azanucleosides can modulate drug sensitivity in cancer cells, providing a means to overcome chemoresistance. Pre-treatment with AZA has been found to sensitize CRC cells to irinotecan, as well as other topoisomerase I/II inhibitors like etoposide, doxorubicin, or mitoxantrone. This combination leads to reduced cell viability and increased apoptosis. The sequential use of AZA and irinotecan has shown to induce long-term sensitization effects, offering a promising approach to combat chemoresistance [113]. To further investigate this synergism, Sharma et al. conducted *in vivo* xenograft models. The combined treatment of AZA and irinotecan exhibited a synergistic response, resulting in significant tumor regression and improved OS compared to control mice. These encouraging findings have led to the initiation of a randomized international phase 2 clinical trial (NCT01896856) [114] in metastatic CRC patients, using guadecitabine, a second-generation DNA methylation inhibitor. Unfortunately, no differences were detected in terms of response between the group given guadecitabine plus irinotecan and the group given regorafenib or TAS-102.

The methylation status of *UGT1A1* has been extensively studied due to its role as an enzyme responsible for metabolizing irinotecan into its active form, SN-38. Several studies have shown that hypermethylation of the *UGT1A1* promoter leads to a repression of its expression, thereby contributing to irinotecan resistance [115–117]. However, irinotecan resistance involves mechanisms beyond those related to its metabolism. For instance, the upregulation of the p16 gene, leading to cell cycle arrest, has been identified as a critical modulator in *RAS*-activated oncogenic stress in CRC [118,119]. In the context of combined irinotecan with 5-FU (FOLFIRI) treatment, the methylation status of p16 has shown to play an interesting role in predicting the clinical outcome for metastatic CRC patients. Specifically, low methylation patterns of p16 have been associated with significantly longer TTP and OS. On the other hand, hypermethylation of p16 correlates with poorer treatment outcomes [120].

The impact of acetylation agents on irinotecan action and metabolism remains poorly understood. Consequently, there is limited information available regarding the influence of these enzymes in the modulation of irinotecan's activity. Similar to the observations in 5-FU therapy, some HDACs may also play a role in resistance to irinotecan-based chemotherapies. A study conducted by Meisenberg and

Table 3
DNA and histone epigenetic modifications in irinotecan and FOLFIRI treatments.

Epigenetic mark	Status	Target gene	Model of study	Effect	Reference
DNA methylation	Hypermethylation	**CHFR**	RKO, HT29, HCT116, SNU-81, SW480, DLD1, SNU407, CACO2, LOVO, SW620, SNU-C4, and SNU-C5, 102 metastatic patients, and TCGA data	Better treatment outcome and increased sensitivity	[112]
	Hypomethylation	**p16**	49 metastatic or recurrent patients (FFPE)	Longer Time To Progression and Overall Survival in response to FOLFIRI treatment	[120]
Histone modification	Acetylation	**H4K16ac**	RKO, and DLD1 cell lines, and 36 metastatic patients (FFPE)	Inhibition of H4K16 acetylation through Trichostatin A (TSA) promoted sensitivity.	[121]

colleagues investigated this aspect using *in vitro* models. They found that inhibition of histone H4K16 deacetylation through the use of Trichostatin A promoted sensitivity to irinotecan. Based on their findings, they also proposed that global H4K16 acetylation decrease could serve as a potential biomarker for the initiation of CRC. The mechanism proposed links lower H4K16 acetylation with increased 53BP1 accumulation and reduced fork stalling, which ultimately leads to cell survival and resistance. However, further research is needed to better understand the intricate relationship between acetylation agents, HDACs, and irinotecan treatment [121].

3.4. Epigenetic features and their role in immune checkpoint inhibitors

Methylation has been identified as a mechanism that can modulate immune responses in CRC patients undergoing ICI; as such, it is not surprising that tumor methylation signatures could give important prognostic information not only in CRC, but also in other type of tumors such as lung or breast cancers [122,123]. Moreover, researchers have explored methylation pattern abnormalities in immune cells infiltrating the tumor that could predict patients' response and prognosis to IT [122]. For instance, CD8 + tumor-infiltrating lymphocytes (TILs) are known to play a crucial role in immune responses, survival outcomes, and prognosis in CRC [124,125]. Accordingly, DNA methylation of CD8 + T-cells has been extensively studied to identify a signature of differentially methylated sites that could be associated with antitumor immunity, T-cell activation, immune response to tumor cells, and cell killing. These signatures were found to be negatively correlated with exhaustion and senescence markers. Taking into account these signatures, patients with high methylation levels of CD8 + T-cells tended to have poorer survival outcomes. However, patients with MSI tumors, which coincide with high methylator phenotype (CIMP), showed a modest objective response. Remarkably, a subset of low-methylated CD8 + T-cells was identified in microsatellite-stable CRC patients. However, whether these MSS low-methylated CD8 + TIL tumors could respond favorably to ICIs remained to be addressed [126].

Not only global profiles, but also specific genes related to checkpoint inhibitors have been implicated in IT response as well. The correlation between *PD-L1* expression, *MLH1* expression, *BRAF* mutations, and MSI status was observed in CRC tumor samples. For instance, when *PD-L1* is methylated and repressed, it was associated with worse outcomes in terms of OS and RFS. Thus, the methylation status of *PD-L1* may serve as a predictive biomarker of response to IT in CRC patients [127]. The clinical trial NCT02260440 [128] evaluated the activity of pembrolizumab (a PD-1 inhibitor) in combination with DNA demethylating agent, AZA, in chemotherapy-refractory metastatic CRC patients. This trial observed modest clinical benefits in patients with higher CD8 + TILs density after treatment, and there was a trend of increased TIL infiltration due to demethylation caused by AZA administration. However, whether this benefit was due to an increased expression of PD-1/PD-L1 axis was not clear; essentially tumor PD-L1 was increased upon treatment (in 11 of 17 pairs of pre-treated *vs* treated samples), but TIL PD-L1 was already detectable in all 25 pre-treatment samples and increased on 7 of 17 pairs (41%) upon treatment. These findings suggest a potential link between DNA methylation and immunomodulation, though further studies are needed to better understand and overcome immune resistance in metastatic CRC patients [129].

In recent years, there has been increasing evidence suggesting that HDAC inhibitors can play a role in regulating the expression of immune checkpoints such as PD-L1 or CTLA-4. In the context of CRC, several studies have been conducted to investigate the potential of HDAC inhibitors in re-sensitizing tumors to IT. Differentially expressed genes related to antigen presentation and natural killer cells were identified by RNA-seq when human CRC cell lines were treated with CXD101, a HDAC inhibitor. Similar results were obtained in *in silico* analysis of CRC datasets treated with several HDAC inhibitors. Further research investigated the combination of CXD101 with anti-PD-L1 or anti-CTLA-4 in *in vivo* models. The results demonstrated a more robust reduction in tumor growth, better OS, and improved tolerance compared to the administration of the same drugs in monotherapy. This indicates that CXD101 may enhance the antitumoral response of anti-PD-L1 and anti-CTLA-4 immunotherapies [138]. Based on these promising preclinical findings, CXD101 is currently being evaluated in a phase I clinical trial in combination with Nivolumab (anti-PD-1 antibody) in patients with metastatic CRC (NCT03993626) [139]. Moreover, additional ongoing clinical trials in CRC are assessing the potential benefits of combining other HDAC inhibitors with ITs. For instance, trials are investigating combinations of Entinostat and Pembrolizumab (NCT02437136) [140], Panobinostat and Spartalizumab (NCT02890069) [141], and Romidepsin and Pembrolizumab (NCT02512172) [142].

Furthermore, Romidepsin, as an HDAC inhibitor, has been extensively studied in *in vivo* models to understand its role in promoting an immunogenic tumor response. The findings suggest that Romidepsin treatment leads to increased apoptosis, inhibition of cell proliferation, and reduced tumor growth. Additionally, Romidepsin treatment results in increased acetylation of histone proteins H3 and H4, which is associated with the upregulation of PD-L1 expression in CRC cells. These observations have sparked interest in exploring the combination of Romidepsin with ICIs targeting PD-L1. The rationale behind this combination is that Romidepsin's ability to enhance PD-L1 expression could potentially complement the effects of anti-PD-L1 IT, leading to enhanced tumor-killing effects [143]. The potential benefit of combining checkpoint blockade IT with HDACi has been evaluated in other tumor types. For instance, there are trials evaluating the combination of Romidepsin with anti-PD-L1 immunotherapy in breast cancer [144] or lung cancer [145].

In recent times, the relationship between IT and histone methylation alterations in CRC remains relatively understudied. However, a study has shed light on the impact of H3K79me2 modification, which upregulates *FOXM1* expression. This upregulation, in turn, hinders the maturation of bone marrow-derived dendritic cells, leading to a deficiency in mature antigen-presenting cells. Consequently, the immune system fails to activate T cells in the presence of tumor antigens. The *FOXM1* promoter's H3K79me2 dimethylation ultimately contributes to enhanced tumoral growth by evading immune system recognition [146]. While *FOXM1*'s implication in IT response has been extensively studied in various tumor types, the underlying mechanisms behind its differential expression remain unclear. Further research is warranted to fully comprehend the significance of histone methylation alterations in the context of IT for CRC treatment.

ARID1A is the unique member of the SWI/SNF complex that has shown significant correlation with IT in CRC. Mutations in *ARID1A* have been strongly associated with a favorable immune infiltration profile in CRC patients. These mutations lead to an increase in infiltrating cytotoxic T lymphocytes, which, in turn, is correlated with higher PD-L1 expression. Consequently, it has been proposed that the absence of *ARID1A* could serve as a potential predictive biomarker for IT in CRC. Tumors with *ARID1A* mutations may exhibit higher sensitivity to IT [147,148], as supported by various studies across a wide range of tumors, including non-small cell lung cancer [149,150], pancreatic cancer [151], and ovarian cancer [152].

Information regarding IT and epigenetic modifications (including Box 3), is summarized in Table 4. Fig. 3 summarizes the different epigenetic alterations found to be involved in sensitivity or resistance to the different CRC treatments mentioned in the present review.

4. Discussion and future perspectives

Predicting responses to cancer treatments and overcoming resistance are crucial goals in modern oncology, aiming to develop precise and personalized therapies for improved outcomes in cancer patients. As reviewed here, epigenetics has emerged as a valuable field, providing relevant data that links specific modifications controlling gene

Box 3
N6-methyladenosine (m6A).

It is becoming increasingly evident that not only DNA methylation but also RNA modifications, such as N6-methyladenosine (m6A), play a crucial role in immune response and tumor behavior, including in CRC. m6A is a common modification found in mRNA and has been associated with various cellular processes, including tumor proliferation, carcinogenesis, and metastasis [130]. Recent research has linked m6A to the regulation of T cell activation and anti-tumor immune response by modulating the IL-7/STAT5/SOCS pathways and METTL3 depletion [131]. This suggests that m6A modification may influence the efficacy of IT in a subset of CRC patients. Indeed, the efficacy of immunotherapy in CRC can vary widely among patients, and only a subset of individuals with specific genetic characteristics seem to benefit significantly from these treatments. As a result, researchers have been actively investigating the role of m6A RNA modifications in relation to IT response in CRC.

The analysis of m6A regulators' signatures based on TCGA data has provided valuable insights into the immune tumor landscape and its impact on patient outcomes, specifically in the context of CRC. Chong and colleagues' research proposed three distinct m6A-related signatures that explain the immune tumor landscape in CRC patients. The first signature identified was the "immune-inflamed" phenotype, characterized by lower m6A levels. Patients with this signature displayed high immune activation and the presence of tumor-infiltrating cells. Importantly, this signature was associated with a reduction in PD-L1 expression, reinforcing the role of PD-L1 as a predictive value of immune response. Moreover, patients with the "immune-inflamed" signature showed better OS. In contrast, Chong et al. *also identified an "immune-excluded" signature that was associated with higher m6A levels and characterized by the activation of EMT, TGF-β, and Wnt signaling pathways. The presence of immune and stroma cells in the tumor microenvironment contributed to the "immune-excluded" phenotype, blocking the infiltration of lymphocytes and impairing anti-tumor immunity. Consequently, patients with this signature experienced a worse outcome. The third type of signature, named the "immune-desert," was also characterized by high m6A levels in CRC patients. However, unlike the "immune-excluded" signature, the "immune-desert" phenotype exhibited a strong immune-suppression of the tumor microenvironment, which in turn modulates IT efficacy [132,133].*

In addition to m6A-related signatures, alterations in m6A regulators, such as mutations or copy number variations, have been found to impact the prognosis of CRC patients treated with IT. These alterations can lead to aberrant immune cell infiltration and are associated with worse OS and DFS outcomes [134,135]. In vitro models have provided valuable insights into the role of m6A writers, such as METTL3 and METTL14, in sensitizing CRC cells to anti-PD-1 treatment. Depletion of these key m6A writers has been shown to increase the presence of CD8 + TILs, which are important for mounting effective anti-tumor immune responses [136]. Similarly, the absence of ALKBH5, an m6A eraser, has been shown to significantly deregulate mRNA expression and splicing of a cascade of genes. Consequently, the depletion of ALKBH5 has been found to modulate the content of TILs in the tumor microenvironment. This modulation results in decreased tumor growth and improved survival outcomes in patients treated with PD-1 inhibitors [137], further highlighting the impact of m6A-related mechanisms on immunotherapy response in CRC.

Table 4
Epigenetic modifications in immunotherapy.

Epigenetic mark	Status	Target gene	Model of study	Effect	Reference
DNA/RNA methylation	Hypermethylation	**PD-L1**	TCGA data	Associated with worse outcome in Overall Survival and Regression Free Survival	[127]
	Hypermethylation (m6A)	**m6A Signature**	NCBI and TCGA data	Low m6A signature score correlated with better response and clinical benefits	[132, 133]
		m6A regulators	TCGA data	Alterations in m6A regulators correlate with worse OS and DFS	[134, 135]
		METTL3/ METTL14	CT26 cell line, *in vivo* models, and 59 patients (FFPE)	Depletion of these m6A mark writers promotes sensitazion to anti-PD-1	[136]
		ALKBH5	CT26 cell lines, and *in vivo* models	Absence of this m6A mark eraser promotes deregulation of mRNA expression and splicing, together with the modulation of the content of TILs promoting survival in patients treated with PD-1 inhibitors	[137]
Histone modification	Acetylation	**HDACs**	SW620, HCT116 cell lines, xenografts and TCGA data	The combination between HDACs inhibitors and immunotherapies increase anti-tumoral response.	[138]
	Methylation	**H3K79me2**	CT26, HCT116 cell lines, and xenografts	FOXM1 dimethylation enhances tumoral growth by eluding the immune system recognition.	[146]
SWI/SNF complex		**ARID1A**	NGS, WES and IHC from TCGA datasets	Mutations correlate with higher sensitivity.	[147, 148]

expression and DNA repair to the efficacy of various treatments in CRC.

We observed that resistance to drugs impacting DNA biology such as fluoropyrimidines, oxaliplatin and irinotecan share some features, such as reduced expression of tumor suppressors (FOXP1, RASSF1A), inducers of apoptosis (MEIS2, ARID1A), drug metabolism players (UGT1A1), HATs (PCAF), and cell cycle arrest inducers (p16). In many cases, the associated epigenetic repressive mechanism is DNA hypermethylation at promoter and enhancer regions, but also H3K27me3 accumulation and loss of histone acetylation are reported as an effective manner to suppress expression of these genes. Of note, aberrant promoter hypermethylation has intrigued cancer epigenetic researchers for decades; while it is accepted that DNMTs should be recruited, most of the signaling pathways controlling DNMT recruitment are poorly understood. Moreover, since hypermethylation not only occurs at CIMP markers, a common hypermethylation mechanism seems unlikely, and the causative means remain poorly understood. Nevertheless, recent work on epigenetic clocks and ageing suggest that a mixture of stochastic and age-related factors might be at play [33].

Epigenetic drugs have undergone extensive investigation both as single-agent treatments and within combinations of multiple drugs, yielding remarkably favorable outcomes in cases of liquid tumors. Roughly 500 clinical trials have showcased the effectiveness of incorporating such drugs into the treatment of acute myeloid leukemia, T-cell lymphoma, and myelodysplastic syndrome, promoting the decrease of abnormal epigenetic modifications in malignant cells. In consequence, Vorinostat and AZA were the first epidrugs approved by the FDA in the

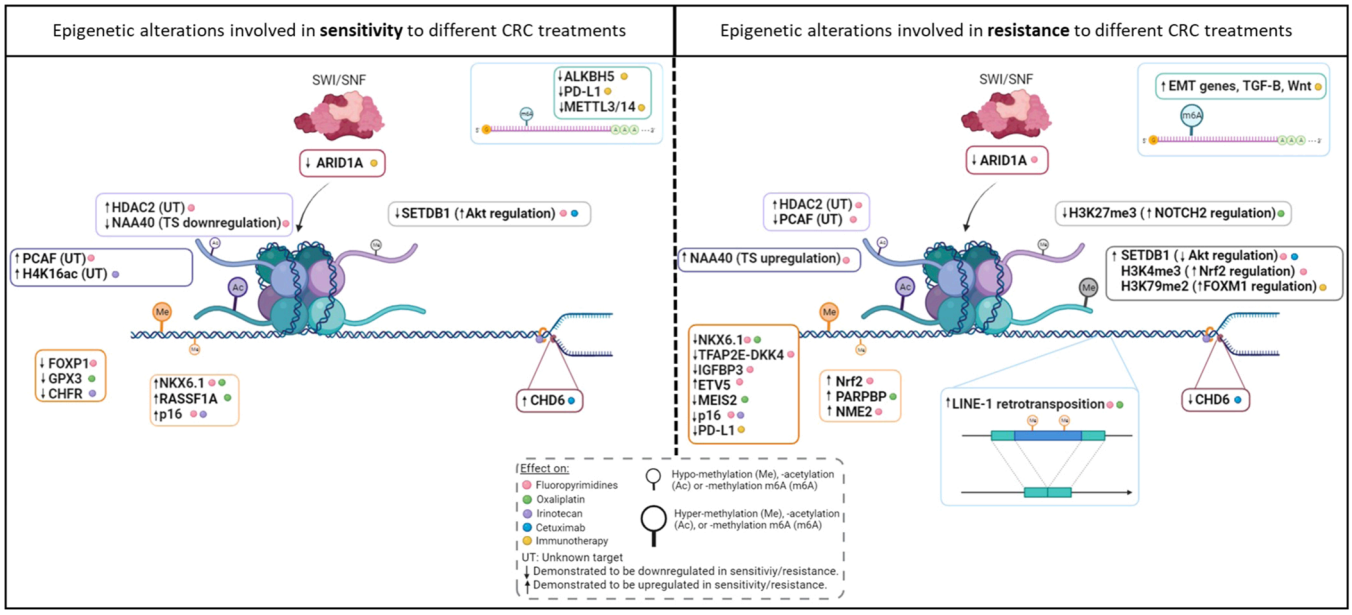

Fig. 3. Chromatin states integrate responses to CRC treatments. Epigenetic modifications are represented in lollipops with different colors and sizes (type and magnitude respectively). Therapies are indicated with round-colored spheres. Arrows indicate upregulated and downregulated genes or histone modifications. In parenthesis, there is the associated epigenetic modification and target. Created with BioRender.com.

early 2000's [153,154].

These encouraging results were promising avenues for further clinical trials focused on solid tumors. However, using epigenetic drugs like DNMT and HDAC inhibitors to modulate gene expression, mainly with the goal of overcoming treatment resistance in solid tumors, has proven partially effective. The combination of these drugs with chemotherapy and immunotherapy has constituted the focal point of numerous clinical trials conducted within the past decade across diverse solid cancer types. In the context of CRC, clinical trials designed to evaluate the clinical significance of these combined interventions have yielded inconclusive outcomes (as presented in Table 5).

The fact that these drugs have broad genomic effects inducing the expression of tumor suppressor genes, apoptotic stimulators and alike, helps to sensitize cells to chemotherapy. Simultaneously, the concomitant overexpression of resistance-contributing genes such as oncogenes, apoptotic repressors, DNA repair machinery factors, invasiveness contributors (HDACs, NME, SETDB1), as well as stemness genes (NOTCH2) promotes opposite effects. This interplay between contradictory molecular signals might explain the moderate effectiveness observed in these therapeutic approaches. Altogether, it is still a challenge to predict which chromatin landscapes are going to be responders or resistant to treatment with 100% accuracy.

Still, it is noteworthy to highlight that these clinical trials are currently situated within the early stages (phase I/II). Consequently, there exists a necessity to progress to subsequent phases which hold the potential to yield definitive outcomes in terms of efficacy. Furthermore, while these clinical trials are investigating promising drug combinations, it is unfortunate that certain completed trials have not published yet their results, which could contribute to enrich the existing knowledge.

Nonetheless these complexities, we remain optimistic about the potential of the proposed combinatory strategies explored in clinical trials to enhance overall clinical outcomes for CRC patients, even if they just turn to be beneficial for a subgroup of mCRC patients. Idealistically, directing these drugs to the specific altered genomic loci would be the best therapeutic approach.

As indicated in this review, aberrant overexpression in tumors correlates with promoter hypomethylations, decreased H3K27me3 and increased histone acetylation levels. Mechanisms driving aberrant hypomethylation have also puzzled researchers for years. It was reasonable to expect mutations in DNMTs that could explain some of the observed methylation impairments. Unfortunately, hypomethylation is observed in tumors with unaltered DNMTs, suggesting that additional proteins or signaling enzymes may be responsible for global DNA demethylation in cancer. Importantly, passive mechanisms have been proposed to drive global hypomethylation, which is observed at repetitive regions in all tumor types. Regardless of the cause, efforts to revert aberrant hypomethylations include to localize DNA methylation activity by engineered CRISPR-Cas9 approaches [155], representing a novel selective epigenetic treatment. Of note, cellular heterogeneity in the tumor, with subclones showing opposing epigenetic profiles, defies to devise straightforward treatment strategies. Finally, we also must consider that these concomitant subpopulations, with distinct genetic and epigenetic changes, evolve over the course of the disease, and are also heavily influenced by the selective pressure exerted by therapies themselves.

Regarding checkpoint inhibitor treatments, PD-1 and PD-L1 expression levels do not always predict responses. High expressing tumors can bear other inhibitory molecules, which prevent ICI therapy to unlock T-cells, while unexpectedly, low PD-tumors may respond well to ICI therapy, indicating that ICIs may inhibit additional molecules (beneficial off-targets). Moreover, if the tumor has downregulated expression of MHCII, a crucial component for activating T-cells, ICI therapy will not work regardless of PDs expressions [156]. Altogether this data indicates that best responses and synergisms with chemotherapy depend on the concomitant expression of several factors. Therefore, integrating as many layers as possible of –omic data (epigenomic, transcriptomic, proteomic, metabolomics) would seem the best manner to accurately predict an effective response. Related to this idea, alterations of chromatin remodeler factors from the SWI/SNF family seem to synergize with immunotherapy by activating an intracellular innate immune response. This results in upregulation of interferon pathways and boosting T-cell activity, cooperating with ICI therapy to unleash T-cell tumor killing.

We are moderately optimistic about future therapies that aim to monitor these dynamic alterations and understand which combinatorial treatments are necessary at different disease stages to block its progression. The present review summarized several of the latest and most

Table 5
Clinical Trials in colorectal cancer assessing combinations of chemotherapy or immunotherapy and epigenetic drugs.

Treatment	Epigenetic mark	ID	Official title	Phase	Status	Results
5FU-based treatments	DNA methylation	**NCT00897819**	Evaluation of the Association Between DNA Methylation and Shortened Survival in Patients With Advanced Colorectal Cancer Treated With 5-FU/ Oxaliplatin-Based Regimens in E3200	Observ	Completed	OS and PFS for caucasian better with low CIMP values. Afroamerican showed better OS and PFS with high CIMP values.
		NCT04689347	Phase 1b Trial of 5-fluorouracil, Leucovorin, Irinotecan in Combination With Temozolomide (FLIRT) and Bevacizumab for the First-line Treatment of Patients With MGMT Silenced, Microsatellite Stable Metastatic Colorectal Cancer.	1	Recruiting	Not reported
	Histone acetylation	**NCT00413322**	A Phase I Safety, Pharmacodynamic, Anti-Tumor Activity, and Pharmacokinetic Study of PXD101 Alone and in Combination With 5-Fluoro-uracil in Patients With Advanced Solid Tumors	1	Completed	Not reported
		NCT01238965	Phase I Clinical Trial With LBH589 and Infusional 5-FU/LV in Patients With Metastatic Colorectal Cancer Who Failed 5-FU Based Chemotherapy	1	Terminated	Not reported
		NCT00336141	Phase I/II Clinical Trial With Vorinostat and Infusional 5-FU/LV in Patients With Metastatic Colorectal Cancer Who Failed 5-FU-Based Chemotherapy	1	Completed	Not reported
		NCT00138177	Phase I Study of Suberoylanilide Hydroxamic Acid (Vorinostat) in Combination With 5-Fluorouracil, Leucovorin, and Oxaliplatin (mFOLFOX) in Patients With Colorectal Cancer and Other Solid Tumors	1	Completed	Not reported
		NCT01277406	A Phase I/II Study to Evaluate Safety, Tolerability, Pharmacokinetics and Efficacy of Resminostat (4SC-201) in Combination With a Second-line Treatment in Patients With K-ras Mutated Advanced Colorectal Carcinoma	1/2	Completed	Not reported
		NCT00942266	A Randomized Phase II Study of Two Dose-Levels of Vorinostat in Combination With 5-FU and Leucovorin in Patients With Refractory Metastatic Colorectal Cancer	2	Completed with Results	Vorinostat and 5-FU did not present overall toxicity, however it did not show clinically relevant activity.
anti-EGFR	DNA Methylation	**NCT02022995**	Investigation of Methylation of EGFR in the Response of the Cetuximab in Metastatic Colorectal Cancer Patients	Observ	Completed	Not reported
		NCT00879385	Targeted Demethylation to Enhance Response or Overcome Resistance to EGFR Blocking Agents in KRAS Wild-type Metastatic Colorectal Cancer Patients Using Sequential Decitabine and Panitumumab	1	Completed	The combination of decitabine and panitumumab was well tolerated and showed activity in wild-type KRAS patients.
OXA	DNA methylation	**NCT01193517**	Phase I/II Study of Azacitidine and CAPOX (Capecitabine + Oxaliplatin) in Metastatic Colorectal Cancer Patients Enriched for Hypermethylation of CpG Promoter Islands	1/2	Completed	Azacitidine and CAPOX were well tolerated. High rates of stable disease in CIMP-high patients, however, no objective responses were observed.
Irino	DNA methylation	**NCT01896856**	A Phase I Study of SGI-110 Combined With Irinotecan Followed by a Randomized Phase II Study of SGI-110 Combined With Irinotecan Versus Regorafenib or TAS-102 in Previously Treated Metastatic Colorectal Cancer Patients	1/2	Completed with Results	Guadecitabine and irinotecan with growth factor support were safe and tolerable, with early indication of benefit.
IT	DNA methylation	**NCT03182894**	A Phase IB/II Study of Epacadostat (INCB024360) in Combination With Pembrolizumab (MK-3475) and Azacitidine in Subjects With Chemo-refractory Metastatic Colorectal Cancer	1/2	Withdrawn	Not reported
		NCT02260440	A Phase 2 Study of Pembrolizumab (MK-3475) in Combination With Azacitidine in Subjects With Chemo-refractory Metastatic Colorectal Cancer	2	Completed with Results	The combination of pembrolizumab and azacitidine is safe and tolerable with modest clinical activity, suggesting that tumor DNA demethylation and immunomodulation occurs.
		NCT02959437	A Phase 1/2 Study Exploring the Safety, Tolerability, Effect on the Tumor Microenvironment, and Efficacy of Azacitidine in Combination With Pembrolizumab and Epacadostat in Subjects With Advanced Solid Tumors and Previously Treated Stage IIIB or Stage IV Non-Small Cell Lung Cancer and Stage IV Microsatellite-Stable Colorectal Cancer (ECHO-206)	1/2	Terminated with results	Both epigenetic lead-in and concurrent therapy were moderately well-tolerated. However, overall efficacy was limited.
		NCT03576963	A Phase Ib/II Study of Guadecitabine (SGI-110) Plus Nivolumab in Refractory CIMP+ Metastatic Colorectal Cancer	1/2	Withdrawn	Not reported
	Histone acetylation	**NCT03993626**	A Phase Ib/ II Trial to Assess the Safety and Efficacy of CXD101 in Combination With the PD-1 Inhibitor Nivolumab in Patients With Metastatic, Previously-Treated, Microsatellite-Stable Colorectal Carcinoma	1/2	Unknown status	The combination therapy was well tolerated. The study demonstrated anti-tumour efficacy in 3rd line and above.
		NCT02437136	A Phase 1b/2, Open-label, Dose Escalation Study of Entinostat in Combination With Pembrolizumab in Patients With Non-small Cell Lung Cancer, With	1/2	Active, not recruiting	ENT + PEMBRO demonstrates acceptable safety and encouraging preliminary activity.

(*continued on next page*)

Table 5 (*continued*)

Treatment	Epigenetic mark	ID	Official title	Phase	Status	Results
			Expansion Cohorts in Patients With Non-small Cell Lung Cancer, Melanoma, and Mismatch Repair-Proficient Colorectal Cancer			
		NCT02890069	Phase Ib, Open-label, Multi-center Study to Characterize the Safety, Tolerability and Pharmacodynamics (PD) of PDR001 in Combination With LCL161, Everolimus (RAD001) or Panobinostat (LBH589)	1	Completed	The evaluated combinations show high tolerability, however, in preliminary results reported by Novartis, none of the combinations showed clear superior activity compared to RAD001 monotherapy.
		NCT04708470	A Phase I/II Study of Combination Immunotherapy for Advanced Cancers Including HPV-Associated Malignancies, Small Bowel, and Colon Cancers	1/2	Recruiting	Not reported
	DNA methylation and histone acetylation	**NCT02512172**	A Study of Using Epigenetic Modulators to Enhance Response to MK-3475 in Microsatellite Stable Advanced Colorectal Cancer	1	Completed	The combination of 5-azacitidine and romidepsin with pembrolizumab is considered safe and generally tolerable.

5FU: 5-Fluorouracil; OXA: Oxaliplatin; Irino: Irinotecan; IT: Immunotherapy; Observ: Observational. The highlighted IDs correspond to the clinical trials that are related to the chromatin factors mentioned in the review.

extensively studied epigenetic changes associated with better or worse responses to major CRC treatments. It also explored their potential as predictive biomarkers. While standardizing the use of some of these markers remains a challenge, the increasing availability of vast -omic data, even at the single-cell level, offers new opportunities to integrate this knowledge and make it therapeutically effective.

Indeed, artificial intelligence (AI) will play a crucial role in pursuing this daunting yet exciting task. By harnessing the power of AI and integrating multi-dimensional data, researchers will unlock deeper insights into cancer biology and develop more effective, targeted therapies to combat CRC and other malignancies.

5. Conclusions

Chromatin factors are valuable markers for treatment response in some mCRC patients, however, as often occurs in cancer, concomitant alterations can modulate and shift expected behaviors. As such, novel classifications, based on selective chromatin factor expressions and mutations could be instrumental to better predict responses to current mCRC treatments such as chemotherapy, targeted therapies and immunotherapy. While epidrugs were expected to revert many of the chromatin alterations observed in tumors, their broad effects result in conflicting pathways, which are resolved in unexpected outputs and as such, they are difficult to predict. Obtaining precise and global epigenetic maps of the tumors with recorded outcomes could yield novel classifications that will help to treat patients more effectively.

CRediT authorship contribution statement

Cristina Moreta-Moraleda: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing - original draft. **Cristina Queralt:** Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing - original draft. **Carla Vendrell-Ayats:** Methodology, Conceptualization, Formal analysis. **Sonia Forcales:** Conceptualization, Supervision, Funding acquisition, Formal analysis, Writing - review & editing. **Eva Martínez-Balibrea:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

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

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used CHATGPT in order to improve language and readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Background

CRC is mainly treated with chemotherapy drugs aimed at damaging DNA; yet treatment resistance still is a major challenge in the clinics. Epigenetics affects gene expression by modulating chromatin accessibility and as such, specific chromatin factors may facilitate or prevent the effectiveness of these drugs.

Translational significance

Chromatin factors and epigenetic alterations that associate with treatment outcome may be considered as novel predictive biomarkers or therapeutic targets contributing to overcome resistance to the conventional CRC treatments.

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