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Predictors of chemotherapy efficacy in non-small-cell lung cancer: a challenging landscape

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Background: Conventional cytotoxic chemotherapy (CCC) is the backbone of non-small-cell lung cancer (NSCLC) treatment since decades and still represents a key element of the therapeutic armamentarium. Contrary to molecularly targeted therapies and immune therapies, for which predictive biomarkers of activity have been actively looked for and developed in parallel to the drug development process ('companion biomarkers'), no patient selection biomarker is currently available for CCC, precluding customizing treatment.

Materials and methods: We reviewed preclinical and clinical studies that assessed potential predictive biomarkers of CCC used in NSCLC (platinum, antimetabolites, topoisomerase inhibitors, and spindle poisons). Biomarker evaluation method, analytical validity, and robustness are described and challenged for each biomarker.

Results: The best-validated predictive biomarkers for efficacy are currently ERCC1, RRM1, and TS for platinum agents, gemcitabine and pemetrexed, respectively. Other potential biomarkers include hENT1 for gemcitabine, class III β-tubulin for spindle poisons, TOP2A expression and CEP17 duplication (mostly studied for predicting anthracyclines efficacy) whose applicability concerning etoposide would deserve further evaluation. However, none of these biomarkers has till now been validated prospectively in an appropriately designed and powered randomised trial, and none of them is currently ready for implementation in routine clinical practice.

Conclusion: The search for predictive biomarkers to CCC has been proven challenging. If a plethora of biomarkers have been evaluated either in the preclinical or in the clinical setting, none of them is ready for clinical implementation yet. Considering that most mechanisms of resistance or sensitivity to CCC are multifactorial, a combinatorial approach might be relevant and further efforts are required.

Key words: predictive biomarkers, NSCLC, platinum, antimetabolites, topoisomerase inhibitors, spindle poisons

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introduction

Innovation and research in the field of conventional cytotoxic chemotherapy (CCC) have markedly slowed down since the advent of targeted anticancer therapy and immune checkpoint inhibitors. Although these therapies have significantly improved the outcome of some selected patients with non-small-cell lung cancer (NSCLC), ~60% of tumours do not present targetable driver mutations, and only 15%–25% of NSCLC patients derive benefit from immunotherapy [1–3]. CCC, which benefits clinically to a majority of patients, particularly in the adjuvant setting [4], and costs 10 to 1000 times less than targeted or immune therapies, still has a full role to play and remains the cornerstone of the treatment of hundreds of thousands lung cancer patients worldwide. Despite all recent therapeutic advances, NSCLC remains the leading cause of cancer death, and improvements are urgently needed [5]. Several factors explain this high mortality rate, including late patient diagnosis, preventing local curative approaches (surgery, radiotherapy). Inner biological aggressiveness, tumour heterogeneity, primary and acquired resistance mechanisms concur to restrict the potential of systemic treatments. Also, contrary to targeted therapies, CCC is unfortunately still used in a historical 'one-size fits all' approach, which is clearly suboptimal. Although several predictive biomarkers for CCC efficacy have been explored, none of them has gone through clinical implementation for routine daily practice, and predictive biomarkers or molecular tools designed to customise CCC to the patient's tumour molecular profile are crucially lacking. Such biomarkers would not only help identifying chemosensitive patients and selecting appropriate drug combinations upfront, but it would also avoid useless toxicities, decrease overall costs, and eventually improve patient outcome. Noteworthy, the remarkable failure rate in the development of biomarkers predicting CCC efficacy reflects how challenging this task is.

Here, we present molecular mechanisms involved in either sensitivity or resistance to CCC, and review the main biomarkers studied in the field of NSCLC. Their analytic validity, scientific robustness and potential for clinical implementation will also be discussed.

cytotoxic drugs and their clinical activity in NSCLC

Four main classes of cytotoxic agents are commonly used to treat NSCLC patients (Figure 1): (i) Alkylating agents—including cisplatin and carboplatin—which directly damage DNA

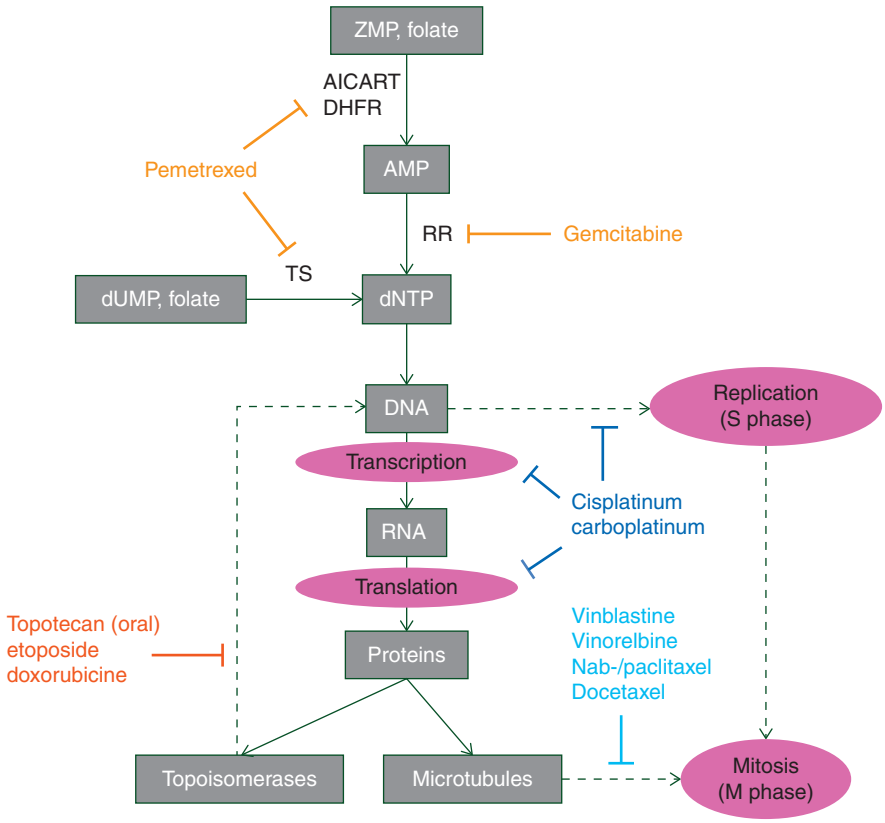

Figure 1. Main cellular targets of CCC drugs used in NSCLC. Antimetabolites (in orange) exert their effect by targeting key enzymes that regulate deoxynucleotide bioavailability or acting as decoys by being misincorporated into nucleic acids. Platinum agents (in blue) induce DNA damage that disrupt replication and transcription. Moreover, their capacity to link with RNA interferes with the translation process. Spindle poisons (in green) disrupt the polymerisation and depolymerisation dynamics of microtubules. Topoisomerase inhibitors (in red) induce cell death by blockading the necessary DNA relaxation during replication and transcription. CCC, conventional cytotoxic chemotherapy; NSCLC, non-small-cell lung cancer; AICART, aminoimidazolecarboxamide ribonucleotide formyltransferase; DHFR, dihydrofolate reductase; RR, ribonucleotide reductase; TS, thymidylate synthase; ZMP, 1-β-D-ribofuranosyl-5-Aminoimidazole-4-carboxamide-5′-phosphate.

thereby disrupting its replication and transcription; (ii) antimetabolites (pemetrexed, gemcitabine), which block nucleic acid synthesis by acting as decoys that either limit deoxyribonucleoside triphosphates (dNTPs) availability or get misincorporated into nucleic acids; (iii) inhibitors of topoisomerases—key enzymes that relax DNA supercoiling during replication and transcription—including topoisomerase I (topotecan) and topoisomerase II (etoposide); (iv) spindle poisons, which disrupt the polymerisation or depolymerisation of the microtubule of the mitotic spindle and include vinorelbine, paclitaxel, and docetaxel [6]. Among these, platinum salts represent the backbone of NSCLC treatment.

Historically, median time to progression and overall survival of metastatic NSCLC with platinum-based doublets were 3.5 and 8 months, respectively [7]. The historical benchmark of 1-year overall survival was reached in non-squamous NSCLC patients in 2006 by adding the antiangiogenic antibody bevacizumab to carboplatin and paclitaxel [8] and in 2008 thanks to the development of the multi-target antifolate pemetrexed [9, 10].

Other advances have been brought by non-cytotoxic agents: a further 10–12 months gain in median survival was obtained in molecularly selected populations presenting tumour *EGFR* activating mutations or *ALK* translocations treated with specific corresponding tyrosine kinase inhibitors (TKIs) [11, 12]. After these druggable biomarkers, several other targets have been uncovered (e.g. ROS1, MET, BRAF, NTRK, etc.) [13]. Most recently, immune checkpoint blockers have demonstrated long-time benefit in 20%–35% of metastatic NSCLC patients [2, 3, 14].

Contrary to these latter agents for which predictive biomarkers of efficacy—such as *EGFR* or *BRAF* mutations, *ALK* translocations, or PD-L1 positivity—have been actively looked for and developed almost in parallel of the drug development ('companion biomarkers'), no single biomarker is currently approved for customising the choice of CCC. However, several pharmacodynamic, pharmacokinetic, or other molecular targets have been identified that could potentially serve as selection biomarkers.

overview of global pharmacodynamic and pharmacokinetic resistance mechanisms to cytotoxic drugs in NSCLC

The first mechanisms of resistance to CCC are the alteration or the absence of the drug's target. Several cytotoxic drugs act indeed at specific phases of the cell cycle and are sometimes qualified as 'cell cycle targeted compounds'. For instance, antimetabolites are mainly active during G1 and S phases, and topoisomerase inhibitors target the S phase, whereas spindle poisons are only active on mitotic cells. Therefore, CCC is mostly active on rapidly growing tumours, whereas tumour-initiating cells or tumour stem cells (which are quiescent and not engaged into the cell cycle) are in most cases resistant to CCC [15].

Beyond pharmacodynamic resistance mechanisms, insensitivity to CCC can also be explained by tumour-specific pharmacokinetic features, including low drug influx or increased efflux through the cell membrane, intracellular drug inactivation, lack of activation, or detoxification (Figure 2). For example, high activity of the detoxification protein gluthatione S-transferase protein GSTP1 has been involved in resistance to platinum agents [16]. Genetic germinal gene polymorphisms affecting the detoxifying enzyme cytidine deaminase (CDA) have been shown to determine bioavailability of gemcitabine both in the tumour and in the liver, as recently reviewed elsewhere [17].

Intrinsic characteristics of the cancer cell can also play a significant role in drug resistance. These include enhanced ability to repair DNA damage—which removes chemo-induced lesions—[18], increased expression of survival signalling pathways (e.g. HER2 overexpression or PI3K/AKT pathway activation) [19, 20], and alteration of the DNA damage or apoptosis signalling cascades (e.g. loss of Chk1 or Chk2 function, or interference with caspases' activation) [21, 22].

However, most resistance mechanisms are multifactorial. For example, resistance to taxanes has been explained by a 'multidrug resistance (MDR) phenotype' resulting from overexpression of the ATP-binding cassette (ABC) transporter family combined with the overexpression of the target tubulin [23–25]. Concomitant decrease in the intracellular concentration (independent of MDR phenotype), increased levels of glutathione or metallothioneins, and a better ability to repair DNA damage can cause resistance to platinum agents [26].

Overall, many candidate biomarkers of chemosensitivity or resistance have been studied, including drug transporters, targets and associated proteins, together with elements involved in metabolic detoxification processes, DNA repair ability, cell cycle regulation, apoptotic or survival signals, and related transcription factors [27]. However, only a few biomarkers harbour the potential for clinical implementation.

critical overview of predictive biomarkers of CCC efficacy in NSCLC

grading the evidence level of predictive biomarkers

In 2006, a literature review focused on predictive biomarkers predicting response to cytotoxic chemotherapies in NSCLC [28]. It revealed that out of 80 *in vitro* identified genes of interest, only 13 had been evaluated in 27 clinical studies. Among these, only four were deemed to be robust enough for further clinical development, namely the transmembrane pump ABCB1 (P-glycoprotein) expression, GSTP1 expression, ERCC1 alterations, and *TP53* mutations. Ten years after, none of these has been implemented in clinical practice, and, with the exception of *TP53* mutational status in some cases, none of them is even looked at by clinicians. Several reasons explain the absence of further clinical implementation: (i) the lack of technical homogenisation and standardisation between different studies (IHC versus RT–PCR versus polymorphisms or missense mutations at the DNA level), thereby preventing any reproducibility of the results; (ii) the variability of judgment criteria and end points (types of criteria, thresholds, choice of statistical tests); (iii) the lack of analytical validation; and (iv) the inadequate study designs and heterogeneous cohorts (retrospective approaches, limited and statistically underpowered size of the study populations).

Several working groups have proposed different grading systems for establishing the analytical validity of clinical biomarkers, including the USA Preventive Services Task Force (USPSTF) [29, 30], The Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) working group [31], and the Strength

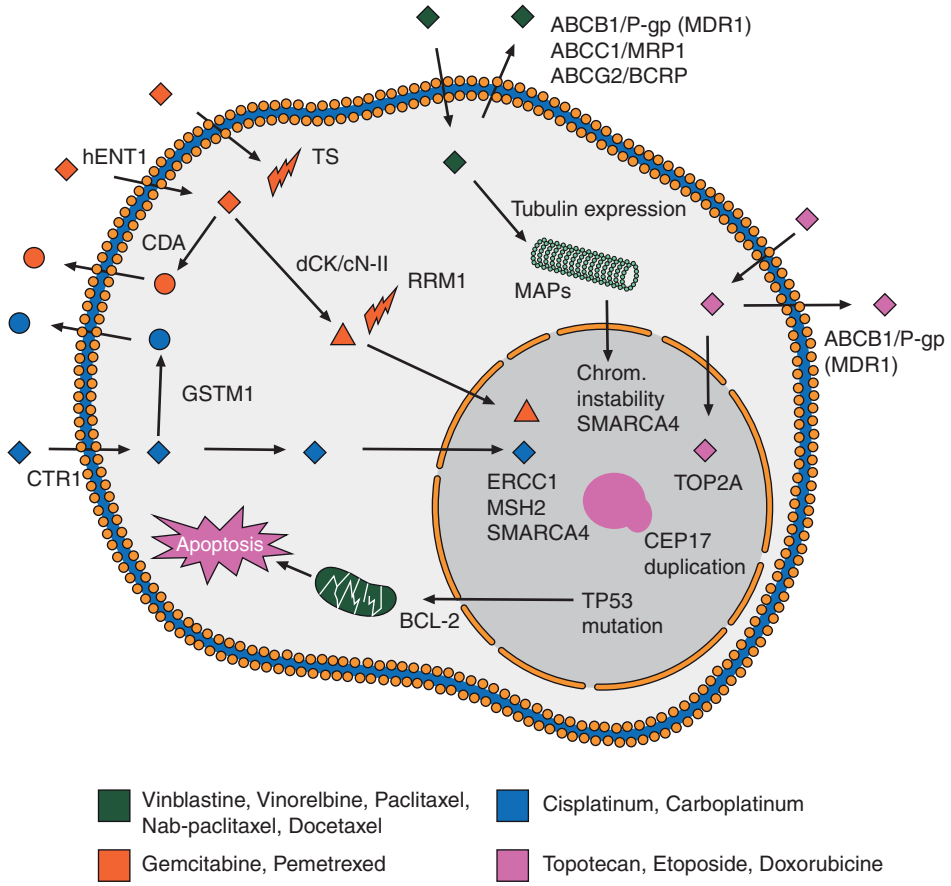

Figure 2. Principal molecular determinants of CCC efficacy investigated in NSCLC. CCC resistance mechanisms find explanation in multiple mechanisms not necessarily mutually exclusive, such as drug influx/efflux efficacy, activation/detoxification processes, target expression levels, DNA repair capacity, and DNA lesion tolerance mechanisms such as failure to induce apoptosis. Platinum drug (in blue) efficacy depends on the CTR1 transporter, the GSTM1 detoxification enzyme, the ERCC1 and MSH2 repair proteins, as well as on the anti-apoptotic protein BCL-2 and the chromatin remodelling regulator SMARCA4. Therapeutic efficacy of antimetabolites (in orange) such as gemcitabine also relies on influx (hENT1), deactivation (CDA), or activation (dCK and cN-II). However, overexpression of the target, RRM1, largely participates in gemcitabine resistance. Resistance to spindle poisons (in green) can be attributed to the increase of drug efflux due to ABC transporter proteins (PGP, BCRP, and MRP1), overexpression of MAPs or tubulin itself, chromosomal instability, or to high SMARCA4 expression. Studies on topoisomerase inhibitor-related biomarkers (in red) have identified MDR1 and TOP1 expression, as well as TP53 mutations, but also CEP17 duplication. CCC, conventional cytotoxic chemotherapy; NSCLC, non-small-cell lung cancer; BCL-2, B-Cell CLL/Lymphoma 2; CDA, cytidine deaminase, CEP17, pericentromeric alpha satellite repeat on chromosome 17; CTR1, copper transporter 1; GSTM1, glutathione S-transferase M1; ERCC1, excision repair cross-complementation group 1; MSH2, MutS homologue 2; hENT1, human equilibrative nucleoside transporter 1; dCK, deoxycytidine kinase; cN-II, cytosolic nucleotidase II/NT5C2; RRM1, ribonucleotide reductase M1; ABCB1/P-gp, ATP-binding cassette B1/P-glycoprotein; ABCC1/MRP1, ATP-binding cassette C1/multidrug resistance-associated protein 1; ABCG2/BCRP, ATP-binding cassette G2/breast cancer resistance protein; MAPs, microtubule-associated proteins; MDR1, multidrug resistance 1; TOP2A, topoisomerase II alpha.

of recommendation taxonomy (SORT) group [32]. As no grading system can perfectly recapitulate the robustness of all biomarkers of interest, here we propose, for the purpose of this review, the use of a simple grading system using three categories: great, intermediate, and low promise with regards to clinical utility (Table 1).

platinum compounds

Platinum compounds form both intra- and inter-strand DNA adducts (or crosslinks), which impair DNA replication and transcription that eventually leads to cell death. Several resistance mechanisms to platinum-based chemotherapy have been identified [26]. Briefly, transport mechanisms (Copper transporter 1, CTR1), detoxification proteins such as glutathione S-transferase (GSTM1 expression or variants), and tolerance-related mechanisms such as apoptotic impairment (e.g. BCL-2 expression) have been reported as mediators of resistance [33–35]. Overexpression of XIAP, a cytoplasmic caspase-inhibiting protein induced by cisplatin, has also been described [36]. However, none of these candidates have been clinically assessed in an appropriately designed prospective trial, and their clinical utility is therefore limited. More recently, low expression of SMARCA4 (member of the ATP-dependent chromatin remodelling complex SNF/SWI) was associated with improved efficacy of platinum-based adjuvant chemotherapy in NSCLC, which

Table 1. Candidate biomarkers predicting CCC efficacy in NSCLC.

		Biomarker level of action				
		Receptor or transporter	Metabolic activation or detoxification	Target modification	DNA repair or genome maintenance	Other processes (apoptotic, epigenetic, etc.)
Drug class	Cisplatinum Carboplatinum	CTR1 expression	GSTM1 variants or expression	n.a.[a]	MSH2 expression ERCC1 expression (++)	BCL-2 expression SMARCA4 expression
	Vinblastine Vinorelbine Paclitaxel Nab-paclitaxel Docetaxel	ABCB1/P-gp (MDR1) expression ABCG2/BCRP expression ABCC1/MRP1 expression	n.a.	α- or β-tubulin expression pattern or level (+) Class I tubulin resistance mutations (nucleotide 810 or 1092) Expression profile of MAPs	Chromosomal instability	SMARCA4 expression
	Oral topotecan	ABCG2/BCRP expression	n.a.	TOP1 mutations TOP1 phosphorylation (S506)	SLFN11 expression	Suppression of apoptosis Activation of survival pathways (ERBB pathway)
	Etoposide Anthracycline (Doxorubicine)	ABCB1/P-gp (MDR1) expression	n.a.	TOP2A expression TOP2A copy number alterations CEP17 duplication	n.a.	ERBB2 and TOP2A co-amplification TP53 mutations
	Gemcitabine	hENT1 expression[a]	dCK expression CDA expression cN-II expression	RRM1 expression (++)	n.a.	n.a.
	Pemetrexed	n.a.	n.a.	TS expression (++)	n.a.	n.a.

The other biomarkers might be considered as 'low' promise.
++, 'great' promise with regard to clinical utility; +, 'intermediate' promise.
[a]n.a., not available.

observation needs to be confirmed in independent validation studies [37].

The most promising biomarkers for predicting response to platinum agents are currently the proteins involved in DNA repair processes. Indeed, the ability of the cell to remove platinum adducts is inversely proportional to the platinum sensitivity. If intra-strand crosslinks can be removed by activation of the nucleotide excision repair (NER) pathway, several factors of different DNA repair pathways must cooperate to repair inter-strand crosslinks, including the FANC family of proteins (Fanconi pathway), BRCA1, BRCA2, ATM and ATR (homologous recombination pathway), DNA polymerase ν (translesion synthesis pathway), as well as other protein complexes such as BTR (Bloom's syndrome complex containing BLM and TOPIIIα) [38].

ERCC1 is a pivotal endonuclease in the NER repair pathway. The gene presents a frequent conservative single-nucleotide polymorphism (SNP) at the third position of codon 118 (rs11615, AAC/AAT). Although both alleles are coding for asparagine, the variant T allele is associated with an ∼50% reduction in platinum DNA adduct repair capacity, probably secondary to a reduced production of ERCC1 mRNA [39]. It was not correlated with outcome after cisplatin-based therapy, contrary to what was observed for another polymorphism in linkage disequilibrium (C8092A) [40].

Other investigators have focused on ERCC1 mRNA expression and reported that higher expression was associated with clinical resistance to platinum in NSCLC, as well as in other tumour types including stomach and ovarian cancer [41–43]. In 2006, ERCC1 protein expression was reported as a predictive marker of outcome on platinum-based chemotherapy in the large International Adjuvant Lung Trial (IALT) [44]. The underlying basis for ERCC1 as a key determinant of platinum sensitivity was further highlighted by Friboulet et al. [45], who showed that ERCC1-deficient NSCLC cell lines were unable to eliminate platinum-DNA adducts *in vitro* and *in vivo*, and that ERCC1_202 isoform only was fully able to restore platinum resistance. However, this work, which used samples from the LACE-bio study and a more recent batch of the ERCC1 antibody, failed to revalidate the initial IALT results. It also demonstrated that all current commercially available antibodies recognised multiple isoforms of ERCC1, thereby potentially leading to misclassification of the ERCC1-proficient and ERCC1-deficient populations secondary to overexpression of inactive isoforms. Following this finding, the only randomised phase III trial designed to evaluate prospectively ERCC1 as predictive biomarker (the ET trial) was halted for futility, and its results would soon be available. However, ERCC1 remains a highly promising biomarker, and the development of a relevant assay for determining ERCC1 status is the matter of intense work in several research teams worldwide [46].

predictors related to spindle poison efficacy

Tubulin, the target of spindle poisons, constitutes an interesting candidate as a predictive biomarker in NSCLC. In particular, the overexpression of the class III β-tubulin alpha (α-) or beta (β-) was described as responsible for resistance to taxanes in breast and ovarian cancers, but also in lung malignancies [47–54]. However, when class III β-tubulin (TUBB3) expression was tested for cross-validation by IHC in the LACE-bio study on 1149 patients, no predictive effect for vinorelbine efficacy could be confirmed, although the prognostic effect was validated (HR = 1.27 for death with high TUBB3 expression) [55]. Other highly investigated biomarkers for spindle poison efficacy are the expression of ABC transporters (PGP, BCRP, MRP-1) [56] and microtubule-associated proteins (MAPs); chromosomal instability is sounded out too [57, 58]. However, as most of these studies were conducted on cell lines or small-sized retrospective cohorts, these biomarkers currently do not get enough consensus for clinical validation and should be graded as low level of promise. Of notice, confirmatory studies of recent reports on docetaxel and chromatin regulators such as SMARCA4 are highly awaited [59].

antimetabolites

gemcitabine. Gemcitabine is a pyrimidine analogue that inhibits the ribonucleotide reductase (RR) class IA—the main human enzyme for biosynthesis of deoxyribonucleotides. RR is allosterically regulated by ATP (activator) and dATP (inhibitor) to maintain balanced NTP versus dNTP pools in the cell, thereby protecting from toxic and mutagenic effects that can arise from dNTP overproduction [60]. RR is a large oligomer consisting of its catalytic subunit RRM1 and one of its two regulative subunits: RRM2 or p53R2 (a p53-regulated paralog of RRM2). The binding of gemcitabine diphosphate, the active metabolite, to the (RRM1)6/(RRM2)2 or (RRM1)6/(p53R2)2 oligomers inhibits the function of the enzyme [61, 62].

The two most promising predictive biomarkers of gemcitabine efficacy are the transporter human equilibrative nucleoside transporter 1 (hENT1) and RRM1 [63]. Among many, one recent study on 110 pancreatic cancer patients in the preoperative setting correlated higher survival rates with increased gemcitabine tumour exposure, which itself correlated with hENT1 expression [64]. In NSCLC, only few consistent retrospective clinical studies have been conducted on hENT1, but the data remain less impressive [65–67]. Overall, hENT1 is not ready for clinical routine use in NSCLC and would classify as being of intermediate promise.

As RRM1 contributes to the synthesis of dNTPs—the building blocks necessary to any DNA repair process—high RRM1 expression associates with increased DNA damage repair capacity and vice versa [68]. Of notice, both RRM1 and RRM2 expressions have been associated with gemcitabine resistance in cell lines and in patients, although most studies have focused on RRM1 [69, 70]. High RRM1 expression was first retrospectively correlated with resistance to gemcitabine in 67 stage IIB-IIIB NSCLC patients in the neoadjuvant setting [71]—a finding that was further confirmed in the metastatic setting in several retrospective studies, using either platinum-based [72–74] or non-platinum-based doublets such as gemcitabine–pemetrexed [75] (reviewed in [76, 77]). Several studies confirmed the feasibility of prospectively analysing RRM1 status using RT–PCR [78], antibody-based techniques [79, 80], or RRM1 SNP analysis (−37C/A and −524T/C) [81], which places RRM1 as the most robust and promising biomarker for gemcitabine efficacy. Beyond IHC or PCR, an interesting technology based on fluorescent RRM1 and ERCC1 antibodies with automated quantitative image analysis (the 'AQUA' system) created initially a

large excitement. However, its prospective evaluation for chemotherapy assignment in a large randomised phase III trial in stage IIIB/IV NSCLC failed to demonstrate a superiority of the customised arm [80, 82].

pemetrexed. Pemetrexed is a multi-target antifolate compound that primarily targets the thymidylate synthase (TS)—an enzyme responsible for maintaining the dTMP pool—thereby reducing the amount of thymidine available for DNA replication and repair. Two other enzymes required for *de novo* purine biosynthesis are also inhibited by this drug: dihydrofolate reductase (DHFR) and aminoimidazolecarboxamide ribonucleotide formyltransferase (AICART) [83]. Several studies have suggested better survival and response rates following pemetrexed treatment in patients with tumours harbouring low TS expression, which was confirmed by several large meta-analyses [84, 85]. Even if these data derived from retrospective and non-randomised studies, it is clear that TS represents a robust biomarker for pemetrexed activity. In clinical practice, although levels of TS are not directly assessed, pemetrexed is already the preferred treatment of non-squamous NSCLC, which harbour lower levels of TS compared with squamous NSCLC [10]. Therefore, TS could be considered as displaying a 'high' level of promise as predictive biomarker pemetrexed efficacy if a prospective validation existed. Other potential biomarkers, but with less convincing data in NSCLC, are DHFR, glycinamide ribonucleotide formyltransferase (GART), proton-coupled folate transporter (PCFT), folylpolyglutamate synthase (FPGS), and deoxycytidine kinase (dCK) that activates gemcitabine [66, 67, 69, 86, 87].

topoisomerase II inhibitors

Etoposide, although less frequently used in NSCLC, acts by trapping Top II on to DNA, thereby preventing DNA replication and transcription, and causing DNA single- and double-strand breaks (DSBs) which in turn result in apoptosis when not adequately repaired [88].

Most of the work on topoisomerase II inhibitors comes from work carried out in breast cancer research and relates to anthracyclines sensitivity. In this context, topoisomerase IIα (TOP2A) expression and in a lesser degree CEP17 duplication (pericentromeric alpha satellite repeat on chromosome 17) or chromosome instability (CIN) are the most robust candidate predictors of efficacy [89, 90], but they still need prospective validation. Other published hits are MDR1 expression [91], (TOP2A) copy number alterations [92], TOP2A/ERBB2 co-amplification [93], tissue inhibitor of metalloproteases 1 (TIMP-1), and decreased apoptosis *via* BCL-2 interaction [94] or mutated TP53 [95]. However, none of these biomarkers has been robustly evaluated in NSCLC and for etoposide treatment, and hence do not represent promising candidates in this context.

topoisomerase I inhibitors

Even more rarely prescribed in NSCLC is the oral form of topotecan that has shown some clinical activity (5% response rate) with acceptable tolerability in relapsed, locally advanced, unresectable NSCLC [96]. This topoisomerase I inhibitor prevents religation of DNA. The DNA/topo-I/drug complex collides with replication forks during S phase, which results in DSBs and apoptosis only in dividing cells [97].

The most recent data on biomarkers of topoisomerase I inhibitors concern the phosphorylation level of serine 506 (PS506) on topoisomerase-I (TOP1) that seems related to irinotecan sensitivity by increasing the capacity of TOP1 to bind DNA [98]. The other candidate predictors such as drug efflux transporters (ABCG2/BCRP) [99, 100], topoisomerase I mutations [101], suppression of apoptosis [102], or SLFN11 [103]. However, no translational or clinical study has been initiated based on these results yet.

inhibition of growth signalling pathways and impact on CCC sensitivity

Depicting the mechanisms underlying some associations between inhibition of growth signalling pathways and sensitivity to CCC has been the matter of intense research. However, most of the research carried out *in vitro* has not been validated in retrospective clinical translational studies yet and has not generated any hypothesis-driven trial. Therefore, we will only describe a few examples. Enhanced activity of pemetrexed has been described in ALK-positive and EGFR-mutated NSCLC tumours [104, 105]. The mechanism is unclear since adenocarcinomas intrinsiqually underexpress TS compared with their squamous counterpart, but there is generally a weaker expression of TS in ALK-positive cells compared with ALK-negative cells [106]. A spillover effect of pemetrexed to mTORC1 due to AICART inhibition could also explain its enhanced effect in ALK-positive and EGFR-mutated NSCLC tumours [107, 108]. Further, gemcitabine transport inhibition has been linked to TKIs exposure (including erlotinib, gefitinib, and vandetanib) in yeast and cell lines [109]. As tentative explanations, the influence of pro-survival transcription factors such as STAT3, anti-apoptotic proteins like c-FLIP, or the expression of DNA repair proteins like Rad51 is currently being investigated [110–112]. Also, FGFR4 up-regulation has been associated with resistance to the DNA-damaging agent doxorubicin, and the targeting of FGFR4 enhances sensitivity to 5-FU and oxaliplatin in colon cancer cell lines [110, 113]. Overall, none of these 'signalling pathway-related' biomarkers has been robustly linked to activity of CCC in clinical samples, and their use as predictive biomarker out of the scope of TKIs sensitivity appears today rather unlikely and premature. Further, and contrasting with these preclinical observations, none of the trials associating a tyrosine kinase inhibitor to a cytotoxic agent (in a concomitant setting) demonstrated superiority to the cytotoxic regimen alone [114]. A potential blockade of the cells in the G1 phase by the TKI—which would reduce sensitivity to cytotoxic agents targeting cycling cells—has been hypothesised for these negative results. Therefore, a smarter scheduling of the TKI and CCC (e.g. sequential administration) might allow a stronger therapeutic impact.

practical issues and challenges to consider for successful biomarker development

Mandatory key steps have to be followed for ensuring successful biomarker development (Figure 3). If only 3%–5% of candidates will eventually reach the clinic [115–117], the attrition rate for

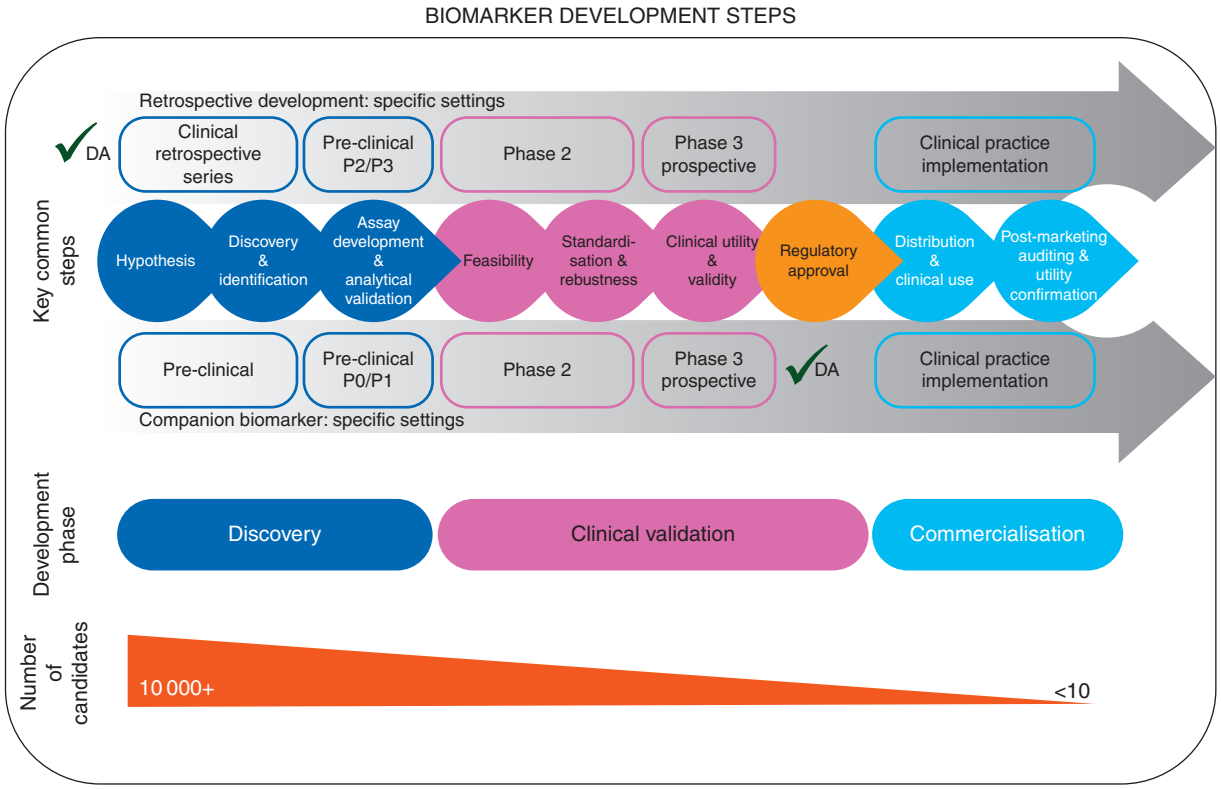

Figure 3. Biomarker development steps. A successful biomarker development follows three consecutive phases: discovery, clinical validation, and commercialisation. Each of these phases can be divided into several steps, shared by the development of all biomarkers ('key common steps'). However, the discovery phase can occur in different contexts according to the biomarker development setting. For a 'retrospective' biomarker development (i.e. when biomarkers are developed after drug approval and commercialisation; top grey arrow), the biomarker discovery usually takes place in retrospective clinical series, while the assay development and analytical validation is carried out preclinically and validated in phase 2 and 3 trials. For companion biomarkers (i.e. biomarkers that are developed in parallel to the drug; bottom grey arrow), the discovery and assay development usually happen in the preclinical setting and are tested in early phase trials (phase 0 and phase 1). The following steps are common and independent of the biomarker development setting: the feasibility of the biomarker assessment and its robustness are evaluated in phase 2 trials, whereas its clinical utility and validity are validated prospectively in dedicated randomised phase 3 trials, for example randomising patients in customised versus non-customised arms of treatment. After regulatory approval, the test can be implemented in clinical practice. The attrition rate of biomarkers is very high, and <1/1000 of all studied biomarkers eventually reaches clinical significance and implementation. DA, drug approval; P0, phase 0; P1, phase 1; P2, phase 2; P3, phase 3; NPV, negative predictive value; PPV, positive predictive value.

CCC-related biomarkers has been particularly high. Beyond historical reasons—the mechanism of action of some agents was unknown at the time of their first clinical administration—one important cause of failure is the lack of standardisation in biological, technical, and clinical approaches, harbouring intrinsic complexity. As CCC has proven efficacy and is the standard of care in almost all stages of NSCLC, the design of randomised clinical trials appropriately addressing the validation of a biomarker is challenging, as a placebo arm is no longer an option. The design of 'customised' trials (where chemotherapy choice is guided by the biomarker) is therefore precious, but these require large number of patients to avoid being underpowered, and data interpretation is impacted by the fact that most chemotherapies are prescribed in combination. The difficulty and potential lack of motivation of most drug companies and academics to work on CCC biomarker development are well illustrated by the very low number of clinical trials currently investigating such biomarkers (Table 2). Tangible challenges include the intra-tumour heterogeneity, the spatial and temporal biological variability, the scientific relevance of the biomarker, and the multiplicity of interdependent mechanisms underlying sensitivity/resistance. As these first challenges are somehow inherent to clinical practice and tumour biology—and as such cannot be influenced—most attention should be put on technical standardisation, for which precise guidelines should be established. These include the pre-analytical standardisation (tissue sampling, handling, etc.), the analytical standardisation (material to be studied, thresholds for significance and scoring systems used, etc.), and the post-analytical standardisation (learning processes, inter-centre reproducibility, etc.) [118–120]. Importantly, all these 'fit-for-purpose' assay validation steps are interconnected and should be regularly re-evaluated to best fit therapeutic and technological advances.

lessons learned and future challenges

Despite all efforts that have been put on identifying predictive biomarkers for sensitivity to CCC, results have been disappointing so far and an extremely high attrition rate between promising preclinical data and negative clinical results has been observed. Only two or three major biomarkers (RRM1, ERCC1,

Table 2. Ongoing clinical trials evaluating biomarkers of response to cytotoxic conventional chemotherapy in all tumour types

Biomarker	Analysis	Study	Tumour type	Setting	Therapeutic intervention
ERCC1, RRM1, TS	Prospective	NCT01784549 (CONTEST)	NSCLC	Stage IIIA (N2)	**Cisplatin** Vinorelbine **Gemcitabine** Docetaxel **Pemetrexed**
ERCC1, TS, TOP2A	Retrospective	NCT02535325	NSCLC	Advanced/metastatic	Radiotherapy **Cisplatin** Methoxyamine
ERCC1, RRM1, TS	Retrospective	NCT01574300 (CASTLE)	NSCLC	Any	Any
ERCC1	Retrospective	NCT02128906	HNSCC	Locally advanced	Radiotherapy **Cisplatin** Docetaxel-cetuximab
ERCC1	Retrospective	NCT00953511 (CERP-study)	Oesophageal cancer	Neoadjuvant	Radiotherapy **Cisplatin** Fluorouracil
ERCC1	Prospective	NCT01703390	Colorectal cancer	Metastatic	**Oxaliplatin** Fluorouracil cetuximab Irinotecan
ERCC1	Retrospective	NCT01748825	Solid tumours	Metastatic	**AZD-1775**
ERCC1	Retrospective	NCT01989546	Breast and Ovarian cancer	Advanced/metastatic	**BMN763** (talazoparib)
TOP2A	Prospective	NCT02339532	Breast cancer	Neoadjuvant	Docetaxel Trastuzumab Pertuzumab **Carboplatin**
TUBB3	Retrospective	NCT01865045	Pleural mesothelioma	Advanced/metastatic	**Vinorelbine**
hENT1	Retrospective	NCT02486497	Pancreatic cancer	Adjuvant	**Gemcitabine** Fluorouracil
hENT1	Retrospective	NCT01586611 (Panc001)	Pancreatic cancer	Metastatic	**Gemcitabine** Fluorouracil Oxaliplatin
hENT1	Prospective	NCT01411072	Pancreatic cancer	Metastatic	**Gemcitabine** Fluorouracil

Retrospective analyses correspond to trials where the potential role of the biomarker will be analysed after trial completion; prospective analyses correspond to trials where the biomarker will be analysed before starting treatment and guide treatment allocation. Conventional cytotoxic chemotherapies of interest are highlighted in bold.
HNSCC, head and neck squamous cell carcinoma; NSCLC, non-small-cell lung cancer; SCLC, small cell lung cancer.

TS) might be considered as still in the race in NSCLC. One major aspect has hampered the selection of biomarkers, which is that chemotherapeutic agents are usually given in combination. For instance, cisplatin resistance may be mediated by enhanced DNA repair, which is initially meant to be overcome by co-administration of gemcitabine that induces an attrition of available dNTPs, thereby preventing chain elongation during the DNA repair process [121, 122]. Therefore, future studies will have to better integrate multiple markers to develop biologically meaningful predictive algorithms that explain treatment failure.

Several other caveats can explain the disappointing results in the field: (i) the limited interest for analytical validity (lack of procedures standardisation, excessive number of techniques and methods explored); (ii) the experiences being run on small retrospective patient series instead of prospective 'on-purpose' designed trials; (iii) the difficulty in initiating well-designed randomised trials including a control arm (as CCC is now a standard in almost all settings in NSCLC); (iv) the lack of interest of industrials and academics (either for functional validation of known candidates or for the discovery of novel targets), as the cost of developing a biomarker would ostensibly overcome the current cost of CCC; however, the recent work carried out on ERCC1 isoforms and the variability of the 8F1 antibody nicely illustrates how well-designed functional studies can explain repeated failures in clinical trials aimed at biomarker validation; and (v) the exclusive focus on DNA repair capacity of cancer cells. Indeed, recent evidence demonstrates that epigenetic factors can also play a role in response to CCC [123], as well as microenvironmental elements (including stromal and immune cells) [124, 125].

There is nevertheless hope in this challenging field. Examples include recent work on DNA signatures for predicting sensitivity to DNA-targeting agents (e.g. PARP inhibitors), which actually have the advantage of working on a reliable material (DNA) that yields reproducible results [110, 111]. Also, the decreasing cost of targeted gene panel sequencing opens promising future for identifying deleterious mutations in DNA repair genes that would predispose to CCC sensitivity. Moreover, the ability to use such techniques on circulating biomarkers (ctDNA) represents an attractive non-invasive opportunity for stratifying patients and monitoring tumour evolutions [126]. Recent work focusing on epigenetic regulators should also be encouraged [37, 59]. At a more preliminary level, other techniques such as RNAseq or methylome analysis could also bring interesting promises. Finally, it is important to remember that assessing a target at the protein expression level (rather than DNA or RNA) is still the most relevant in most cases, and novel techniques allowing the analysis of hundreds of samples by IHC on automated instruments followed by rapid image analysis raise new hopes.

In conclusion, despite the absence of validated predictive biomarker for CCC customisation, novel technological advances open encouraging perspectives for performing analytical validation of some promising candidates that have been identified so far, or for developing novel types of predictive biomarkers—such as DNA signatures. Most importantly, patients are eager to know as much as possible about their tumour and to benefit from a customised treatment, even within the frame of an exploratory clinical trial. Clinicians and academic researchers should therefore be committed to pursue investigations in this field.

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disclosure

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