Genetic and Epigenetic Biomarkers of Immune Checkpoint Blockade Response

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Abstract: Checkpoint inhibitor therapy constitutes a promising cancer treatment strategy that targets the immune checkpoints to re-activate silenced T cell cytotoxicity. In recent pivotal trials, immune checkpoint blockade (ICB) demonstrated durable responses and acceptable toxicity, resulting in the regulatory approval of 8 checkpoint inhibitors to date for 15 cancer indications. However, up to ~85% of patients present with innate or acquired resistance to ICB, limiting its clinical utility. Current response biomarker candidates, including DNA mutation and neoantigen load, immune profiles, as well as programmed death-ligand 1 (PD-L1) expression, are only weak predictors of ICB response. Thus, identification of novel, more predictive biomarkers that could identify patients who would benefit from ICB constitutes one of the most important areas of immunotherapy research. Aberrant DNA methylation (5mC) and hydroxymethylation (5hmC) were discovered in multiple cancers, and dynamic changes of the epigenomic landscape have been identified during T cell differentiation and activation. While their role in cancer immunosuppression remains to be elucidated, recent evidence suggests that 5mC and 5hmC may serve as prognostic and predictive biomarkers of ICB-sensitive cancers. In this review, we describe the role of epigenetic phenomena in tumor immunoediting and other immune evasion related processes, provide a comprehensive update of the current status of ICB-response biomarkers, and highlight promising epigenomic biomarker candidates.

Keywords: immunotherapy; predictor; resistance; epigenetics; stroma; melanoma; non-small-cell lung cancer

1. Introduction

Immunotherapy constitutes a major breakthrough in cancer treatment. In particular, approaches based on inhibiting programmed death-1 (PD-1)/PD-L1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) are the most rapidly growing drug class [1]. In recent years, the reprogramming of immune checkpoint receptor (ICR) expression on the surface of T cells emerged as a critical mechanism of tumor cell immune-evasion [2]. ICRs are a class of co-stimulators (CD27, CD28, and CD137) and co-inhibitory receptors (e.g., PD-1, CTLA-4, lymphocyte activation gene 3 (LAG-3)) that regulate T cell response quality [3]. The most widely used immune checkpoint blockade (ICB) therapeutic strategies target the PD-1/PD-L1 and CTLA4 axes to regulate anti-tumor immune activity with demonstrated
clinical benefits [4]. The interaction between PD-1 in the cytotoxic T lymphocyte and PD-L1 in the cancer cell decreases the activity of T cells by several mechanisms, including the inhibition of the T cell receptor downstream signaling [5,6], the enhancement of regulatory T cells [7], and the decrease in B cells and natural killer activities [8]. Another important ICR is CTLA-4, which impairs T cell activation by outcompeting the co-stimulatory receptor CD28 [9]. PD-1 and CTLA-4 blockade restores the anti-tumor immune response by inducing the expansion of exhausted-like tumor-infiltrating CD8 T cells; in addition, CTLA-4 blockade rescues Th1-like CD4 effector T cells and could be implicated in the enhancement of CD8 infiltration and cytolytic activity as well as the formation of memory T cells [10].

ICB is effective in multiple malignancies with strong immunogenicity including melanoma and non-small-cell lung cancer (NSCLC). Indeed, the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved the clinical use of ICB drugs for melanoma, NSCLC, renal cell carcinoma, head and neck squamous cell cancer, Hodgkin’s lymphoma, urothelial carcinoma, gastric cancer, cervical cancer, hepatocellular carcinoma, primary mediastinal large-B-cell lymphoma, microsatellite instability-high/deficient mismatch repair cancer, and Merkel cell carcinoma. In 2019, the first-line anti-PD-1 treatment was approved for patients with stage III NSCLC that were not susceptible to surgery, definite chemoradiation, or present metastasis, and that complied with an epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK) wild-type phenotype and PD-L1 expression (Table 1). Interestingly, more than 20 clinical trials are currently ongoing for various novel oncological indications (Table 2). Although a considerable fraction of patients demonstrate an objective clinical response, the majority of patients do not respond appropriately to ICB therapy because of primary or acquired treatment resistance [11,12]. The least efficient line of treatment so far was anti-CTLA4, with ~85% of non-responding patients [13–15], followed by anti-PD-1 approaches with a response of ~40% [16,17]. Combination regimens are related to better percentages of response (~50%) but also higher toxicity [18,19]. Hence, biomarkers predicting ICB response are imperatively needed.

| Drug | Approval Date | Mechanism | Sample Size | Reference Clinical Trial | Cancer Type | Indications |
|------|---------------|-----------|-------------|--------------------------|-------------|-------------|
| Ipilimumab (YERVOY®) | 28/10/2015 | CTLA4 | 951 | EORTC (NCT00636168) | Melanoma | Adjuvant treatment of cutaneous melanoma patients with pathologic involvement of regional lymph nodes of more than 1 mm who have undergone complete resection |
| Ipilimumab (YERVOY®) | 25/3/2011 | CTLA-4 | 676 | MDX010-20 (NCT00094653) | Melanoma | Unresectable or metastatic melanoma with previous systematic treatment previously |
| Pembrolizumab (KEYTRUDA®) | 04/09/2014 | PD-1 | 173 | KEYNOTE-001 (NCT01299227) | Melanoma | Unresectable or metastatic melanoma and disease progression following Ipilimumab and, if BRAF V600 mutation positive, a BRAF inhibitor |
| Pembrolizumab (KEYTRUDA®) | 18/12/2015 | PD-1 | 834+540 | KEYNOTE-006 (NCT01866319); KEYNOTE-002 (NCT01704287) | Melanoma | Unresectable or metastatic melanoma |
| Nivolumab | 30/09/2015 | PD-1, CTLA4 | 142 | CheckMate-069 (NCT01927419) | Melanoma | BRAF V600 wild-type, unresectable or metastatic melanoma |
| Nivolumab + Ipilimumab (OPDIVO® + YERVOY®) | 22/12/2014 | PD-1 | 120 | CheckMate-037 (NCT01721746) | Melanoma | Unresectable or metastatic melanoma and disease progression following Ipilimumab and, if BRAF V600 mutation positive, a BRAF inhibitor |
| Pembrolizumab (KEYTRUDA®) | 15/02/2019 | PD-1 | 1019 | KEYNOTE-054 (NCT02362594) | Melanoma | Melanoma with involvement of lymph node(s) following complete resection |
| Drug                          | Approval Date | Mechanism | Sample Size | Reference Clinical Trial | Cancer Type                                      | Indications                                                                 |
|-------------------------------|---------------|-----------|-------------|---------------------------|-------------------------------------------------|-----------------------------------------------------------------------------|
| Nivolumab (OPDIVO®) *         | 20/12/2017    | PD-1      | 906         | CheckMate-238 (NCT02388906) | Melanoma                                        | Adjuvant treatment of advanced melanoma                                      |
| Nivolumab + Ipilimumab (OPDIVO®+ YERVOY®) | 16/04/2018    | PD-1, CTLA4 | 847         | CheckMate-214 (NCT02231749) | Hepatocellular carcinoma                        | Intermediate or poor risk advanced hepatocellular carcinoma without prior treatment |
| Pembrolizumab (KEYTRUDA®)     | 09/11/2018    | PD-1      | 104         | KEYNOTE-224 (NCT02702414)  | Hepatocellular carcinoma                        | Hepatocellular carcinoma previously treated with Sorafenib                   |
| Nivolumab (OPDIVO®)           | 22/09/2017    | PD-1      | 154         | CheckMate-040 (NCT0168878) | Hepatocellular carcinoma                        | Hepatocellular carcinoma previously treated with sorafenib                   |
| Pembrolizumab (KEYTRUDA®) *   | 15/03/2017    | PD-1      | 210         | KEYNOTE-087 (NCT02453594)  | Lymphoma                                        | Recurrent classical Hodgkin lymphoma patients, or those who have relapsed after three or more prior lines of therapy |
| Nivolumab (OPDIVO®) *         | 17/05/2016    | PD-1      | 95          | CheckMate-205 (NCT02181738); CheckMate-039 (NCT01592370) | Lymphoma                                        | Refractory classical Hodgkin lymphoma following autologous hematopoietic stem cell transplantation and post-transplantation Brentuximab Vedotin |
| Pembrolizumab (KEYTRUDA®)     | 13/06/2018    | PD-1      | 53          | KEYNOTE-170 (NCT02576990)  | Lymphoma                                        | Refractory primary mediastinal large B-cell lymphoma patients, or who have relapsed after two or more prior lines of therapy |
| Cemiplimab-rwlc (LIBTAYO®) *  | 28/09/2018    | PD-1      | 108         | R2810-ONC-1423 (NCT02383212); R2810-ONC-1540 (NCT02760498) | Cutaneous squamous cell carcinoma               | Metastatic or locally advanced cutaneous squamous cell carcinoma patients who are not candidates for curative surgery or curative radiation |
| Pembrolizumab (KEYTRUDA®) *   | 05/08/2016    | PD-1      | 174         | KEYNOTE-012 (NCT01848834)  | Squamous cell carcinoma of the head and neck     | Recurrent or metastatic squamous cell carcinoma of the head and neck with progression on or after platinum-containing chemotherapy |
| Nivolumab (OPDIVO®) *         | 10/11/2016    | PD-1      | 361         | CheckMate-141 (NCT02105636) | Squamous cell carcinoma of the head and neck     | Advanced squamous cell carcinoma of the head and neck with progression on after a platinum-based therapy |
| Nivolumab (OPDIVO®)           | 31/07/2017    | PD-1      | 74          | CheckMate-142 (NCT02060188) | Colorectal                                      | Treatment of patients 12 years and older with mismatch repair deficient and microsatellite instability high metastatic colorectal cancer that has progressed following treatment with Fluoropyrimidine, Oxaliplatin, and Irinotecan |
| Nivolumab + Ipilimumab (OPDIVO®+ YERVOY®) | 10/07/2018    | CTLA4     | 82          | CheckMate-142 (NCT02060188) | Metastatic colorectal cancer with high microsatellite instability or mismatch repair deficiency | Unresectable or metastatic, microsatellite instability-high or mismatch repair deficient solid tumors patients that have progressed following prior treatment and who have no satisfactory alternative treatment options or with microlitally instability-high or mismatch repair deficient colorectal cancer that has progressed following treatment with Fluoropyrimidine, Oxaliplatin, and Irinotecan |
| Pembrolizumab (KEYTRUDA®)     | 23/05/2017    | PD-1      | 149         | KEYNOTE-016 (NCT01876511); KEYNOTE-164 (NCT02460198); KEYNOTE-012 (NCT01848834); KEYNOTE-028 (NCT02054806); KEYNOTE-158 (NCT02628067) | Colorectal                                      | Recurrent or metastatic cervical cancer patients with progression on or after chemotherapy whose tumors express PD-L1 as determined by an FDA-approved test |
| Pembrolizumab (KEYTRUDA®)     | 12/06/2018    | PD-1      | 98          | KEYNOTE-158 (NCT02628067)  | Cervical                                        | Recurrent or metastatic cervical cancer patients with progression on or after chemotherapy whose tumors express PD-L1 as determined by an FDA-approved test |
Table 1. Cont.

| Drug | Approval Date | Mechanism | Sample Size | Reference Clinical Trial | Cancer Type | Indications |
|------|---------------|-----------|-------------|--------------------------|-------------|-------------|
| Pembrolizumab (KEYTRUDA®) * | 11/04/2019 | PD-1 | 1274 | KEYNOTE-042 (NCT02220894) | Lung | First-line treatment of patients with stage III non-small-cell lung cancer who are not candidates for surgical resection or definitive chemoradiation or metastatic non-small cell lung cancer. Patients’ tumors must have no EGFR or ALK genomic aberrations and express PD-L1 (Tumor Proportion Score [TPS] ≥1%) determined by an FDA-approved test. |
| Pembrolizumab (KEYTRUDA®) * + chemotheray | 06/12/2018 | PD-L1 | 1202 | IMpower150 trial (NCT02366143) | Lung | Metastatic non-squamous, non-small-cell lung cancer with no EGFR or ALK genomic tumor aberrations. |
| Pembrolizumab (KEYTRUDA®) + pemetrexed and carboplatin * | 18/10/2016 | PD-L1 | 1137 | POPLAR (NCT01903993); OAK (NCT02088227) | Lung | Metastatic non-small-cell lung cancer patients whose disease progressed during or following platinum-containing chemotherapy. |
| Pembrolizumab (KEYTRUDA®) + pemetrexed and carboplatin * | 10/05/17 | PD-1 | 123 | KEYNOTE-021 (NCT0203674) | Lung | Previously untreated metastatic non-squamous non-small-cell lung cancer. |
| Nivolumab (OPDIVO®) * | 09/10/2015 | PD-1 | 582 | CheckMate-057 (NCT01673867) | Lung | Metastatic non-small-cell lung cancer with progression on or after platinum-based chemotherapy. |
| Pembrolizumab (KEYTRUDA®) + carboplatin/paclitaxel * | 30/10/2018 | PD-1 | 559 | KEYNOTE-407 (NCT0275435) | Lung | Metastatic squamous non-small-cell lung cancer. |
| Pembrolizumab (KEYTRUDA®) * | 24/10/2016 | PD-1 | 305 + 1033 | KEYNOTE-024 (NCT0142738); KEYNOTE-010 (NCT01905657) | Lung | Metastatic non-small-cell lung cancer patients whose tumors express PD-L1 as determined by an FDA-approved test. |
| Nivolumab (OPDIVO®) * | 04/03/2015 | PD-1 | 272 | CheckMate-017 (NCT01642004) | Lung | Metastatic squamous non-small-cell lung cancer with progression on or after platinum-based chemotherapy. |
| Pembrolizumab (KEYTRUDA®) + pemetrexed and carboplatin * | 20/08/2018 | PD-1 | 616 | KEYNOTE-189 (NCT02576680) | Lung | Metastatic, non-squamous non-small-cell lung cancer, with no EGFR or ALK genomic tumor aberrations. |
| Durvalumab (IMFINZI®) * | 06/02/2018 | PD-L1 | 713 | PACIFIC (NCT02125461) | Lung | Unresectable stage III non-small cell lung cancer patients whose disease has not progressed following concurrent platinum-based chemotherapy and radiation therapy. |
| Pembrolizumab (KEYTRUDA®) * | 02/10/2015 | PD-1 | 61 | KEYNOTE-001 (NCT01295827) | Lung | Metastatic non-small-cell lung cancer patients whose tumors express programmed death ligand 1 as determined by an FDA-approved test, with disease progression on or after platinum-containing chemotherapy. |
| Pembrolizumab (KEYTRUDA®) + carboplatin and etoposide * | 18/03/2019 | PD-L1 | 403 | IMpower133 (NCT02763579) | Lung | Extensive-stage small cell lung cancer. |
| Nivolumab (OPDIVO®) | 16/08/2018 | PD-1 | 109 | CheckMate-032 (NCT01928394) | Lung | Progressive metastatic small cell lung cancer with progression after platinum-based chemotherapy and other lines of therapy. |
| Drug | Approval Date | Mechanism | Sample Size | Reference Clinical Trial | Cancer Type | Indications |
|------|---------------|-----------|-------------|--------------------------|-------------|-------------|
| Nivolumab (OPDIVO®) * | 02/02/2017 | PD-1 | 270 | CheckMate-275 (NCT02387996) | Urothelial | Locally advanced or metastatic urothelial carcinoma patients who have disease progression during or following platinum-containing chemotherapy or have disease progression within 12 months of neoadjuvant or adjuvant treatment with a platinum-containing chemotherapy. |
| Durvalumab (IMFINZI®) | 01/05/2017 | PD-L1 | 182 | Study 1108 (NCT01693562) | Urothelial | Locally advanced or metastatic urothelial carcinoma patients who have disease progression during or following platinum-containing chemotherapy or who have disease progression within 12 months of neoadjuvant or adjuvant treatment with platinum-containing chemotherapy. |
| Atezolizumab (TECENTRIQ®) * | 18/05/2016 | PD-L1 | 310 | IMvigor210 (NCT02108652) | Urothelial | Locally advanced or metastatic urothelial carcinoma patients who have disease progression during or following platinum-containing chemotherapy or have disease progression within 12 months of neoadjuvant or adjuvant treatment with platinum-containing chemotherapy. |
| Avelumab (BAVENCIO®) | 09/05/2017 | PD-L1 | 242 | JAVELIN Solid Tumor (NCT01772004) | Urothelial | Locally advanced or metastatic urothelial carcinoma patients whose disease progressed during or following platinum-containing chemotherapy or within 12 months of neoadjuvant or adjuvant platinum-containing chemotherapy. |
| Pembrolizumab (KEYTRUDA®) * | 18/05/2017 | PD-1 | 542 | KEYNOTE-045 (NCT02256436) | Urothelial | Locally advanced or metastatic urothelial carcinoma patients who have disease progression during or following platinum-containing chemotherapy or have disease progression within 12 months of neoadjuvant or adjuvant treatment with platinum-containing chemotherapy. |
| Pembrolizumab (KEYTRUDA®) | 19/12/2018 | PD-1 | 50 | KEYNOTE-017 (NCT02256763) | Merkel cell carcinoma | Recurrent locally advanced or metastatic Merkel cell carcinoma |
| Pembrolizumab (KEYTRUDA®) | 19/12/2018 | PD-1 | 50 | KEYNOTE-017 (NCT02256763) | Merkel cell carcinoma | Recurrent locally advanced or metastatic Merkel cell carcinoma |
| Pembrolizumab (KEYTRUDA®) | 19/12/2018 | PD-1 | 50 | KEYNOTE-017 (NCT02256763) | Merkel cell carcinoma | Recurrent locally advanced or metastatic Merkel cell carcinoma |
| Avelumab (BAVENCIO®) * | 23/3/2017 | PD-L1 | 1738 | JAVELIN Merkel 200 (NCT02155647) | Merkel cell carcinoma | Metastatic Merkel cell carcinoma |
| Nivolumab (OPDIVO®) * | 23/11/2015 | PD-1 | 821 | CheckMate-025 (NCT01668764) | Renal | Advanced renal cell carcinoma in patients with previous anti-angiogenic therapy |
| Atezolizumab (TECENTRIQ®) * | 08/03/2019 | PD-L1 | 902 | IMpassion130 (NCT02425891) | Breast | Unresectable locally advanced or metastatic triple-negative breast cancer patients whose tumors express PD-L1 (PD-L1 stained tumor-infiltrating immune cells [IC] of any intensity covering ≥ 1% of the tumor area), as determined by an FDA-approved test |
| Pembrolizumab (KEYTRUDA®) | 22/09/2017 | PD-1 | 259 | KEYNOTE-059 (NCT02335411) | Gastric/gastroesophageal junction adenocarcinoma | Recurrent locally advanced or metastatic, gastric or gastroesophageal junction adenocarcinoma patients whose tumors express PD-L1 as determined by an FDA-approved test |

* Drug administration also approved by the European Medicines Agency (EMA) for the same cancer type.
Table 2. Active clinical trials of immune checkpoint blockade for novel oncological indications.

| Drug                | Targeted IC | Sample Size | Cancer Type                                      | Response Rate | Phase | Trial Number     |
|---------------------|-------------|-------------|--------------------------------------------------|---------------|-------|------------------|
| Ipilimumab          | CTLA-4      | 100         | Melanoma (stage III/IV)                          | 10.9%         | III/IV| NCT00946763     |
| Pembrolizumab       | PD-1        | 31          | Hodgkin Lymphoma (recurred)                     | 65%           | I     | NCT01958692     |
| Pembrolizumab       | PD-1        | 26          | Locoregional Merkel-cell carcinoma (advanced)   | 56%           | II    | NCT02267603     |
| Nivolumab           | PD-1        | 240         | Squamous-Cell Carcinoma (relapsed or advanced)  | 13.3%         | III   | NCT02105636     |
| Nivolumab           | PD-1        | 410         | Renal-Cell Carcinoma (advanced)                 | 25%           | III   | NCT01668784     |
| Pembrolizumab       | PD-1        | 270         | Urothelial Carcinoma (advanced)                | 21.1%         | III   | NCT02256436     |
| Pembrolizumab       | PD-L1       | 27          | Triple-Negative Breast Cancer (advanced)        | 18.5%         | I     | NCT01848834     |
| Nivolumab           | PD-1        | 39          | Hepatocellular carcinoma (advanced)             | 23%           | I/II  | NCT01658878     |
| MDX1105-01 (anti-PD-L1) | PD-L1   | 207         | Non-small-cell lung cancer, melanoma, colorectal cancer, renal cell carcinoma, prostate cancer, ovarian cancer, gastric cancer, breast cancer | 12.6%         | I     | NCT00729664     |
| Atezolizumab        | PD-L1       | 175         | Non-small-cell lung cancer, renal cell carcinoma, melanoma, other tumors | 18%           | I     | NCT01375842     |
| Tremelimumab        | CTLA-4      | 17          | Hepatocellular carcinoma (advanced with chronic hepatitis C) | 17.6%         | II    | NCT01088358     |
| Avelumab            | PD-L1       | 88          | Merkel cell carcinoma (chemotherapy-refractory stage IV) | 31.8%         | II    | NCT02155647     |
| Atezolizumab        | PD-L1       | 116         | Triple-negative breast cancer (metastatic)      | 9.5%          | I     | NCT01375842     |
| Atezolizumab        | PD-L1       | 32          | Head and neck cancer                           | 22%           | I     | NCT01375842     |
| Atezolizumab        | PD-L1       | 95          | Urothelial cancer (metastatic)                  | 26%           | I     | NCT01375842     |
| Nivolumab           | PD1         | 296         | Melanoma (advanced), non-small-cell lung cancer, prostate cancer (castration-resistant), renal-cell cancer, colorectal cancer | 18% in non-small-cell lung cancer, 28% in melanoma, 27% in renal-cell cancer | I     | NCT01354431     |
| Pidilizumab         | PD-1        | 66          | Diffuse large B-cell lymphoma                   | 51%           | II    | NCT00322529     |
| Pidilizumab         | PD-1        | 32          | Follicular lymphoma (relapsed)                  | 66%           | II    | NCT00904722     |
| Nivolumab           | PD-1        | 23          | Hodgkin’s lymphoma (relapsed or refractory)    | 87%           | I     | NCT01952370     |
| Lambrolizumab       | PD-1        | 135         | Melanoma (advanced)                            | 38%           | I     | NCT01295827     |
| Nivolumab           | PD1         | 107         | Melanoma (advanced)                            | 30.8%         | I     | NCT00730639     |
| Nivolumab           | PD1         | 418         | Melanoma (untreated without BRAF mutation)      | 40.0%         | III   | NCT01721772     |
| Nivolumab           | PD1         | 631         | Melanoma (advanced that progressed after anti-CTLA-4 treatment) | 31.7%         | III   | NCT01721746     |
| Pembrolizumab       | PD1         | 495         | Non-small-cell lung cancer                      | 19.4%         | I     | NCT01295827     |
| Nivolumab           | PD1         | 272         | Squamous-cell non-small-cell lung cancer (advanced) | 20%           | III   | NCT01642004     |
| Nivolumab           | PD1         | 129         | Non-small-cell lung cancer (previously treated advanced) | 17%           | I     | NCT00730639     |

There is increasing evidence of the important role of epigenetic marks, such as 5mC and 5hmC, in carcinogenesis [20–22]. Interestingly, 5mC is also of critical importance for regulating T cell proliferation and maintaining differentiation in cytotoxic and helper T cells [23], whereas 5hmC dynamically changes during T cell differentiation [24]. Recently, a specific role of 5hmC deposition in key immune genes has been reported for the activation and differentiation of T lymphocytes after antigen presentation.
Interestingly, the variations in 5hmC were more dynamic than those in 5mC [25]. Importantly, tumor cells such as hepatocellular carcinoma cells show a cancer-specific methylome and hydroxymethylome profile [26], indicating the potential of these epigenetic marks to serve as diagnostic or prognostic biomarkers [27]. Indeed, we have recently shown that specific patterns of DNA methylation, named as the “EPIMMUNE” signature, that can be further subrogated to a single CpG variant in FOXP1, have been associated with clinical benefit in NSCLS patients receiving ICB [28]. Here, we will revisit the current research on and clinical use of ICB biomarkers, and we will critically review 5mC and 5hmC as potential biomarkers of response to cancer immunotherapy.

2. Induction of Inhibitory Immune Checkpoints (ICs) as a Major Mechanism of Tumor Immune Evasion

The “immunoediting” hypothesis conceptualizes the evolution of the tumor cells under immune pressures towards expansion and immune surveillance escape of the tumor [29]. Several factors contribute to this immune evasion. The tumor microenvironment (TME) can be immunosuppressive per se, facilitating tumor progression with cytokines, chemokines, and inhibitory factors [30]. For example, VEGFα can upregulate PD-1 expression on CD8+ T cells, while TGF-β enhances PD-L1 expression on tumor cells [31,32]. In addition, immune-cold tumors could prevent effector T cells from entering into the tumor, in which case the patients are mostly unresponsive to cancer immunotherapy. Moreover, the TME can also recruit immunosuppressive immune cells including regulatory T cells, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages to evade immune clearance [33–35].

Besides, tumor cells could diminish the number of T-cell-recognized neoantigens by reducing their gene expression or losing the mutant allele in a selection process triggered by the pressure of the immune attack [36]. Other mechanisms related to tumor immunoediting include the downregulation of interferon-γ (IFN-γ) or antigen presentation and recruitment pathways [37–39].

Finally, one of the major mechanisms of immunosuppression that occurs in the context of tumorigenesis and tumor growth is the upregulation of multiple inhibitory co-receptors (ICRs) that create a series of interactions on the tumor–stroma interface and within the stroma itself, leading to the blockade of the immune attack and exhaustion of the T cells [12]. T cell exhaustion was first observed in mice infected with certain strains of lymphocytic choriomeningitis virus (LCMV) [40]. Strikingly, these viruses escaped elimination by rapidly inducing most of the CD8+ effector T cells (T_{eff}), thereby resulting in the depletion of this specific antiviral T cell population within a few days and, consequently, persistent infection. The key hallmarks of T cell exhaustion are the expression of ICR, leading to loss of effector functions and failure to transition into the memory T cell pool [41].

Importantly, recent research demonstrated that T cell exhaustion is of central importance in various cancers similarly to the exhaustion occurring during chronic infection. In both cases, chronic antigen stimulation triggers co-expression of high levels of multiple inhibitory receptors, including PD-1, CTLA-4, LAG-3, and T-cell immunoglobulin and mucin domain-3 (TIM-3) [42]. The PD-1/PD-L1 signaling axis plays a predominant role in negative regulation of immune response. For instance, when co-expressed with TIM-3, PD-1 decreases the secretion of various pro-inflammatory cytokines, such as IL-2, IFN-γ, and TNF, and results in T cell tolerance towards tumor cells in acute myelogenous leukemia, colon adenocarcinoma, and melanoma [43–45]. Another clinically relevant ICR is CTLA4 that, as PD-1, but non-redundantly, establishes immune inhibitory interactions for the blockade of the co-stimulation of T cell activation, and it maintains the peripheral immune tolerance. When CTLA-4 and PD-1 are co-blocked in B16 melanoma cells vaccinated with B16-Flt3-ligand (Fvax), these agents synergistically increase the ratio of T_{eff} to T_{reg} and myeloid-derived suppressor cells, as well as the production of T cells that secrete IFN-γ and TNF-alpha. This triggers an inflammatory cascade that enhances tumor rejection and diminishes tumor-induced immune suppression [46]. Given this prominent role of the PD-1/PD-L1 and CTLA4 pathways in cancer immune evasion, anti-PD-1/PD-L1 and anti-CTLA4 drugs, and their combinations, have become the current paradigm of IBC-based cancer immunotherapy.
3. Mechanisms of Clinically Targeted ICR Signaling pathways

3.1. PD-1 Signaling

PD-1 is broadly expressed on T-lymphocytes, B-lymphocytes, antigen-presenting cells, NK cells, and macrophages [47,48]. PD-1 is usually deemed as a dominant inhibitory ICR. Unlike CTLA-4, activation of the PD-1 signaling pathway mainly occurs in the effector phase of the adaptive immune suppression cascade, and it blocks the capacity of cytotoxic T cell to eliminate cancer cells [9].

Binding of PD-1 to PD-L1 moreover blocks CD28 [49] and T cell receptor (TCR) signaling activation and the contact of T lymphocytes and dendritic cells (DCs) [50]. In tumor-associated macrophages, a high PD-1 expression level causes decreased macrophage phagocytosis [48]. Expression of its ligand PD-L1 on tumor cells leads to Teff cytolysis resistance and reduced transcript levels of granzyme A and perforin [51,52]. Activated PD-1 signaling inhibits conversion of Teff into the memory T cell pool by pro-apoptotic activities of Teff with upregulation of BCL-2-interacting mediator of cell death (BIM) [41]. Furthermore, PD-1 signaling paralyses CD4+ and CD8+ T cell motility during exhaustion by stabilizing the immunological synapse formation [53]. Besides, PD-1 promotes the suppression of melanoma antigen-specific CTL that is mediated by CD4+CD25Hi regulatory T cells [54]. This relevance of PD-1 signaling in tumor immunity is exemplified by the identification of PD-1 expression in CD8+ T cells as a biomarker of resident tumor reactive T cell subpopulations in advanced melanoma and cervical cancer patients [55,56].

3.2. CTLA-4 Signaling

In contrast to the broad expression of PD-1, cytotoxic T lymphocyte antigen-4 (CTLA-4) is mainly expressed on Treg cells and controls immunological self-tolerance and Treg-induced immunosuppression [57,58]. On one hand, CTLA-4 inhibits CD28-dependent T cell activation and survival, resulting in reduced levels of IL-2, IL-4, TNF-α, and IFN-γ as well as diminished proliferation of CD8+ and CD4+ T cells [49,59,60]. Also, CTLA-4 interaction with CD80 and CD86 expressed by conventional T cells (Tconv) increases their susceptibility to Treg-mediated suppression [61]. Furthermore, CTLA-4 inhibits CD86/80 expression on DC and impairs antigen priming (initial T cell activation) by excluding Treg physical attachment with DCs and conventional T cells (Tconvs) [62]. In addition, CTLA-4+ CD4+ T cells have shorter-term interactions with DCs under antigen exposure than that of CTLA-4- CD4+ T cells, causing a decrease in IL-2 levels and proliferation [63]. Lastly, CTLA-4 restricts follicular helper T cell (Tfh) differentiation by controlling the level of CD28 engagement [64].

4. Molecular Underpinnings of ICB Failure

Although ICB has revolutionized the therapy of cancer, significant fractions of patients are insensitive, or eventually develop resistance to ICB [39]. Overall, 9% of patients receiving anti-PD-1/PD-L1 monotherapy shows hyper-progressive tumor aggression with poor overall survival [65]. Resistance is generally driven by intra-tumor heterogeneity coupled to selection of resistant cells. Generally, intratumor heterogeneity results in molecularly different cancer cell subpopulations, among which a fraction is insensitive to cancer therapy [66]. With sensitive tumor cells being killed, the surviving resistant cells drive tumor progression. In the context of ICB, this tumor heterogeneity can be particularly important, given that many different tumor intrinsic and stromal factors collude in the final clinical outcome. Tumor heterogeneity has been described for key modulators of the ICB response such as PD-L1 [67], and the neoantigens and tumor clonality has already been reported as a predictor of enhanced response to anti-CTLA4 and anti-PD-1 treatment in NSCLC [68].

Resistance Mechanisms

A crucial tumor-intrinsic factor leading to ICB resistance is the low neoeptope load that generally leads to minimal immune reinvigoration with both CTLA-4 and PD-1/PD-L1 blockade [69–72]. Interestingly, alterations in the epitope or mutation load during treatment with ICB has been reported as
related to response. In the context of NSCLC patients at the moment of response to anti-PD-1 treatment, a reduction of the number of clonal mutations and T cell repertoire evenness is proportional to the response, being 19% the average fraction of remaining variants in those patients with complete and partial response, and 101% the fraction for patients presenting disease progression [73]. Interestingly, the tumor immunoediting induced by anti-PD-1 or anti-PD-1/anti-CTLA-4 therapies has also been related to a loss of dominant mutation-associated neoantigens in initially responding patients that developed acquired resistance, suggesting the further evolution to tumors with diminished immunogenicity [74].

In addition, there are a number of specific genetic and transcriptomic aberrations that have been proposed as candidates for response biomarkers (Table 3). Prototypic oncogenic pathways include amplifications in the MDM2 gene family, alterations in EFGR alterations, which are associated to hyper-progressive disease after anti-CTLA-4 or PD-1/PD-L1 treatment [75], together with alterations that imply the activation of the canonical Wnt/β-catenin signaling pathway [39]. The latter marks a “non-T-cell inflamed” tumor microenvironment. In addition, Wnt/β-catenin signaling could directly inhibit T cell activation [76].

Table 3. Candidate response biomarkers for immune checkpoint blockade.

| Biomarker                                      | Type          | Target of the Test | Cohort Size | Predictive Power | Assay/Predictive Value                                                                 |
|-----------------------------------------------|---------------|--------------------|-------------|------------------|----------------------------------------------------------------------------------------|
| Amount and clonality of TCR repertoire        | Genetic       | Immune             | 25          | p = 0.004        | TCR sequencing                                                                         |
|                                               |               |                    |             |                  | In metastatic melanoma, high clonality of TCR repertoire significantly correlated with clinical response to pembrolizumab treatment [77] |
| Tumor neoantigen clonality                    | Genetic       | Tumor              | 139         |                  | Whole exome sequencing                                                                 |
|                                               |               |                    |             |                  | In melanoma patients treated with ipilimumab or tremelimumab, overall survival was significantly better in tumors with low neoantigen intratumor heterogeneity (ITH) and high clonal neoantigen burden [68] |
| Tumor mutational burden (TMB)                 | Genetic       | Tumor              | 16, 49      | HR = 0.19, p = 0.01 | Whole exome sequencing targeted next generation sequencing                              |
|                                               |               |                    |             | HR = 0.13, p = 0.24 | High TMB associated with clinical benefit [71,78,79]                                  |
| ctDNA                                         | Genetic       | Tumor              | 28          | p = 0.03         | DNA level by next-generation sequencing                                                |
|                                               |               |                    |             | Overall survival, HR = 0.17, p = 0.007 | High value of ctDNA drop indicates good response [80]                                  |
| JAK1, JAK2                                    | Genetic       | Immune             | 4           |                  | JAK1/JAK2 mutation by whole genome sequencing                                           |
|                                               |               |                    |             |                  | JAK1/JAK2 mutation indicates bad response [37,39,81]                                   |
| β2 microglobulin (B2M)                        | Genetic       | Tumor              | 40, 34      | p = 0.009, p = 0.004 | B2M mutation by whole-genome sequencing                                                |
|                                               |               |                    |             |                  | B2M mutation indicates bad response [69]                                               |
| Germinal SNPs −1577G/G and CT60G/G in CTLA4   | Genetic       | Germinal           | 173         | −1577G>A, OR = 0.04 and 0.24 CT60G>A, OR = 0.07 and 0.28 | SNP by genotyping, −1577G>A and CT60G>A indicates good response [82]                     |
|                                               |               |                    |             |                  | SNP by genotyping, −1577G>A and CT60G>A indicates good response [82]                     |
| BRCA1/2                                       | Genetic       | Tumor              | 38          | OR = 6.2, p = 0.002 | BRCA1/2 mutation by whole-genome sequencing                                              |
|                                               |               |                    |             |                  | BRCA1/2 mutation indicates good response [70,82,84]                                     |
| KRAS, TP53                                    | Genetic       | Tumor              | 54          | pTP53 mut = 0.042 and pKRAS mut = 0.003 | TP53 and KRAS mutation by whole genome sequencing                                       |
|                                               |               | (immunotherapy cohort) |            |                  | TP53 and KRAS mutation indicates good response [85]                                     |
| MDM2, EFGR                                    | Genetic       | Tumor              | 155         | OR (MDM2) = 10.8 OR (EGFR) = 8.36 | Targeted sequencing, MDM2/EGFR amplification indicates bad response [75] |

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| Biomarker | Type          | Target of the Test | Cohort Size | Predictive Power | Assay/Predictive Value |
|-----------|---------------|-------------------|-------------|------------------|------------------------|
| rs17388568 | Genetic       | Germinal          | 169         | OR = 0.26, p = 0.0002 | Genotyping by Sequenom MassArray. rs17388568 associated with response [86] |
| FOXPI BS-5mC | Epigenetic   | Immune            | 61          | Progression-free survival, HR = 0.415, p = 0.0063 Overall survival, HR = 0.409, p = 0.0094 | FOXPI methylation by EPIC array and pyrosequencing FOXPI methylation indicates bad response [28] |
| CTLA4, PDCD1 | Epigenetic   | Tumor             | 18          | p < 0.01         | Array-based CpG-methylation assessment |
| 68 genes  | Epigenetic    | Tumor             | 18          | p < 0.05        | Differential DNA methylation pattern between durable clinical benefit vs. no clinical benefit [88] |
| LAMA3     | Transcriptional | Tumor            | 26          | p = 0.003       | RT-PCR In patients with metastatic melanoma, LAMA3 is differentially expressed in regressing versus progressing metastases [89] |
| IFN-γ-associated gene-expression score | Transcriptional | Tumor | 19, 62, 43, 33 | p < 0.05 | Expression score by NanoString gene expression profiling High value of expression level indicates better response [1,90] |
| KRT1, KRT5, KRT10, KRT15, KRT78 (keratin genes) LOR, FLG2, DSC1, DSC3, LGALS7, LAMA3, KLK7 (cell adhesion genes) WNT3, WNT5A (Wnt pathway genes) | Transcriptional | Immune/tumor | 10 | FC ≥ 1.5 | Gene expression by whole genome microarray High values indicate bad response [89] |
| Melanoma Antigen Gene (MAGE)-A cancer-germline antigens | Transcriptional/histopathological | Tumor | 55 | p = 0.011 | Expression of MAGE-A cancer-germline antigens by RT-PCR and IHC. High value indicates bad response [91] |
| PD-L1     | Histopathological | Immune/tumor | 455, 305, 26 | Overall survival, p = 0.06 (≥1% PD-L1), p < 0.001 (≥5% and ≥10% PD-L1), Progression-free survival, p = 0.02 (≥1% PD-L1), p < 0.001 (≥5% and ≥10% PD-L1), Objective response rate, p = 0.02 (≥1%, ≥5% and ≥10% PD-L1); Overall survival HR for death, 0.60, p = 0.005; p = 0.006. | PD-L1 IHC In advanced non-small-cell lung cancer patients treated with Nivolumab, PD-L1 expression predicts overall survival, progression-free survival, and objective response rate, with increasing interaction p-values with increasing % of PD-L1 expression [92] In PD-L1 negative metastatic non-small-cell lung cancer patients, ICB efficacy is equivalent to chemotherapy [93] In advanced non-small-cell lung cancer, first line setting pembrolizumab in monotherapy is correlated with better progression-free survival (PFS) and overall survival (OS) than platinum-doublet chemotherapy, only if PD-L1 expression is equal to or above 50% [94] In metastatic melanoma treated with Pembrolizumab, the responders presented significantly higher numbers of PD-L1+ cells when compared to the patients that progressed (p = 0.006) [77] |
### Table 3. Cont.

| Biomarker | Type | Target of the Test | Cohort Size | Predictive Power | Assay/Predictive Value |
|-----------|------|-------------------|-------------|------------------|------------------------|
| **CD8**  | Histopathological | Immune | 46 | *p < 0.0001* | CD8 IHC In metastatic melanoma treated with Pembrolizumab, the responders presented significantly higher numbers of CD8+ cells when compared to the patients that progressed [77] |
| **PD-1** | Histopathological | Immune | 41 | *p = 0.0002* | PD-1 IHC In metastatic melanoma treated with Pembrolizumab, the responders presented significantly higher numbers of PD-1+ compared to the patients that progressed [77] |
| **Immunoscore** | Histopathological | Immune | 475 | | CD3 and CD8 or CD8 and CD45RO IHC In colorectal cancer patients treated with anti-PD-1, immunoscore is a better response biomarker than microsatellite instability. Multivariate analysis shows a significant correlation of Immunoscore with disease-specific survival, disease-free survival, and overall survival despite their microsatellite status [95] |
| CD63, E-cadherin, CXCL4, CXCL12 | Histopathological/protein | Immune/tumor | 8 | pCD63 = 0.013, pE-cadherin = 0.005, pCXCL4 = 0.04, pCXCL12 = 0.041 | All of them indicate better response [96] |
| **PTEN** | Histopathological | Tumor | 39 | *p = 0.029* | PTEN IHC High value indicates bad response (p = 0.029) [77] |
| Circulating CD8+ T cells | Cellular | Immune | 43 | % survival, HR = 0.21, *p = 0.00063* | Circulating CD8+ T cells by flow cytometry. High value indicates response [98] |
| Circulating monocytic MDSCs (CD14+) | Cellular | Immune | 43 | Overall survival, HR = 2.89, *p = 0.002203* | Circulating monocytic MDSCs (CD14+) by flow cytometry. High value indicates bad response [98] |
| Circulating PD-1+ CD8+ T cells | Cellular | Immune | 25 | *p = 0.02* | Circulating PD-1+ CD8+ T cells by flow cytometry. High value indicates response [99] |
| Neutrophils/lymphocytes ratio | Cellular | Immune | 58 | Overall survival (NLR ≥ 4), HR = 2.2, *p = 0.0009* | Neutrophils and lymphocytes by flow cytometry. High value indicates bad response [100] |
| Circulating Bim+PD-1+CD8+ T cells | Cellular | Immune | 13 | *p < 0.05* | Bim+PD-1+CD8+ T cell by flow cytometry. High value indicates better response [101] |
| Total tumor infiltrating lymphocytes (TILs) | Cellular | Immune | 64 | *p = 0.005* | Total TILs by IHC. High value indicates response [102,103] |
| Total eosinophils | Cellular | Immune | 29 | Progression-free survival *p < 0.0001*, overall survival *p = 0.017* | Absolute eosinophil counts by blood tests. High values indicate better response [104] |
| **Lactate Dehydrogenase (LDH)** | Secreted | Serum | 66 | Overall survival *p = 0.0292* | LDH ELISA. Elevated value indicates bad response [105] |
| sCD25 | Secreted | Serum | 262 | % survival, HR = 1.26, *p < 0.0165* | sCD25 level by sIL-2 Receptor EIA assay. High value indicates bad response [106] |
Table 3. Cont.

| Biomarker             | Type     | Target of the Test | Cohort Size | Predictive Power                          | Assay/Predictive Value                                                                 |
|-----------------------|----------|--------------------|-------------|-------------------------------------------|----------------------------------------------------------------------------------------|
| CXCL11                | Secreted | Serum              | 247         | Overall survival, HR = 1.88, p = 0.014    | CXCL11 level examined by bead-based multiplexed immunoassay. High value indicates bad response [107] |
| CXCL9 and CXCL10      | Secreted | Plasma             | 18          | p < 0.001                                 | CXCL9 and CXCL10 levels examined by ELISA. Levels after anti-PD1 + anti-CTLA4 treatment are higher in responders vs. non-responders [108] |
| C-reactive protein    | Secreted | Serum              | 196         | p = 0.028                                 | CRP by immunofiltration. High value indicates response [109]                                      |

Among the immune-related mechanisms (Figure 1), loss-of-function mutations of Janus kinase (JAKs) desensitize the T cells to the IFN-γ exposure and dramatically decrease the expression level of PD-L1, which is normally transcribed in response to IFN-γ via Signal Transducer and Activator of Transcription (STAT) activation. This decrease in PD-L1 leads to both primary and acquired resistance of PD-1 blockade therapy, given that the reinvigoration capacity of T cells through reactivation of the PD-1/PD-L1 axis has been abrogated [37,110]. Disruption of the IFN-γ pathway can also occur through a transcriptional dysregulation of several genes used to build the “IFN-γ-associated gene expression score”, which indicates to which extent the tumor microenvironment is “T cell inflamed”. This score is predictive of response to pembrolizumab (anti-PD-1 ab), and the lack of IFN-γ-associated gene expression is associated with lack of clinical benefit in ICB treatment of melanoma, NSCLC, and gastric cancers [39]. This scenario is associated with response to treatment with anti-PD-1 antibodies [90], and such transcriptomic signatures are considered both prognostic and predictive [111]. Indeed, impairment of the IFN-γ pathway through knockdown of Ifgr1 after anti-CTLA4 treatment shows enhanced tumor growth and reduced survival in mice [112]. In addition, deleterious mutations in the gene encoding β2 microglobulin (a MHC class I subunit) have also been described in anti-PD-1-antibody-resistant patient samples and cell lines [37,113].

Another immune-related proposed mechanism of resistance to PD-1 inhibition is the propensity of tumor-related PD-1 macrophages to take up the anti-PD-1 monoclonal antibodies, even those that are already PD-1-engaged on the membrane of the PD-1+CD8+ T cells [114]; in such a scenario, the co-receptor PD-1 and PD-L1 interaction between the T and tumor cells cannot not be disrupted and the immune blockade not unleashed.

Finally, upregulation of other co-inhibitory ICs is among other possible causes of acquired resistance in PD-1 blockade [115], as well as in CTLA-4 therapy, where the activation of tumor indoleamine-2,3-dioxygenase (IDO) constitutes an important resistance mechanism that results in suppression of T cells and NK cells in the TME, stimulation of regulatory T cells, and enhancement and expansion of myeloid-derived suppressor cells (MDSCs) [116,117]. Indeed, IDO-deficient mice increase CD4+ and CD8+ effector T cell infiltration in the tumor microenvironment and show better anti-CTLA-4 therapy effects than that of the wild-type [118].
Despite substantial progress in our understanding of immune checkpoints and the development of specific immune checkpoint inhibitors, many patients with immunogenic tumors are insensitive to ICB. In addition to this lack of efficacy, serious adverse effects and high treatment cost incentivize the search for biomarkers that allow a preemptive identification of ICB responders [119]. Combination of static biomarkers obtained in pre-treatment and dynamic biomarkers for monitoring and further clinical stratification are supposed to be incorporated into ICB treatment regimens [120]. Currently, many patients with immunogenic tumors are insensitive to ICB. In addition to this lack of efficacy, serious adverse effects and high treatment cost incentivize the search for biomarkers that allow a preemptive identification of ICB responders [119]. Combination of static biomarkers obtained in pre-treatment and dynamic biomarkers for monitoring and further clinical stratification are supposed to be incorporated into ICB treatment regimens [120].

**5. ICB Response Biomarker Candidates**

Despite substantial progress in our understanding of immune checkpoints and the development of specific immune checkpoint inhibitors, many patients with immunogenic tumors are insensitive to ICB. In addition to this lack of efficacy, serious adverse effects and high treatment cost incentivize the search for biomarkers that allow a preemptive identification of ICB responders [119]. Combination of static biomarkers obtained in pre-treatment and dynamic biomarkers for monitoring and further clinical stratification are supposed to be incorporated into ICB treatment regimens [120]. Currently, many patients with immunogenic tumors are insensitive to ICB. In addition to this lack of efficacy, serious adverse effects and high treatment cost incentivize the search for biomarkers that allow a preemptive identification of ICB responders [119]. Combination of static biomarkers obtained in pre-treatment and dynamic biomarkers for monitoring and further clinical stratification are supposed to be incorporated into ICB treatment regimens [120].

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**Figure 1.** Representative mechanisms of resistance to anti-PD-1 immune checkpoint blockade. (A) A low epitope load in the tumor cells normally drives to a minimal immune reinvigoration due to a lower capacity of antigen presenting cells (APC) to present antigen to T cells (low priming) and a lower cytotoxic T cell recognition of the tumor cell antigen. (B) Loss-of-function mutations of Janus kinase (JAKs) desensitize the T cells to the IFN-γ exposure and dramatically decrease the expression level of PD-L1 through lack of activation of the transcription factor STAT. This decrease in PD-L1 leads to both primary and acquired resistance of PD-1 blockade therapy, given that the reinvigoration capacity of T cell through reactivation of the PD-1/PD-L1 axis is abrogated. (C) Deleterious mutations in the gene encoding β2 microglobulin (an MHC class I subunit) lead to loss of antigen presentation, producing resistance to anti-PD-1 drugs. (D) The propensity of the tumor-related PD-1 macrophages to take up anti-PD-1 monoclonal antibodies causes the capture of the anti-PD-1 antibody even from the surface of the PD-1+CD8+ T cells that already bound the drug. This impedes or reverts the anti-PD-1/PD-1 interaction at the cytotoxic T cell provoking resistance to the treatment. (E) In the “escape” phase of the tumor immunoediting, when the tumor is clinically manifested, tolerogenic dendritic cells, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages secrete indoleamine-2,3-dioxygenase (IDO), which decreases tryptophan and increases kynurenine. These molecules inhibit effector T cells and NK functions and stimulate regulatory T cells, provoking immunosuppression and enhancing the tolerogenicity of macrophages and dendritic cells. IDO1 also enhances the expansion and activation of MDSCs. All previous alterations suppress the activity of anti-tumor effector T cells.

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**Table 1.** Summary of clinical biomarker candidates for ICB response prediction.

| Biomarker | Function | Clinical Value |
|-----------|----------|----------------|
| TMB       | Mutation  | High TMB correlates with better response to ICB |
| PD-L1     | Expression| High PD-L1 expression predicts resistance to ICB |
| MSI       | Genotype | MSI high predicts sensitivity to ICB |
| TME       | Landscape | TME rich predicts sensitivity to ICB |
| ctDNA     | Circulating DNA | ctDNA alterations predict resistance to ICB |

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**Figure 2.** Schematic representation of ICB treatment in different tumor environments. (A) In the “elimination” phase of the tumor immunoediting, the tumor is killed by the immune system due to high antitumor T cell recognition. (B) In the “equilibrium” phase, the tumor is resistant to treatment due to low antitumor T cell recognition. (C) In the “escape” phase, the tumor is resistant to treatment due to high immunosuppression and low antitumor T cell recognition.
response biomarker candidates for ICB have been discovered on several levels, including genomic, transcriptomic, and proteomic levels, along with immunological parameters [121,122] (Table 3).

5.1. Solid Biopsy Biomarker Candidates

Clinical biomarkers that might be useful for the prediction of ICB response have been uncovered at different biological levels (cellular, protein, transcript, gene), in different locations (tumor, peripheral blood), and associated to the biology of both immune and tumor-related cell populations.

5.1.1. Genetic and Epigenetic Markers

Various studies have addressed the relationship between overall tumor mutation load/neoantigen burden and ICB response in NSCLC and melanoma, in the context of anti-PD-1 and anti-CTLA-4 monotherapy [69–72]. Importantly, neoantigen burden was more strongly associated with tumor immunogenicity than mutation load, reflecting the selection process of the tumor antigens that are finally recognized by the T cell receptors and trigger a sufficient immune activation. Thereby, specific genetic mutations in genes, such as JAK1/2 and BRCA1/2, emerge to predict ICB clinical efficacy, likely due to the failure to activate the IFN-γ target genes and the increased mutation load in tumors deficient in the DNA repair machinery, respectively [37,70,110,123]. Loss-of-function mutations of JAK family members render melanoma resistant to IFN-γ stimulation with insensitivity to IFN-γ-triggered growth arrest [37] and potentially down-regulate PD-L1 expression, which is a possible mechanism for desensitization towards PD-1 blockade [110]. Indeed, melanomas with mutations in IFN-γ signaling are resistant to anti-CTLA-4 blockade [112]; also, this pathway is activated in responders to anti-PD-L1 treatment [124]. In addition, IFN-γ-induced IDO expression is increased in melanoma patients responding to CTLA-4 and PD-L1 blockade [102,124]. Mutations in the DNA double strand repair enzyme BRCA2 result in drastically increased mutational burden, leading to increased responsiveness to PD-1 blockade [70]. In relation with this, mismatch repair deficiency tumors of different origins with germline alterations of MSH2, MSH6, PMS2, or MLH1 were associated with high neoantigen burden, and indicative of recognition by tumor-specific T cells [83]. However, similar mutation and neoantigen profiles were observed in responders and non-responders under ICB treatment [89,110,125]. Other genetic variants that can constitute ICB response biomarkers are the genotypes 1577G/G and CT60G/G in CTLA4, which demonstrate a favorable overall response (OS) in patients receiving anti-CTLA4 therapy [82]. Also, higher TCR clonality identified by sequencing of the β-chain of the TCR is observed in responders to PD-1 blockade rather than CTLA-4 blockade [77,125]. With respect epigenetic biomarkers of response, we reported for the first time a signature of DNA methylation in 301 CpGs, EPIMMUNE, that could be downscaled to the unmethylated state of a single CpG site in the transcription factor FOXP1, which regulates both quiescence in naive CD4+ cells [126], and Th follicular cells [127], as predictive of response to ICB in NSCLC patients [28].

5.1.2. Transcriptional Biomarkers

Transcriptional signatures can be also informative of response to PD-1 blockade, and in particular when DNA mutation profiles and immunological features are similar [89]. Several gene expression signatures related to IFN-γ [1,90] and the Wnt/β-catenin signaling pathways [39] have been associated to response to ICB. Other signatures associated with clinical outcomes after ICB introduce novel putative resistance mechanisms such as the action of extracellular matrix components like laminins, which might create a barrier excluding immune cells from penetrating the tumor and thus impairing immunotherapy, or the neutrophil infiltration or activation in progressing [89]. Another recently reported panel of expression markers has proven useful to associate survival advantage with moderate tumor proliferation in comparison with tumors that are highly/poorly proliferative in NSCLC patients treated with ICB [128]. Also, expression of endogenous retroviruses (ERV) RNA is correlated with clinical response to anti-CTLA-4 and PD-L1 treatment [129]. Finally, a novel expression signature based on the overexpression of MAGE-A cancer germline antigens has been reported as a putative specific
predictor of resistance to anti-CTLA4 treatment. The expression of these germline antigens is typically restricted to immune-privileged gonadal tissues and several types of tumors, and the MAGE-A types are often targets of anti-tumor T cells in melanoma [91].

5.1.3. Histopathological Biomarkers

At the protein level, histopathological biomarkers include PD-L1 expression, which is a biomarker candidate during anti-PD-1 and PD-L1 monotherapy in the context of melanoma, NSCLC, renal cell carcinoma (RCC), and bladder cancer [130], and several other proteins, in their majority marking the presence of relevant immune cell populations to the efficacy of ICB. PD-L1 so far is the only ICB biomarker for which FDA has approved a companion test for pembrolizumab (anti-PD-1) treatment in patients with NSCLC, gastric or gastroesophageal junction adenocarcinoma, cervical cancer, and urothelial carcinoma (PD-L1 IHC 22C3 pharmDx).

5.1.4. Cellular Biomarkers

Indeed, the immune cell panorama within the tumor differentiates clinical response and resistance in both CTLA4 and PD-1 blockade [131]. PD-1 intratumor CD8+ T cell density prior to therapy indicates shrinking radiographic tumor size [77]. More specifically, within the tumor-infiltrating CD8+ T cells, the presence of the particular population of CD8+ T cells of PD-1+CTLA4+T cells is associated with progression-free survival (PFS). Melanoma patients with frequency higher than 20% have a PFS of 31.6 months, while those with less than 20% have a PFS of 9.6 months [132]. These cells represent the tumor-associated T cells with a partial exhaustion phenotype; hence, they are more susceptible to reinvigoration via the blockade of the co-inhibitory receptor interaction. On the other hand, the rescue of CD8+ T cells alone is not necessarily correlated with clinical response. However, when related to tumor burden, circulating rejuvenated PD-1+Ki67+CD8+ T cells are a better predictor of PFS after PD-1 blockade than rejuvenated cell counts alone [99]. Also, the ratio of CD8+ T cells and Treg cells is linearly associated with tumor necrosis in CTLA-4 blockaded melanoma [133].

5.2. Liquid Biopsy Biomarker Candidates

Circulating components are of great potential for the identification of response biomarkers that can be tested non-invasively and dynamically in bodily fluids [134]. So far, the detection of circulating free DNA (cfDNA) provides clinical guidance for multiple cancer treatment strategies [135]. The mutations identified in cfDNA constitute a reliable surrogate of tumor biopsy, and the increase of post-treatment cfDNA levels might be related to progressive disease in melanoma patients. Moreover, cfDNA levels are informative of response before its clinical manifestation and predict the tumor burden in melanoma patients treated with ICB [136]. Also, copy number instability quantified in cfDNA predicts disease progression and shows better overall accuracy than cfDNA concentration alone in patients with diverse tumors treated with immunotherapy [137]. In addition, a recent proof-of-concept work demonstrated the added value of screening 5mC and 5hmC variants in cfDNA for the diagnosis and prognosis of several types of cancers [138].

Several recent studies also indicate that the detection and quantification of circulating tumor cells (CTCs) can be considered a promising circulating biomarker candidate in ICB. A recently published case report [139] associated the detection of CTCs in peripheral blood with the metastatic process. In addition, PD-L1 was highly expressed in CTCs in advanced head and neck cancer patients, indicating that PD-L1+ CTCs could serve as a predictive biomarker of ICB response.

Other proposed biomarkers of response include several proteins and cell populations such as serum level of interleukin-8 (IL-8), which is secreted by the tumor and is inversely correlated with OS in NSCLC and melanoma patients under PD-1 blockade [140]. Serum baseline and post-treatment level of angiopoietin-2 is also inversely related to OS in both anti-CTLA4 and PD-1 therapy [141].

Proteins that belong to the ICR pathways have also been detected in liquid biopsies and are correlated with response; patients with higher ICB pre-treatment soluble PD-L1 levels are more likely
to progress, while interestingly the post-treatment increase of PD-L1 level is correlated with partial response in ICB [142]. Co-inhibitory IC T cell immunoglobulin mucin 3 (TIM3) and PD-1 and IL-15 serum level are negatively associated with long survival after CTLA-4 blockade, in which context, IL-15 increases TIM3 and PD-1 expression [143].

With regards to circulating immune populations, it has been reported that the amount of circulating PD-1$^\text{+}$ CD4$^\text{+}$ Teff cells is inversely related to OS in anti-CTLA-4 treated prostate cancer patients, with no significant difference in the case of PD-1$^\text{+}$ CD8$^\text{+}$ T cells [144]. Also, pre-treatment levels of circulating CD45RO$^\text{+}$CD8$^\text{+}$ T cells are positively correlated with patient survival after CTLA-4 blockade. In addition, a higher fraction of circulating CD4$^\text{+}$ICOS$^{\text{hi}}$ T cells indicates longer survival after CTLA-4 blockade [145]. Low baseline LDH level, high relative/absolute eosinophil counts, and relative lymphocyte counts are correlated with prolonged OS in melanoma anti-PD-1 and CTLA-4 treatment [146,147]. Finally, possibly because the BIM level positively reflects PD-1 expression and PD-1/PD-L1 interaction, an increased frequency of circulating Bim$^\text{+}$PD-1$^\text{+}$CD8$^\text{+}$ T cell has been found to be correlated with anti-PD-1 efficacy [101].

6. DNA Methylation and Hydroxymethylation as Potential Biomarkers of Response to Cancer Immunotherapy

6.1. Involvement of DNA Methylation and Hydroxymethylation in Tumor Immune Evasion

The fact that 5mC and 5hmC are dynamic marks that correlate with tumor immune evasion and T cell exhaustion opens new avenues for clinical biomarkers research, where epigenetic variants or signatures can represent a new class of biomarkers for ICB response.

In the context of T cell exhaustion, it can be hypothesized that DNA methylation can be involved in the maintenance and reinforcement of exhaustion gene expression signatures. Indeed, a progressive Dnmt3a-mediated de novo methylation has been observed in murine antigen-specific CD8 T cells that underwent exhaustion via repression of key genes implicated in the effector function, the proliferation, metabolism, and tumor recruitment of immune cells, impairing T cell expansion and clonal diversity under treatment with anti-PD-1 [148]. These findings are corroborated by observations in chronic lymphocytic choriomeningitis virus (LCMV)-infected mice, where the preservation of a specific chromatin configuration was associated with a transient reinvigoration of T cells induced by treatment with PD-1 [149]. This reinvigoration was likely mediated mainly by NFκB signaling, and the preserved chromatin configuration that sustains the transient nature of the anti-tumor immune reactivation could be related to the post-treatment static expression of key transcription factors implicated in the exhaustion phenotype such as T-bet and Eomes [149]. Indeed, in primary human CD4$^\text{+}$ T cells that were TCR-stimulated in vitro using antibodies against CD3 and CD28, the genomic binding regions of specific enhancers and transcription factors involved in the activation of the T cells overlap with regions of accessible chromatin in the post-treatment remodeled scenario [150]. Interestingly, in some individuals, these regions have mutations associated with autoimmune diseases and enhancer specific of different T cells. In addition, correlations between specific SNPs and local regions of accessible chromatin have been delineated, raising the possibility that interindividual genetic variation is affecting the chromatin remodeling after treatment with ICB [150]. Combined, the highlighted studies provide evidence of an important role of epigenetic mechanisms in relapse risk of patients post ICB therapy [151,152].

The involvement of DNA methylation in the transcriptional reprogramming of the T cells has also been reported in the context of T cell exhaustion following viral infections, where the PD-1 promoter undergoes extensive de-methylation resulting in permanent CD8$^\text{+}$ T cell exhaustion [153]. By contrast, in the acute setting, it is subsequently re-methylated in the transition of Teff to Tmem [153]. Ten-eleven translocation (TET) dioxygenases-dependent oxidation of DNA methylation has been related to reprogramming processes in differentiated cells [154]. Indeed, this form of active de-methylation is underlying the dynamics of 5mC and 5hmC in the promoter of the murine gene coding for PD-1 (Pdcd1) in CD4$^\text{+}$ autoimmune T effector cells. The deposition of 5hmC seems to mark a poised state. Only in
the context of permanent induction of PD-1 provoked by peptide immunotherapy, 5hmC is erased in mouse [155]. In view of this information, 5hmC constitutes and good candidate for monitoring the phenotypic reprogramming of T\text{eff}\ cells during exhaustion or ICB resistance.

Regarding the tumor-intrinsic reprogramming that features the immunosuppressive TME, it has been reported that DNA methylation at the promoter regions of the tumor Th1-type chemokines CXCL9 and CXCL10, mediated by DNMT1, represses their transcript and protein expression in ID8 ovarian cancer in C57/BL6 mice. Consequently, cytotoxic T cell trafficking into the tumor microenvironment decreases. Interestingly, the epigenetic modulation of the expression of these chemokines with azacytidine indicated that the modification of the epigenetic program can improve the T\text{eff} infiltration as well as the response to anti-PD-L1 agents [156]. Another tumor-intrinsic adaptation against the immune anti-tumor activity is the DNA methylation-induced repression of tumor-specific antigens [157]. For example, promoter hypermethylation of cancer/testis antigens abrogates the tumor immunogenicity by nullifying the recognition and response of antigen-specific CD8\text{+} T cells [158–160]. On the other hand, de-methylation increases the level of endogenous retrovirus double-stranded RNA and triggers the activation of the MDA5/MAVS signaling pathway, which stimulates immune-related transcription factors and IFN response, and reduces the tumor growth [161,162].

As highlighted above, epigenetic alterations associated with immune response and evasion in immune cells are extensive, and they set the stage for the identification of 5mC and 5hmC biomarkers of response. Specifically, Tet2 controls the differentiation of naïve CD4\text{+} T cells into several lineages of helper T (Th) cells in mice, thereby directly modulating cytokine production [163]. Moreover, Tet2 has been found to contribute to CD8\text{+} T-lymphocyte effector differentiation [164]. The important role of TET-mediated active de-methylation is furthermore exemplified by its direct control of Foxp3 expression in T\text{reg}, where demethylation gives rise to lineage-specific epigenetic signatures that guide development and maturation of Foxp3\text{+} T\text{reg} within the thymus [165]. In addition, TET activity modulation is related to the maintenance of Foxp3 expression [166], and active de-methylation of the IL2 promoter coincides with increased IL2 expression upon CD4\text{+} cell activation [167].

Even though the field is prolific in determining the roles of 5mC and 5hmC profiles in different tumor immune evasion scenarios, it is important to emphasize that the mechanisms underlying these associations are, so far, poorly understood. Scharer and collaborators identified a step-wise differentiation process of CD8\text{+} T cells triggered by antigen presentation, in which inactive genes, such as Pdcd1 in naïve cells, were progressively demethylated towards CD8\text{+} T\text{eff}-cells [168]. These transitions would start by the DNA binding of transcription factors that do not contain any CpGs in their binding site (e.g., NFATc1). They would induce histone H3 and H4 acetylation, as well as DNA de-methylation. The generated open chromatin landscape is permissive to the binding of DNA methylation-sensitive transcription factors that direct the rewiring of the expression towards the effector phenotype (e.g., Pdcd1). Interestingly, the DNA methylation-sensitive transcription factors c-JUN, JUND, c-MYC, CREB/ATF, CTCF, and ETS1 are expressed ubiquitously in differentiating CD8\text{+} T cells [169].

6.2. Emerging Evidence Supporting the Roles of DNA Methylation and Hydroxymethylation as Epigenetic Predictors of ICB Response

The appreciation of the important role of the 5mC and 5hmC landscape has given rise to the emerging discipline of pharmacoepigenetics. Particularly in tumor cells, epigenomic patterns undergo substantial changes, which has resulted in the discovery of an increasing repertoire of epigenomic biomarkers. For an overview of this field, we refer the interested reader to recent comprehensive reviews [170–174]; however, we would like to emphasize how such epigenetic alterations, when associated to ICB response, could serve useful for monitoring the clinical benefit during the course of the disease by agglutinating in Table 4 the most relevant DNA methylation non-invasive cancer biomarkers. It is important to note that the great majority of identified cytosine methylation biomarkers, so far, pertain to DNA methylation, at least in part because methodologies that discriminate 5mC from
5hmC have only been recently developed [175,176]. By adapting the newly arising technologies that allow distinct typing of 5mC and 5hmC, we have characterized the absorption, distribution, metabolism, and excretion (ADME)-related methylole and hydroxymethylome of the human liver [177], and we have reported a proof-of-principle study where specific 5hmC mapping unmasks a unexpectedly high degree of hypermethylation in human hepatocellular carcinoma tumors and contributes to the identification of novel diagnostic biomarkers [178].

Concerning the epigenetic biomarkers of response to ICB, we have recently reported the association of the methylation state of 301 CpGs that conform to the “EPIMMUNE” signature, and its subrogation to the unmethylated state of a single CpG of FOXP1, a transcription factor involved in the regulation of quiescent CD4⁺ cells, and the regulation of follicular T helper cells, with overall and progression-free survival to anti-PD-1 treatment in NSCLC patients [28]. We speculate that the release of the immunosuppression that had been induced by the PD-1/PD-L1 interaction would give rise to the activation of a remaining pool of naïve CD4⁺ cells and the subsequent enhancement of the anti-tumor immune activity. Interestingly, the most amply studied response predictors, such as CD8, PD-L1 immunodetermined proteins, and the tumor mutational burden, did not separate significantly those patients with better treatment outcome. This constitutes the first reported association of epigenetic variants with the clinical benefit of ICB.

To our knowledge, so far, no specific 5hmC biomarker of response to cancer therapy has been validated, although several lines of evidence suggest the involvement of TET enzymes in the response mechanisms; for example, TET1 knockdown in lung cancer cell lines with EFGF mutations leads to enhanced EFGFR inhibitor resistance, while the responsive tumors show increased TET1 expression [179]. As discussed above, epigenetic remodeling of 5mC and 5hmC signatures controls many aspects of the reprogramming events associated with innate and acquired resistance to ICB, both tumor-intrinsic and extrinsic. Indeed, DNA methylation seems to control PD-1, PD-L1, PD-L2, and CTLA-4 gene expression; when these genes are silenced, the antigen presentation and the immune cytotoxic effects are inhibited [180,181]. The hypermethylation-derived silencing of CTLA-4 and PD-1 was also observed in baseline tumor biopsies compared to their pair-matched tissues in NSCLC patients [87]. In addition, in colorectal cancer, PD-L1 expression is associated with CpG island hypermethylation in a subpopulation of BRAF V600E carriers with high infiltration of CD3⁺ T cells [182]. Moreover, in metastatic melanoma patients treated with CTLA-4 blockers, responders and non-responders have a differential pattern of DNA methylation in specific genes of the nervous system development and neuron differentiation pathways [88]. Because neuron and melanocytes have precursor cells of neural crest origin, these results suggest a process of de-differentiation of the transformed melanocytes refractory to ICB treatment. Noteworthy, de-differentiation induced by inflammation has been already reported as a possible tumor immune evasion mechanism [183].

Stepwise hypermethylation has also been related to the facilitation of tumor escape by repressing expression of the IFN regulator IRF8 [184]. Importantly, the positive effect of de-methylation on transcriptional activity for some immune-related genes, including PD-L1 and genes of the interferon signaling cascade, has been validated in vitro, corroborating that epigenetic modulation might be a useful tool to sensitize patients to anti-PD-L1 ICB, and the facilitation of tumor escape by repressing IFN regulatory factor 8 transcriptional expression [185]. Importantly, the positive effect of de-methylation on transcriptional activity for some immune-related genes, including PD-L1 and genes of the interferon signaling cascade, has been validated in vitro, corroborating that epigenetic modulation might be a useful tool to sensitize patients to anti-PD-L1 ICB [186]. In addition, in a mouse ovarian cancer model, de-methylation triggers the type I interferon signaling pathway, sensitizing mice to anti-CTLA4 therapy [161]. Furthermore, azacytidine and CTLA-4 mAb combination therapy represses tumor growth more strongly than each of the monotherapies, via the upregulation of MHC class I components as the putative mechanism [187]. Other studies also identify the increase of the lymphocyte infiltration and the T helper 1-type chemokines and cytokines as possible cause of the better observed outcome when de-methylation and anti-PD-L1 and CTLA4 agents are used in combination in a murine ovarian cancer model [188]. Interestingly, the inverse interaction between immune signaling pathways and
epigenetic regulation in cancer has also been observed, where NF-κβ interacts with TET1 promoter for its downregulation in breast cancer cells [189].

As a consequence of this ample pre-clinical evidence and the appreciation that epigenetic reprogramming participates in acquired drug resistance, there is a drastic increase of clinical trials that explore the synergy of epidrug combination therapies [170,190]. Indeed, demethylating agents and histone deacetylases are being combined with ICB in numerous clinical trials and types of malignancies. Sun and collaborators have recently reviewed the current clinical trials that combine histone modifications inhibitors with immunotherapy. The majority of combinations include anti-PD-1 drugs and histone deacetylases. Some suggested mechanisms for the synergy of the combinations in enhancing the response and preventing the relapse are the upregulation by inhibitors of histone deacetylases of CD80 and CD86 in the context of anti-CTLA-4 treatment, the regulation of immune checkpoint ligands, and the induction of tumor neoantigens on tumor cells for PD-1/PD-L1 therapy. Consistently, most of the combinatorial strategies with DNA-demethylating and histone modification inhibiting drugs aim at the upregulation of tumor neoantigens and the downregulation of PD-L1 expression. Also, BET/bromodomain 4 inhibitors promote depolarization of macrophages into immunostimulatory ones, leading to the decrease of MDSCs in the tumor microenvironment [191].

### Table 4. Examples of the relevancy of DNA methylation alterations as non-invasive diagnostic and prognostic biomarkers in cancer.

| Type of Biomarker | Gene        | Type of Cancer | Description                                                                 | Accuracy of Panel Including Methylated Gene or p Value |
|-------------------|-------------|----------------|-------------------------------------------------------------------------------|-------------------------------------------------------|
| Diagnostic        | ARF         | Bladder        | Urine ARF promoter detects bladder cancer [192]                              | Δ82%/96%                                               |
| Prognostic        | APC, GSTP1  | Prostate       | Serum methylation of CDH13 was significantly associated with advanced tumor stage, worse survival outcome and relative risk of death [195] | HR 6.132 (95%CI: 3.160–12.187) p = 0.0073             |
| Diagnostic        | BCL         | Bladder        | Urine sediments BCL methylation detects bladder cancer [194]                 | + 78% (29/37)                                         |
| Diagnostic        | CDKN2A      | Bladder        | Urine sediments CDKN2A promoter detects bladder cancer [192]                 | Δ82%/96%                                               |
| Diagnostic        | DAPK        | Bladder        | Urine sediments DAPK methylation detects bladder cancer [194]                | + 78% (29/37)                                         |
| Diagnostic (early)| ERα         | Prostate/blast (primary) | Serum promoter ERα methylation detects early stage prostate and breast cancer [196,197] | Δ75%/70%                                              |
| Diagnostic (early)| ERβ         | Prostate       | Serum promoter ERβ methylation detects early stage prostate cancer [196]     | Δ75%/70%                                              |
| Diagnostic        | FBN1        | Colorectal     | Stool FBN1 methylation detects colorectal cancer [198]                       | Δ84.4%/93.3%                                           |
| Diagnostic        | FBN2        | Colorectal (primary) | Serum methylation of FBN2 detects colorectal cancer in males and hepatic metastasis [199] | Male: p = 0.0167, hepatic metastasis: p = 0.0001 |
| Diagnostic, Prognostic | GSTP1   | Bladder/prostate/castrate-resistant prostate/breast | Urine/serum GSTP1 is hypermethylated in prostate cancer and strongly correlated to adverse pathological features [193,206,201] | Δ82%/96%−Δ82% (28/34)/Δ75%/98%+ Δ6% 7120+/Δ22% 22/101 |
| Diagnostic        | FHIT        | Ductal breast cancer | Serum FHIT is associated with breast cancer [202]                          | p < 0.05                                              |
| Diagnostic        | hMLH1       | Breast         | Serum hMLH1 detects breast cancer [203]                                      | AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN) |
| Prognostic        | HULF        | Colorectal     | Serum HULF methylation is associated with increased risk of recurrence [204] | HR 2.7 (95%CI: 1.2–6.6) p = 0.014                     |
| Diagnostic        | HOXD13      | Breast         | Serum HOXD13 detects breast cancer [203]                                     | AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN) |
| Type of Biomarker | Gene      | Type of Cancer | Description                                                                 | Accuracy of Panel Including Methylated Gene or p Value |
|------------------|-----------|----------------|-----------------------------------------------------------------------------|-------------------------------------------------------|
| Diagnostic (early) | SMCAM    | Prostate       | Serum promoter SMCAM methylation detects early stage prostate cancer [196]     | Δ75%/70%                                              |
| Diagnostic       | MGMT     | Bladder/lung/Colorectal | Clinical response to dacarbazine is restricted to those with MGMT hypermethylation in colorectal cancer [205] | Δ82%/96%                                              |
| Diagnostic       | NID2     | Bladder (primary) | Urine NID2 methylation detects primary bladder cancer [206]                  | + 94% (466/496)                                       |
| Diagnostic       | P16      | Breast         | Serum P16 detects breast cancer [203]                                       | AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN) |
| Prognostic       | PCDH10   | Prostate       | PCDH10 methylation in serum is an independent predictor of worse biochemical recurrence-free survival and overall survival [207] | HR 2.796 (95%CI: 1.431–6.763), p = 0.006            |
| Diagnostic       | PCDH17   | Bladder        | Urine sediment PCDH17 methylation detects bladder cancer [208]               | Δ90%/93.96%                                           |
| Diagnostic       | PHACTR3  | Colorectal     | Stool PHACTR3 methylation detects colorectal cancer [209]                    | Sensitivity: 55%–66%; specificity: 95%–100%          |
| Diagnostic       | POU4F2   | Bladder        | Urine sediment POU4F2 methylation detects bladder cancer [208]               | Δ90%/93.96%                                           |
| Diagnostic       | TERT     | Bladder        | Urine sediments TERT methylation detects bladder cancer [194]                | + 78% (29/37)                                         |
| Diagnostic       | TMEFF2   | NSCLC          | Higher frequency of TMEFF2 methylation in tumors without EGFR mutations than those harboring EGFR mutations [210] | Multivariate adjusted odds ratio = 7.13 (95%CI: 2.05–24.83), p = 0.002 |
| Diagnostic (early) | RARB    | Prostate       | Urine sediments RARB methylation detects early stage prostate cancer [211]   | + 82% (28/34)                                         |
| Diagnostic       | RARβ2    | Breast         | Serum RARβ2 promoter methylation as part of a methylation/specific PCR assay detects breast cancer [203] | + 6% 7/120/122% 22/101 |
| Diagnostic (early) | RASSF1  | Prostate       | Urine sediments RASSF1 methylation detects early-stage prostate cancer [211] | + 82% (28/34)                                         |
| Diagnostic, Prognostic | RASSF1a | Breast/lung/ovarian | Serum RASSF1a promoter methylation as part of a methylation/specific PCR assay detects breast cancer [203] | AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN); 6% 7/120/122% 22/101 |
| Diagnostic, prognostic | SEPT9, TAC, CEA | Colorectal | Serum SEPT9 methylation predicts colorectal cancer; Epstein Colon 2.0 with 2/3 algorithm is the most effective assay [212]. In postoperative serum, SEPT9, CEA or TAC methylation predict recurrence and survival [213] | (Diagnostic) Sensitivity = 0.71, Specificity = 0.92; AUC = 0.88. (Prognostic) Disease-free survival: adjusted hazard ratios of the Δ = 2.58–4.71 p < 0.05; recurrence: sensitivity = 32.6–90%; specificity = 80–90 |
| Diagnostic       | SFN      | Breast         | Urine sediment SFN methylation detects bladder cancer [194]                 | AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN) |
| Diagnostic       | SNCA     | Colorectal     | Stool SNCA methylation detects colorectal cancer [196]                      | Δ84.3%/93.3%                                          |
| Prognostic       | SST      | Colorectal     | High serum SST methylation is an independent prognostic biomarker of colorectal cancer [214] | Multivariate adjusted for cancer-specific survival: HR 1.96 (95%CI: 1.06, 3.62) p = 0.031; for overall survival HR 2.60 (95%CI: 1.37, 4.94) p = 0.003 |
| Diagnostic       | TWIST1   | Bladder (primary) | Urine TWIST1 methylation detects primary bladder cancer [206]                 | + 94% (466/496)                                       |
| Diagnostic, prognostic | VIM     | Colorectal     | Serum VIM methylation correlated with liver metastasis, peritoneal dissemination, and distant metastasis [215] | (Liver metastasis) p = 0.026 (Peritoneal dissemination) p = 0.0029 (Distant metastasis) p = 0.0063 |
### Table 4. Cont.

| Type of Biomarker | Gene | Type of Cancer | Description | Accuracy of Panel Including Methylated Gene or $p$ Value |
|------------------|------|----------------|-------------|-----------------------------------------------------|
| Prognostic       | mir-34b | Colorectal | Mucosal wash fluid mir-34b methylation is associated with invasiveness [216] | Accuracy: 91.3% for the training set and 85.1% for the test set. |
| Prognostic       | MGMT  | Glioblastoma multiforme | Serum and tumor methylation of MGMT is associated with better stable response [217] | Median time to progression: log-rank test, $p = 0.006$, 29.9 weeks with methylated MGMT, 95%CI, 24.3–35.4) vs. 15.7 weeks with unmethylated MGMT (95%CI, 14.3–17.2). |
| Diagnostic, Prognostic (early) | Panel of 6 genes (CDO1, HOXA9, AJAP1, PTGDR, UNCX, and MARCH11) | Lung | Methylation status in the 6 genes analyzed in serum for the detection of stage IA NSCLC. In addition, a prognostic risk category based on the cancer and serum methylation status of CDO1, HOXA9, PTGDR, and AJAP1 refined the risk stratification for outcomes as an independent prognostic factor in early-stage disease [218] | (Serum) Sensitivity: 72.1%; specificity: 71.4%. Combination methylation marker multivariate adjusted $p = 0.035$ |
| Prognostic       | BRMS1 | Lung | Cell-free DNA circulating BRMS1 promoter methylation has a statistically significant influence both on operable NSCLC patients’ disease-free interval (DFI) time and OS and on advanced NSCLC patients’ PFS and OS [219] | Multivariate analysis: for progression-free survival: HR 1.951 (95%CI: 1.175–3.238) $p = 0.01$; for overall survival: HR 2.057 (95%CI: 1.247–3.386) $p = 0.005$ |
| Prognostic       | SOX17 | Lung | SOX17 promoter methylation in plasma cell-free DNA has a statistically significant influence on advanced NSCLC patient overall survival [220] | Univariate analysis for overall survival: HR 1.834 (95%CI: 1.105–3.045) $p = 0.019$ |

† Overall detection level.

### 7. Future Perspectives and Conclusions

The reactivation of the anti-tumor immune response with antibodies that compete with the co-inhibitory immune receptor could be intuitively considered an Achilles heel for tumor immunoediting and evasion from immune surveillance. However, given the absence of good response biomarkers and the complex network of interactions of the TME that influences the efficacy of ICB, a significant fraction of patients experience innate and acquired resistance, and some even hyperprogression. As we have extensively described in this review, much effort has been devoted to the identification of biomarkers that could predict response to ICB. Lately, new approaches use top-down strategies and Next Generation Sequencing to identify novel tumor-intrinsic and -extrinsic mechanisms. However, despite the importance of epigenetic regulation for reprogramming events during tumor immune evasion, only a single study has reported the identification of CpG-site specific epigenetic biomarkers of response to ICB in human samples [28]. Furthermore, DNA methylation stands as the putative mechanism for the maintenance of the exhaustion gene expression program during ICB. Therefore, we anticipate that the search for 5mC and 5hmC signatures associated with differential clinical outcomes of ICB will reveal new biomarkers and give rise to novel mechanistic hypotheses that could be integrated in multimics prediction algorithms to further personalize cancer immunotherapy.

### Author Contributions:

Q.X. drafted and edited the main text, Tables 1–3. A.N. critically revised and complemented the main text. P.P. performed a language revision of the main text and drafted Table 4. M.C. and M.-A.B.-G. complemented the information on clinical trials and outcome. V.M.L. critically revised the review. V.M.L., I.B., and E.A. participated in the design and coordination of the review. I.B. critically revised, complemented the work, and approved the final content. All authors have read and agreed to the published version of the manuscript.

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