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Lead Guest Editor: Carmen Criscitiello
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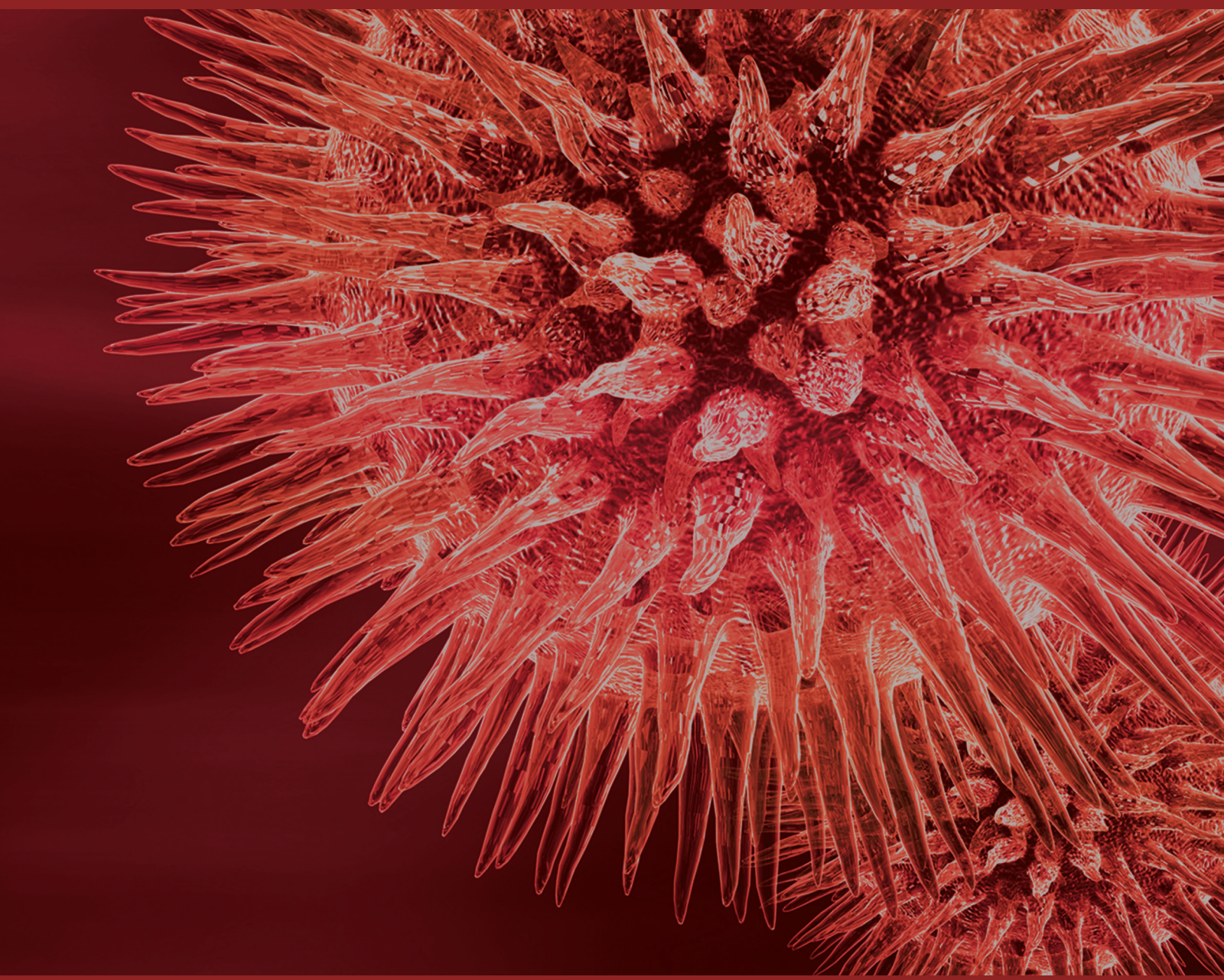

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*Editorial*

# Cancer Immunotherapy and Identification of Prognostic and Predictive Biomarkers

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The immune system is naturally able to detect and destroy abnormal cells thus preventing the development of many cancers. Nevertheless, sometimes cancer cells may avoid detection and destruction by the immune system. Cancer cells may decrease the expression of tumor antigens on their surface, making it harder for the immune system to detect them, express on their surface proteins that induce immune cell inactivation, induce cells in the microenvironment to release factors that suppress immune responses, and promote tumor cell proliferation and survival. In the past few years, the field of cancer immunology has rapidly advanced and led to several new treatment strategies for cancer patients, which may increase the strength of immune responses against tumors. Immunotherapies may work by either stimulating the activities of specific components of the immune system or by counteracting signals produced by cancer cells that suppress immune responses. These advances in cancer immunotherapy have been made possible thanks to huge long-term investments in basic research on the immune system. But, the research in the immunotherapy field is still extremely active. There are still many open questions requiring additional intense research to understand why immunotherapy is effective only in a minority of cancer patients. Prognostic and predictive biomarkers are needed to identify those patients who will derive the greatest benefit from an immunotherapy-based approach. In addition, ongoing research is focusing on expanding the use of immunotherapy to more cancer types. Another crucial area of research aims at increasing the effectiveness of immunotherapy by combining it with other anticancer treatments, such as targeted therapy, chemotherapy, and radiation therapy as well as combinations of different types of immunotherapy agents. In the last year, the US Food and Drug Administration (FDA) approved the first adoptive cell immunotherapy, the so-called chimeric antigen receptor (CAR) T-cell therapy, and granted its first tissue/site-agnostic approval (i.e., a treatment working against different types of cancers that share a common genetic abnormality) for a drug [1]. Indeed, pembrolizumab was approved for use in adult and pediatric patients with locally advanced or metastatic solid tumors that are mismatch-repair deficient or microsatellite instability-high (MSI-H) who have progressed after prior treatment and who have no satisfactory alternative treatment options [2]. This represents a paradigm shift in cancer drug approvals, highlighting the concept that a biomarker may define the disease better than the site.

The first paper of this special issue reports current evidence and future perspectives for advanced urothelial cancer patients treated with immunotherapy. The second paper analyzes current tissue molecular markers in colorectal cancer. The third paper highlights the importance of mismatch repair deficiency as a predictive biomarker for immunotherapy efficacy. Gastric cancer is the topic of the fourth and fifth articles of this special issue: the fourth one reviews preclinical and clinical recent development of immunotherapeutic strategies in gastric carcinoma and the fifth one summarizes which are the diagnostic, predictive, prognostic, and therapeutic molecular biomarkers in third

millennium for this disease. The sixth published paper studies heterogeneous periostin expression in different histological variants of papillary thyroid carcinoma. The seventh paper tries to foresee which might be the clinical applications of immunotherapy combination in the future. The eighth paper reviews evidence on the role of immunotherapy in gastrointestinal cancers.

This special issue wants to highlight how immunotherapy research spans the continuum from basic scientific research to clinical research applications. It is paramount to keep on working to foster the discovery, development, and delivery of immunotherapy approaches to treat cancer.

*Carmen Criscitiello*
*Michele Santangelo*
*Fotios Loupakis*

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*Research Article*

# Heterogeneous Periostin Expression in Different Histological Variants of Papillary Thyroid Carcinoma

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*Background*. Periostin (PN) epithelial and stromal overexpression in tumor pathology has been studied according to tumor growth, angiogenesis, invasiveness, and metastasis, but a limited number of studies address PN in thyroid tumors. *Aim*. Our study aimed to analyze PN expression in different histological variants of PTC and to correlate its expression with the clinicopathological prognostic factors. *Material and Methods*. PN expression has been immunohistochemically assessed in 50 cases of PTC (conventional, follicular, oncocytic, macrofollicular, and tall cell variants), in tumor epithelial cells and intratumoral stroma. The association between PN expression and clinicopathological characteristics has been evaluated. *Results*. Our results show that PTC presented different patterns of PN immunoreaction, stromal PN being significantly associated with advanced tumor stage and extrathyroidal extension. No correlations were found between PN overexpression in tumor epithelial cells and clinicopathological features, except for specific histological variants, the highest risk of poor outcome being registered for the conventional subtype in comparison to the oncocytic type. *Conclusions*. Our study demonstrates differences in PN expression in histological subtypes of PTC. Our results plead in favor of a dominant protumorigenic role of stromal PN, while the action of epithelial PN is less noticeable.

## 1. Introduction

Thyroid cancer represents less than 1% of total number of cancers, but during the last decades its incidence has been continuously growing, showing a dominant involvement of female sex and of young and medium ages [1]. Papillary thyroid carcinoma (PTC) is the most common histological type. Diagnosed in approximate 85% of cases [2], this histological type has a relatively good prognosis, distant metastases, and death being rare events.

Classically, the prognostic assessment of PTC relies, according to the WHO, on the following standard clinical and morphological factors: patients' age and sex, tumor size, histological variant, extrathyroidal extension, completeness of surgical resection, and occurrence of distant metastasis [1].

Tremendous progress has been made by genomics, transcriptomics, and proteomics in all types of cancers, including PTC, resulting in a switch over from traditional clinicopathological prognosis factors to new molecular prognosis markers.

The current trend in thyroid tumor pathology is to improve the grading framework by implementation of new molecular and genetic criteria that could explain the differences between the biological behaviors, quantified by low versus high PTC aggressiveness. Consequently, a large series of molecular markers have been investigated, but none of them has been yet validated and thus they are still considered as candidate prognosis factors. Therefore this issue is remaining a source of heated debate.

As a component of the cellular matrix, periostin (PN) has been recently included in the list of putative prognostic markers. PN is a cellular adhesion molecule, initially identified within the osteoblastic cellular line in mice [3] and named according its identification in periosteum and periodontal ligamentum [4].

PN is secreted by fibroblasts [5–7] and belongs to fasciclin-I family of proteins, functioning in cell-cell and cell-extracellular matrix (ECM) interactions. It is located in fetal and normal adult organs, such as embryonic periosteum, placenta, heart valves, thyroid, adrenal glands, lung, stomach, colon, testicle, prostate, vagina, ovary, breast, and periodontal ligamentum [8–11].

PN epithelial and stromal overexpression in tumor pathology has been studied according to tumor growth, angiogenesis, invasiveness, and metastasis [10–14]. The published data are relatively limited but nevertheless they are supporting PN involvement in tumor progression in different locations, such oral [15] and head and neck carcinomas [16, 17], breast cancer [18–23], ovarian cancer [8, 12, 24–26], prostate cancer [27–30], renal cell carcinoma [31–33], pancreatic carcinoma [34, 35], stomach [36–38], colon [39, 40] and hepatocellular carcinoma [13, 41, 42], non-small-cell lung carcinoma [43–46], malignant pleural mesothelioma [47], neuroblastoma [48], glioblastoma [49–51], and its association with aggressive phenotypes and poor prognosis [7, 10, 13, 14].

To the best of our knowledge, PN expression in thyroid tumors is scarcely reported in the mainstream publications.

Within this context, the present study aims to analyze PN expression in different histological variants of PTC and to correlate its expression with the clinicopathological prognostic factors.

## 2. Material and Method

*2.1. Patients and Tissues.* The study group is comprised of 50 patients diagnosed with PTC in "Sf. Spiridon" County Clinical Emergency Hospital and surgically treated by thyroidectomy with cervical lymph node dissection.

The clinicopathological features have been retrospectively documented from the medical files and included the following data: sex, age (<45 and ≥45 years old, resp.), tumor size, multifocality (number of foci), lymphovascular invasion, extrathyroidal extension (defined as microscopic presence of tumor cells beyond the thyroid capsule, into adipose tissue, skeletal muscle, or sizable vessels and nerves), lymph node metastasis, and tumor stages according to TNM and American Joint Committee on Cancer staging system [52].

All cases have been reassessed by two independent pathologists in order to identify the histological variant of PTC and to confirm the associated thyroid pathology.

The study has been approved by the Ethics Committee of "Grigore T. Popa" University of Medicine and Pharmacy Iasi, complying with the ethical standards of Helsinki declaration that require the patients' informed consent on the usage of their biologic material.

*2.2. Immunohistochemistry.* For each case, a representative paraffin-embedded block has been chosen, and 3 $\mu$m sections have been cut and have been displayed on electrostatically charged polylysine-coated slides.

Tissue samples were dewaxed in xylene and rehydrated in 3 baths of alcohol with progressive decreasing concentrations. Heat induced epitope retrieval technique was used for antigen retrieval. The slides were immersed in sodium citrate pH 6 and boiled in water bath at 98°C for 30 minutes. After cooling at room temperature and inhibition of endogenous peroxidase activity, the samples have been incubated with anti-periostin polyclonal antibody (Santa Cruz, Biotechnology Inc., Santa Cruz, USA) dilution 1 : 100, overnight at 4°C. Immunoreaction has been amplified with the suitable secondary and tertiary antibodies of the LSAB-HRP complex (Dako, Carpinteria, USA) and developed with 3,3′-diaminobenzidine tetrahydrochloride chromogen (DakoCytomation, Carpinteria, USA); the positive reaction has been considered in the presence of a brown cytoplasmic stain. Positive and negative controls have been simultaneously run.

*2.3. Semiquantitative Assessment.* PN expression has been separately assessed in tumor epithelial cells and in intratumoral stroma, using adapted semiquantitative scores based on literature reports [25, 53, 54]. The corresponding non-tumoral thyroid tissue within each PTC specimen has been constantly evaluated. This step allowed us to establish the basal level of thyroid tissue PN immunoreaction, considering the staining of the follicular cells within these areas as absent or weak (+). We have evaluated the staining intensity in the tumor cellular component – $I$ (0 when absent, 1 for weak (+), 2 for moderate (++), and 3 for strong (+++) intensity, resp.) and percentage of positive tumor cells – $P$ (0 for <10%, 1 for 10–30%, 2 for 30–60%, and 3 for >60% positive cells, resp.). The final score has been obtained as a sum between $I$ and $P$, with a minimum value of 0 and a maximum one of 6. We have considered the values between 0 and 3 as low score (corresponding to PN negative or weak expression) and those between 4 and 6 as high score (revealing a high PN expression). The stromal PN reaction has been quantified as 0 for no staining or less than 5% and 1 for >5% of positive intratumoral stroma, respectively.

*2.4. Statistical Analysis.* Statistical analysis has been performed by GraphPad Prism software package (GraphPad Software, San Diego, CA, USA). The association between PN expression and clinicopathological characteristics has been analyzed by applying the $\chi^2$ test, whereas odds ratios (ORs) using logistic regression have been calculated to assess the correlation between PN and outcome variables for tumor aggressiveness. Statistically significant results have been considered when $p < 0.05$.

## 3. Results

*3.1. Clinicopathological Characteristics.* A predominant female sex was observed in our study group, 41 cases (82.0%), compared to male sex, 9 cases (18.5%). The mean age at

diagnosis was 48.24 ± 14.70 years (range 19–76 years), 42.0% (21 patients) being diagnosed at young age, under 45 years. Mean tumor size was 2.18 ± 1.36 cm (range 1.1–7.5 cm). Multifocality was present in 34 cases (68%). We noted lymphovascular invasion in 14 cases (28%), extrathyroidal extension in 23 cases (46.0%), and lymph node metastasis in 7 cases (14%).

Based on TNM and AJCC criteria, the cases were staged as follows: 18 cases (36%), stage I, 6 cases (12%), stage II, 25 cases (50%), stage III, and 1 case (2%), stage IV.

Histologically, there were 10 cases (20%) diagnosed as conventional PTC and 40 cases (80%) as other variants of PTC (follicular, 21 cases (42%), oncocytic, 8 cases (16%), macrofollicular, 7 cases (14%), and tall cell, 4 cases (8%)).

### 3.2. PN Expression

*3.2.1. Qualitative Assessment.* PN immunopositivity has been noticed in both tumor cells and intratumoral stroma.

PN expression exhibited a predominantly cytoplasmic, perinuclear, finely granular pattern, in tumor cells. The distribution was predominantly homogenous, though some heterogenous areas were focally identified. The reaction intensity was predominantly moderate or strong.

The histological variants of PTC showed different patterns of PN immunoreaction. The immunoexpression was diffusely cytoplasmic, with weak apical or basal polarization, in conventional (Figure 1), follicular, and macrofollicular (Figure 2) variants. The tall cell variant was characterized by localized immunoexpression, with predominantly apical distribution, along with focal infranuclear positivity (Figure 3). The immunoreaction was predominantly negative or very weak in oncocytic variant (Figure 4).

The intratumoral stromal PN expression was variable within the histological variants of PTC, from strong positivity in fibroblasts and collagen fibers up to lack of expression.

PN expression has been negative or weak, exhibiting a homogenous and diffuse cytoplasmic distribution in the follicular cells of nonneoplastic thyroid tissue.

*3.2.2. Semiquantitative Assessment.* Tumor cells' PN expression has been evaluated as low score in 14 cases (28.0%) and with high score in 36 cases (72.0%) (Table 1). Intratumoral stroma exhibited PN negativity or weak expression in 16 cases (32.0%), whereas the other 34 cases (68%) showed PN strong positivity (Table 2).

*3.2.3. Correlations with Clinicopathological Prognostic Factors.* The results of the statistical analysis between PN (low versus high expression) in tumor cells and clinicopathological features are summarized in Table 1. Statistically significant differences were registered only between PN immunoreaction and histological variants ($p = 0.0002$). A high PN score was more frequently noted in conventional subtype than in oncocytic subtype (OD = 105, CI 3.73–2948.28, $p = 0.0062$).

Table 2 synthesizes the correlation between PN stromal expression (negative versus positive) and clinicopathological features. Our results show significant differences between stromal PN immunoreaction and tumor stage (early versus advanced stages) ($p = 0.04$) and extrathyroidal extension ($p = 0.008$). Moreover, a high PN score was more frequently observed in advanced tumor stage (OR 0.28, 95% CI 0.07–0.99; $p = 0.0491$) and in the occurrence of extrathyroidal extension (OR 0.16, CI 0.03–0.67, $p = 0.0124$)

We have also noted a very close value to the statistical significant $p$ value for the lymph node metastasis.

## 4. Discussion

PN is encoded by a gene located on chromosome 13 (13q13.3), in humans [55]. Structurally, it is formed by one N-terminal constant domain, one cysteine-rich domain (EMILIN-like), four fasciclin-repetitive-Fas domains, and one C-terminal hydrophilic domain exhibiting a variable structure according to the isoform [3, 4, 55].

Currently, eight PN isoforms are known, only five of them being sequenced and identified in different tissues: isoform 1 or (a) in osteosarcoma, isoform 2 or (b) in human placenta, isoform 3 or (c) in ovarian carcinoma, and 2 (b), 4 (d), and 5 (e) in either normal or tumoral urinary bladder [3, 8, 56–58].

Different PN isoforms may variably influence ECM fibrillogenesis [59] but it is still unknown if their effect on ECM increases the invasiveness or metastatic potential [13, 60, 61].

During the last 15 years, several papers provided evidences that support PN involvement in different malignancies. According to these studies, stromal PN expression is a negative prognostic factor for patients' survival [13, 28, 32, 41, 42] and, in association with epithelial PN, is significantly correlated with different clinicopathological prognostic factors [11, 13, 20, 35, 44, 47, 62, 63]. PN involvement in the epithelial-mesenchymal transition (EMT) has been also a matter of research interest, due to its potential therapeutic target value [8, 13, 39, 64–66]. Therefore, PN expression was analyzed in correlation with EMT (vimentin, elastin, and collagen) and angiogenesis specific markers, demonstrating its involvement as a promoter of this process [12, 15, 39, 63, 67].

Few papers addressed PN in thyroid tumors, predominantly using techniques of molecular biology (cDNA microarrays and real-time PCR) [58, 68–70]. Eight h-periostin isoforms have been identified in both thyroid carcinoma and in corresponding nonneoplastic tissues, all of them being related to thyroid carcinogenesis, invasion, or lymph node metastasis, regardless of differences between their expression pattern [58]. A high PN gene expression is associated with aggressive and poorly differentiated PTCs [68] and is correlated with specific morphological cellular features (loss of polarization and cohesiveness) registered in the invasive front of the tumor [69]. Only one of the four studies from literature has also analyzed PN immunoexpression, within a rather limited number of cases (10 normal thyroids, 10 follicular adenomas, 10 follicular thyroid carcinomas, and 10 PTCs samples, resp.) [70]. No PN staining has been noticed in normal thyroid tissue, in follicular adenoma, and in follicular thyroid carcinoma, and only 4 cases from a total of 10 PTCs showed a diffuse cytoplasmic immunoreaction [70].

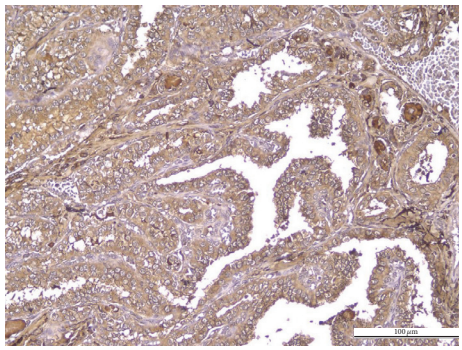

Figure 1: Conventional PTC. Positive PN in tumor cells: diffuse cytoplasmic positive immunoreaction of moderate intensity; negative PN in intratumoral stroma (IHC anti-PN, ×200).

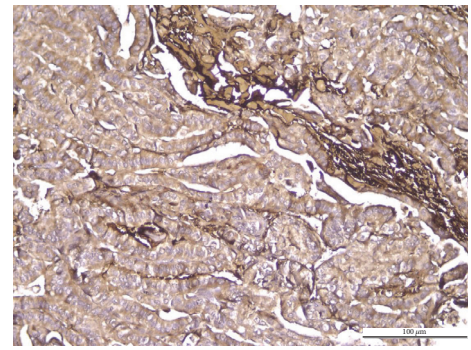

Figure 3: Tall cell PTC. Positive cytoplasmic PN, exhibiting focal apical and basal immunoreaction of moderate intensity; positive PN in intratumoral stroma (IHC anti-PN, ×200).

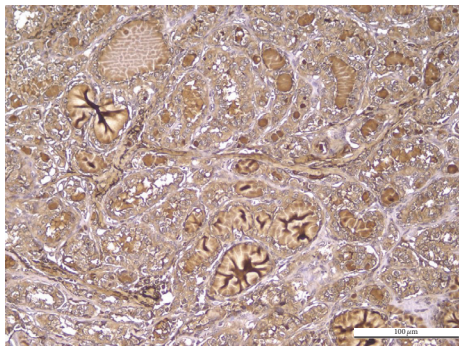

Figure 2: Macrofollicular PTC. Positive PN in tumor cells: diffuse cytoplasmic immunoreaction of moderate intensity; negative PN in intratumoral stroma (IHC anti-PN, ×200).

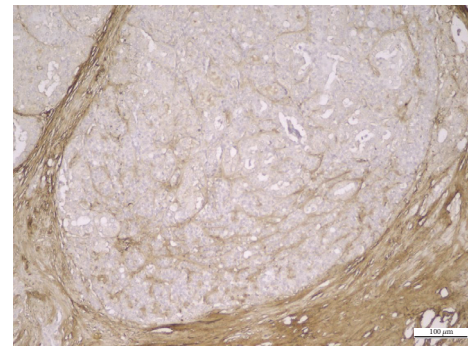

Figure 4: Oncocytic PTC. Negative PN expression in tumor epithelial cells and positive PN expression in tumor stroma (IHC anti-PN, ×200).

Within this context, the present study provides new data regarding the specific PN immunoexpression in epithelial tumor cells and intratumoral stroma, in different histological subtypes of PTC.

To the best of our knowledge, this is the first report of qualitative differences in epithelial and stromal PN expression between conventional, follicular, macrofollicular, tall cell, and oncocytic subtypes. Thus, the idea that PN may be tissue-specific [11] is strengthened by supplementary evidences of its heterogeneity, reported in different histological subtypes of a specific tumor, such as clear cell, papillary, and chromophobe renal cell carcinoma types [33], and conventional and nonconventional osteosarcoma subtypes [71].

The pivotal role of PN synthesis in different malignancies is currently under scrutiny, by comparing the involvement of tumor epithelial cells with that of the tumor stromal component. As a consequence, it has been hypothesized that PN acts in a cell-type-dependent manner related to its expression in stromal versus epithelial cells, as a result of the activity of different PN terminal regions [13].

This hypothesis has been the starting point of our work which has individually quantified PN immunoexpression in tumor cells and in tumor stroma, respectively. We have additionally refined the reported scores already used for PN assessment [25, 53, 54], considering both the percentage of positive cells and the reaction intensity, using a threshold to label the investigated cases into low and high score categories. This modality of semiquantitative evaluation, based on a specific algorithm, has not been yet applied in thyroid tumor pathology.

Our study showed a heterogeneity of PN stromal immunoexpression, comparable to other malignancies reporting either PN positivity [19, 22, 23, 49, 64] or PN negativity [72]. The most papers have reported that stromal PN has a more aggressive potential than the epithelial PN. This aggressiveness can be attributed to the capacity of the PN produced by the stromal components to act not only by intracellular signaling pathways but also by its fibrillogenic potential within ECM, its C-terminal region interacting with ECM molecules [73, 74].

Our results support the dominant protumorigenic role of stromal PN, while epithelial PN action is less evident. We found that the high stromal PN expression is significantly associated with an advanced tumor stage and extrathyroidal extension. Similar results are also reported in renal cell carcinoma [31, 33], prostate [13, 27, 28], penile [75], and breast cancer [20, 23]. There are no available literature data about the stromal PN profile in thyroid tumors.

Table 1: PN expression in tumor epithelial cells and clinicopathological characteristics of PTC.

| Clinicopathologic features | Case number | | PN | | | | $\chi^2$ | OR (95% CI) |
|---|---|---|---|---|---|---|---|---|
| | | | Low score | | High score | | | |
| | # | % | # | % | # | % | *p value* | |
| *Sex* | | | | | | | | |
| Female | 41 | 82 | 12 | 29.27 | 29 | 70.73 | $p = 0.6699$ | 0.69 (0.12–3.81) |
| Male | 9 | 18 | 2 | 22.22 | 7 | 77.78 | | |
| *Age* | | | | | | | | |
| <45 | 21 | 42 | 7 | 33.33 | 14 | 66.67 | $p = 0.4748$ | 0.63 (0.18–2.20) |
| >45 | 29 | 58 | 7 | 24.14 | 22 | 75.86 | | |
| *Tumor stage* | | | | | | | | |
| Stages I, II | 24 | 48 | 7 | 29.17 | 17 | 70.83 | $p = 0.8599$ | 0.89 (0.26–3.07) |
| Stages III, IV | 26 | 52 | 7 | 26.92 | 19 | 73.08 | | |
| *Histologic subtype* | | | | | | | | |
| Conventional | 10 | 20 | 0 | 0 | 10 | 100 | **p = 0.0002** | 10.86 (0.55–211.91) |
| Follicular | 21 | 42 | 7 | 33.33 | 14 | 66.67 | | |
| Macrofollicular | 7 | 14 | 0 | 0 | 7 | 100 | | 1.4 (0.02–78.80) |
| Tall cells | 4 | 8 | 0 | 0 | 4 | 100 | | 1.00 (0.24–4.13) |
| Oncocytic | 8 | 16 | 7 | 87.5 | 1 | 12.5 | | 105.00 (3.73–2948.28) |
| *Multifocality* | | | | | | | | |
| Yes | 34 | 68 | 10 | 29.41 | 24 | 70.59 | $p = 0.7459$ | 0.8 (0.20–3.08) |
| No | 16 | 32 | 4 | 25 | 12 | 75 | | |
| *Tumor size* | | | | | | | | |
| <2.18 cm | 35 | 70 | 10 | 28.57 | 25 | 71.43 | $p = 0.8907$ | 0.90 (0.23–3.53) |
| >2.18 cm | 15 | 30 | 4 | 26.67 | 11 | 73.33 | | |
| *Lymphovascular invasion* | | | | | | | | |
| Absent | 36 | 72 | 12 | 33.33 | 24 | 66.67 | $p = 0.1780$ | 0.33 (0.06–1.73) |
| Present | 14 | 28 | 2 | 14.29 | 12 | 85.71 | | |
| *Lymph node metastasis* | | | | | | | | |
| Absent | 43 | 86 | 12 | 27.91 | 31 | 72.09 | $p = 0.9710$ | 1.03 (0.17–6.06) |
| Present | 7 | 14 | 2 | 28.57 | 5 | 71.43 | | |
| *Extrathyroidal invasion* | | | | | | | | |
| Absent | 27 | 54 | 8 | 29.63 | 19 | 70.37 | $p = 0.7810$ | 0.83 (0.24–2.90) |
| Present | 23 | 46 | 6 | 26.09 | 17 | 73.91 | | |

$\chi^2$: chi-square test; OR: odd ratio; CI: confidence interval.

On the other hand, PN overexpression in tumor epithelial cells was correlated with specific histological PTC variants, the highest risk being registered for the conventional subtype in comparison to the oncocytic one. Our data are supplementing other results in the mainstream publications. Strictly referring to the thyroid pathology, the single published paper on PN immunoexpression in PTC [70] reports a correlation between PN overexpression and clinicopathological features (i.e., extrathyroidal invasion, distant metastasis, and higher grade staging).

Despite the small number of cases, the authors outline the correlation between PN, ETM, and an aggressive tumor behavior [70]. Moreover, they consider that PN could be a stronger negative prognostic marker than B-RAF, regardless of B-RAF mutation [70]. In other types of malignancies, comparable relationships are demonstrated in renal cell carcinoma (mainly for clear cell subtype) where a greater tumor epithelial PN expression is significantly associated with sarcomatoid differentiation, higher tumor stage, lymph node metastases, and poor overall survival [32, 33] and also in hepatocellular carcinoma, where PN correlates with microvascular invasion, multiple tumors, and advanced tumor stage [41, 42].

Taken together, our results are consistent with the complex framework of controversies regarding PN role in carcinogenesis, particularly for the thyroid location, and

Table 2: PN expression in intratumor stroma and clinicopathological characteristics of PTC.

| Clinicopathologic features | Case number | | PN | | | | $\chi^2$ | OR (95% CI) |
|---|---|---|---|---|---|---|---|---|
| | | | Low score | | High score | | | |
| | # | % | # | % | # | % | *p value* | |
| *Sex* | | | | | | | | |
| Female | 41 | 82 | 13 | 31.71 | 28 | 68.29 | $p = 0.9246$ | 1.07 (0.23–4.99) |
| Male | 9 | 18 | 3 | 33.33 | 6 | 66.67 | | |
| *Age* | | | | | | | | |
| <45 | 21 | 42 | 5 | 23.81 | 16 | 76.19 | $p = 0.2907$ | 1.05 (0.55–6.84) |
| >45 | 29 | 58 | 11 | 37.93 | 18 | 62.07 | | |
| *Tumor stage* | | | | | | | | |
| Stages I, II | 24 | 48 | 11 | 45.83 | 13 | 54.17 | **p = 0.0439** | 0.28 (0.07–0.99) |
| Stages III, IV | 26 | 52 | 5 | 19.23 | 21 | 80.77 | | |
| *Histologic subtype* | | | | | | | | |
| Conventional | 10 | 20 | 5 | 50 | 5 | 50 | $p = 0.7522$ | 0.40 (0.08–1.90) |
| Follicular | 21 | 42 | 6 | 28.57 | 15 | 71.43 | | |
| Macrofollicular | 7 | 14 | 2 | 28.57 | 5 | 71.43 | | 1.00 (0.15–6.64) |
| Tall cells | 4 | 8 | 1 | 25 | 3 | 75 | | 3.00 (0.22–39.60) |
| Oncocytic | 8 | 16 | 2 | 25 | 6 | 75 | | 1.20 (0.12–11.86) |
| *Multifocality* | | | | | | | | |
| No | 34 | 68 | 13 | 38.24 | 21 | 61.76 | $p = 0.1683$ | 0.37 (0.08–1.56) |
| Yes | 16 | 32 | 3 | 18.75 | 13 | 81.25 | | |
| *Tumor size* | | | | | | | | |
| <2.18 cm | 35 | 70 | 9 | 25.71 | 26 | 74.29 | $p = 0.1455$ | 2.52 (0.71–8.96) |
| >2.18 cm | 15 | 30 | 7 | 46.67 | 8 | 53.33 | | |
| *Lymphovascular invasion* | | | | | | | | |
| Absent | 36 | 72 | 13 | 36.11 | 23 | 63.89 | $p = 0.3176$ | 0.48 (0.11–2.04) |
| Present | 14 | 28 | 3 | 21.43 | 11 | 78.57 | | |
| *Lymph node metastasis* | | | | | | | | |
| Absent | 43 | 86 | 16 | 37.21 | 27 | 62.79 | $p = 0.0503$ | 0.11 (0.006–2.07) |
| Present | 7 | 14 | 0 | 0 | 7 | 100 | | |
| *Extrathyroidal invasion* | | | | | | | | |
| Absent | 27 | 54 | 13 | 48.15 | 14 | 51.85 | **p = 0.008** | 0.16 (0.03–0.67) |
| Present | 23 | 46 | 3 | 13.04 | 20 | 86.96 | | |

$\chi^2$: chi-square test; OR: odd ratio; CI: confidence interval.

support the interest in understanding its relationship with different tumor behaviors. Further research is needed for the validation of PN current status as a promising biomarker.

## 5. Conclusions

Our study demonstrates a wide variability of PN expression in PTC, both in tumor epithelial component and in tumor stroma. High stromal rather than epithelial PN expression is associated with an aggressive tumor behavior. These results support PN involvement in tumor progression and its possible use as a prognostic marker.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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*Review Article*

# Current Tissue Molecular Markers in Colorectal Cancer: A Literature Review

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*Background*. Colorectal cancer (CRC) is one of the most spread neoplasia types all around the world, especially in western areas. It evolves from precancerous lesions and adenomatous polyps, through successive genetic and epigenetic mutations. Numerous risk factors intervene in its development and they are either environmental or genetic. *Aim of the Review*. Alongside common screening techniques, such as fecal screening tests, endoscopic evaluation, and CT-colonography, we have identified the most important and useful biomarkers and we have analyzed their role in the diagnosis, prevention, and prognosis of CRC. *Conclusion*. Biomarkers can become an important tool in the diagnostic and therapeutic process for CRC. But further studies are needed to identify a noninvasive, cost-effective, and highly sensible and specific screening test for their detection and to standardize their use in clinical practice.

## 1. Introduction

Colorectal cancer (CRC) is the third of all cancers for incidence and mortality, behind prostate and lung cancer in males and behind breast and lung cancer in females [1]. The incidence is similar in both sexes, is slightly greater in males for rectal cancer, and is higher in western countries, especially in the United States, Canada, Europe, and also New Zealand and Australia [2].

It, usually, grows in the lining of colon and rectum in the form of a polyp, a mass protruding in the lumen. Not all the polyps are neoplastic and evolve in cancer, but it is well known that the majority of colorectal cancers progress from adenomatous polyps, in the so-called adenoma-to-carcinoma sequence [3].

Mortality can be reduced through prevention and detection at an early stage; therefore, the ultimate aim should be to implement and improve the screening strategies [4, 5]. The screening techniques can be classified as noninvasive and invasive and their sensitivity and specificity are variable (Table 3). In the latest years, more attention has been paid to numerous biomarkers that could help in the early diagnosis, treatment, and prognosis of CRC. To discover these potential biomarkers, which could be detected in blood and stool through noninvasive methods, it is important to study the genetic and pathogenetic basis of CRC [6, 7].

## 2. Risk Factors

In the development of CRC environmental and genetic factors play a very important role, Tables 1 and 2.

## 3. Biomarkers

A molecular marker or biomarker is a molecule able to be detected in tissue or serum and that allows identifying a particular condition or a disease. Biomarkers have a high

Table 1: Environmental risk factors.

| Environmental factors | |
|---|---|
| Age | The risk of developing CRC increases with age and the majority of the cases are diagnosed in patients older than 50 years [8–10]. A higher prevalence is reported in people aged over 60 years compared to those younger than 40 years [2]. |
| Gender | In the literature the incidence of CRC is the same in males and females. Females are shown to be older and to have right-sided tumors and less advanced diseases [11]. |
| Westernized lifestyle | Long-term smoking is strongly associated with the development of adenomatous polyps and is important for both formation and aggressiveness [12]. Recent meta-analyses point out a statistically significant increase of risk after 30 years of smoking, especially in CRCs displaying MSI. A greater association with rectal and proximal colon tumors is also reported [13, 14].<br>Diet is surely one of the most important risk factors, especially one rich in red meat. This association between red meat and cancer, stronger for the colon cancer, may depend on the presence of heme iron in meat [15–17].<br>Alcohol consumption also is a known risk factor for CRC. The interference on the folate synthesis, with the production of acetaldehyde that degrades folate, may be at the basis of the chromosome damage and so of the carcinogenesis process [18, 19]. |

Table 2: Genetic risk factors.

| Genetic factors | |
|---|---|
| APC | The Adenomatous Polyposis Coli (APC) gene, located on chromosome 5, is a tumor suppressor, which is mutated in most of sporadic cases of colon adenocarcinomas. APC mutation leads to an increased amount of $\beta$-catenin and to the activation of the Wnt signaling pathway that is involved in cellular activation [20–22]. |
| Chromosomal instability | Chromosomal instability is a common factor that intervenes in the adenoma-carcinoma sequence. It causes the inactivation of wild-type allele of tumor suppressor genes, such as SMAD4, APC, and p53, the loss of heterozygosity, and the alteration in chromosome number, like aneuploidy [22–24]. |
| BRAF and RAS | RAS and RAF are two oncogenes which activate the mitogen-activated protein kinase (MAPK) pathway. KRAS has a GTPase activity that activates RAF proteins; BRAF's serine-threonine kinase activity initiates the MAPK signaling cascade, with the activation of several transcription factors. The result is cell survival, proliferation, and metastasis [25].<br>Already small polyps present BRAF mutation, whereas in serrated adenomas, hyperplastic polyps and proximal colon cancer RAS is more often mutated [26, 27]. |
| DCC | Deleted in Colorectal Cancer (DCC) is a tumor suppressor gene sited on the long arm of chromosome 18 (18q21.3). It is a transmembrane protein that stops cell growth in absence of Netrin and its ligand. Its mutation prevents the bond with Netrin-1 and results in abnormal cell survival. Loss of heterozygosity (LOH) of chromosome 18q is seen in more than 70% of advanced CRC [23, 28, 29]. |
| Family history | FAP, Familiar Adenomatous Polyposis, is an autosomal dominant disease caused by germ line mutation of APC gene. Patients affected by FAP develop thousands of polyps in gastrointestinal system, especially in the colon, starting from the second decade of life; if not treated they will develop a CRC in early adulthood [30–35].<br>Hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch Syndrome is the most common hereditary form of CRC (2–4% of all CRC) [30, 36]. A characteristic trait of NHPCC is Microsatellite Instability (MIS) due to the inherited mutation of the Mismatch Repair Genes (MMR) that control the length of microsatellites, short nucleotides' sequences repeated in DNA [37, 38]. |

prognostic and predictive value and are an important instrument for the early diagnosis of CRC, for its treatment, and for the patients' outcome [52].

These markers can be divided into three different groups: diagnostic, predictive, and prognostic.

Diagnostic markers permit an early diagnosis and risk stratification.

Predictive biomarkers are useful for predicting the patient's response to the therapy and so patients can be selected to undergo a particular treatment on the basis of a likely positive response. They can even be used to identify the right drug dose and to prevent its toxicity [53–56].

Prognostic biomarkers allow estimating the natural course of the disease and dividing tumors in two groups: the

Table 3: Current screening options.

| Screening options | |
|---|---|
| Fecal screening tests | These tests search for occult blood in stool, which is nonspecific but can be detected especially in larger polyps and CRC. It is important to collect samples from consecutive bowel movements [39, 40].<br>Guaiac fecal occult blood test (gFOBT) detects qualitatively heme in the stool, using a guaiac material to which hydroperoxidase is added. Heme promotes a process that leads to the guaiac's oxygenation and to a blue discoloration [41, 42]. It has a low sensitivity for the detection of CRC, but when performed every year or two, mortality is reduced [39, 43, 44].<br>Fecal immunochemical tests (FITs) use monoclonal or polyclonal antibodies to detect human haemoglobin. They can give qualitative or quantitative results. FIT is more accurate than gFOBT, because it does not react with nonhuman heme and is less sensitive to upper gastrointestinal tract's bleeding [41, 45]. |
| Endoscopic screening | Flexible Sigmoidoscopy is a screening option that allows examining the rectum and the lower part of the colon. It is an invasive technique that requires simple bowel preparation but cannot detect lesion in the whole colon [41, 45].<br>Colonoscopy is esteemed as the gold standard for CRC screening; it allows exploring the whole colon and removing the suspicious lesions [46, 47]. It is an invasive and expensive exam that must be performed if any other test has a positive result [39].<br>The most common side effect is postpolypectomy bleeding, but also tearing and perforation may be possible [45]. It is recommended that colonoscopy be practiced every 10 years in average-risk patients that underwent to a complete, negative exam [39, 48, 49]. |
| CT-colonography (CTC) | CTC is a noninvasive test that has become a common method for CRC screening. It requires a bowel preparation, but sedation is not needed. The estimated sensitivity and sensibility in detecting polyps > 1 cm are high, above 90%. Limitation of this technique includes low sensitivity for small lesion and serrated polyps, the exposure to radiation, and the need of follow-up for extra colic incidental findings [39, 49–51]. |

ones with a good outcome and the ones with a bad outcome [57]. They can be molecules involved in different process, such as cellular proliferation, differentiation, angiogenesis, invasion, and metastasis [53].

Mutations of KRAS, BRAF, and MSI are the ones most commonly detected during the diagnostic and therapeutic process of CRC to better define the most proper treatment.

### 3.1. Diagnostic

*3.1.1. Microsatellite Instability (MSI).* Microsatellites are short sequences of 1–6 base pairs in the genome that have a major risk of mutations which are corrected by the MMR systems. HNPCC is caused by a germ line mutation of one of the four MMR genes, MSH2, MLH1, MSH6, and PMS2, that leads to Microsatellites Instability (MSI) [37]. MSI is also responsible for sporadic CRC. Five MS markers have been identified: 2 mononucleotides (BAT 25 and BAT 26) and 3 dinucleotides (D2S123, D5S346, and D17S250). These are sought in tissues when HNPCC is suspected and, if positive, in the serum of other family members [52, 58].

It is reported in the literature that MSI has a higher prevalence in stage II CRC and that cancers with MSI have a better prognosis than the ones characterized by microsatellite stability. Therefore, MSI can be not only a diagnostic tool but even a useful prognostic factor [59].

*3.1.2. Insulin-Like Growth Factor Binding Protein 2 (IGFBP2).* IGFBP-2 is a protein that modulates the binding between IGF and IGF-1. In CRC its levels are increased for an overexpression of its mRNA [60].

Serum and plasma levels of IGFBP-2 are significantly higher in CRC patients than in controls and in patients with advanced tumors compared to the ones at early stages [61].

*3.1.3. Telomerase.* Telomeres are specialized terminal structures in eukaryotic chromosomes that consist in repeats of a DNA sequence (TTAGGG), whose length is maintained by the enzyme Telomerase. Numerous studies have demonstrated an increased Telomerase Activity (TA) in CRC samples compared to normal colorectal mucosa. Some authors have also found that TA and telomeres length are independent prognostic elements to predict recurrence and disease-free and overall prognosis [62, 63].

*3.1.4. Pyruvate Kinase M2 (PKM2).* Pyruvate Kinase M2 is a glycolytic enzyme that plays an important role in cellular metabolism of many types of tumors. It can be detected even in normal colic cells, but its level is higher in CRC cells. Mutated PKM2 can be detected in stool, with ELISA technique, but its role as diagnostic marker must be further studied [64, 65].

### 3.2. Predictive

*3.2.1. KRAS.* Mutation of KRAS is of the most common alterations in CRC. The majority of these mutations happen in codons 12 and 13 and are DNA base pair substitutions with subsequent amino acid changes in the protein [66]. They cause an activation of EGFR pathway which becomes independent from EGFR activation. It is reported that these mutations are associated with chemoresistance to Anti-EGFR Antibodies, Cetuximab, and Panitumumab. Thus mutated KRAS is the most important predictive factor of the response to EGFR inhibitors [67, 68].

According to some authors KRAS mutation is also related to a poor prognosis, whereas in other studies it is shown that it has no major prognostic value [66, 69–72].

Recent studies have reported that even mutations of NRAS, which occur in 3–5% of CRC, determine a negative response to anti-EGFR therapy [67].

*3.2.2. BRAF.* BRAF is frequently mutated in CRC; the most common mutation is V600E that leads to a glutamic acid for valine substitution in the protein, causing the constitutional activation of MAPK pathway. BRAF and RAS mutations are, usually, mutually exclusive. BRAF V600E mutation is sought for two reasons: in MSI CRC can exclude Lynch Syndrome and in the MSS (microsatellite stable) ones is associated with a poor prognosis. It determines, in fact, as well as RAS mutation, resistance to the anti-EGFR therapy. Traditionally it has been detected with a PCR analysis, while recently immunohistochemistry has been approved for its research [73–75].

*3.2.3. PIK3CA.* Phosphoinositide-3-kinase is an enzyme of the AKT pathway. Its alteration determines an activation of the pathway and cell proliferation. Mutation in exon 20 is significantly associated with a low response to treatment with the monoclonal antibody anti-EGFR Cetuximab and with a worse prognosis if compared to patients with wild-type PIK3 [67, 76].

*3.2.4. PTEN (Phosphatase and Tensin Homolog Protein).* PTEN is a tumor suppressor gene, whose inactivation causes deregulation of the PI3K pathway. The loss of PTEN has been associated with aggressive CRCs and is predictive of a nonresponse to the treatment with Cetuximab [77, 78].

It is also a predictive factor for tumor with wild-type KRAS treated with anti-EGFR therapy [77, 79].

*3.2.5. ERCC-1.* Excision repair cross-complementing-1 is part of a family of genes that prevent DNA damage by nucleotide excision and repair. Level of its mRNA in cancer cells correlates with response to the therapy with oxaliplatin. Patients with low level show a better outcome than the ones with a higher number of copies of its mRNA; it has been hypothesized that an increased DNA repair antagonized the effect of platinum-based treatments [80].

*3.2.6. Ezrin.* Ezrin is a cytoskeletal protein that plays an important role in cell motility, invasion, and metastasis. Hyperphosphorylation at the site T567 has been sought in liver metastases, but its levels were lower in the primary tumor [81]. An increased cytoplasmatic expression of Ezrin correlates with a greater aggressiveness of CRC and therefore with a poor prognosis. Ezrin could become a target for antimetastatic therapy. Two small molecules, NSC305787 and NSC668394, which bind Ezrin and prevent its phosphorylation and activation, are currently under study [82, 83].

*3.2.7. Cyclooxygenase-2.* Cox-2 is involved in colorectal carcinogenesis. Its level is increased in the majority of CRCs, especially in advanced stages. It could have an important role as prognostic and predictive factor [6].

### 3.3. Prognostic

*3.3.1. APC.* Adenomatous Polyposis Coli is an oncosuppressor gene, whose mutation in germ line is responsible for FAP, but it is also mutated in the majority of sporadic CRCs. Even hypermethylation of APC gene promoter has been implicated in the development of colorectal adenomas and cancers [84, 85]. Both of these mechanisms lead to APC inactivation and this is considered a poor prognostic factor [86].

*3.3.2. p53.* TP53 gene mutation is one of the hallmarks of human tumors and plays an important role in the development of CRC [80]. Numerous studies have reported how its dysfunctions, more often caused by missense mutations, can be used as prognostic markers. It has been demonstrated that in almost half of the patients' serum antibodies anti-p53 can be detected, but the role in tumor screening must be further investigated [87, 88].

*3.3.3. VEGF.* Vascular endothelial growth factor is an angiogenetic factor involved in CRC and indirectly responsible for tumor growth and metastases. Its mutations are associated with a greater aggressiveness and poor prognosis and can be at the basis of resistance to anti-EGFR treatment [52, 89].

*3.3.4. EGFR (Epidermal Growth Factor Receptor).* EGFR is a transmembrane tyrosine kinase receptor, which is overexpressed in various tumors, including CRC. Two monoclonal antibodies, Cetuximab and Panitumumab, are currently used in treatment of CRCs presenting this overexpression, as monotherapy or in combined chemotherapy [77, 90, 91].

*3.3.5. 18q Loss of Heterozygosity (LOH).* Allelic loss of chromosome 18q is observed in up to 70% of CRCs and is associated with a poorer prognosis. Patients with stage II or III cancer that present LOH are shown to have a worse outcome compared to the ones with both allelic copies and could benefit from an adjuvant chemotherapy [92, 93].

*3.3.6. SMAD4.* SMAD4 is an oncosuppressor protein that intervenes in the intracellular pathway of TGF-$\beta$. Its inactivation leads to altered TGF-$\beta$ signaling and is related to tumor invasion, metastases formation, and poor response to chemotherapy. Thus, SMAD4 is a valuable prognostic marker [94, 95].

*3.3.7. Mutated in Colorectal Cancer (MCC).* MCC is a multifunctional protein that enters in the Wnt and NFkB pathways [96]. Mutations or loss of heterozygosity of its gene, located on chromosome 5q21, has been associated with CRC. MMC binds $\beta$-catenin, hindering the Wnt/$\beta$-catenin signaling pathway, and so it could have a prognostic value [97].

*3.3.8. Insulin-Like Growth Factor II mRNA-Binding Protein 3 (IMP3).* IMP3 is a protein expressed during embryogenesis and is almost undetectable in adult tissues but is expressed in neoplastic cells. It is reported that its expression in CRC is related to a more aggressive phenotype. It is considered an important prognostic marker and a predictor for metastases' formation [98].

*3.3.9. TRAF2- and NICK-Interactive Kinase (TNIK).* TNIK is a kinase involved in cytoskeleton organization and neural dendrite extension and is activated by the binding with $\beta$-catenin. High levels of TNIK are present in CRC and they are related to distant metastases in stage II and III tumors [99].

*3.3.10. S100A2 Protein.* S100 calcium-binding protein A2 (S100A2), a protein involved in cell cycle progression, has been demonstrated to be implicated in the distant metastasis of stage II and III CRC. Thus it can be used as a marker for the recurrence's prediction [100].

## 4. Conclusion

Biomarkers can be an important tool for early detection and prevention of CRC and guide the therapeutic process with a personalized therapy, on the basis of the presence of defined markers. Nowadays, there is not still a universal biomarker of CRC that allows a satisfying secondary prevention of this disease. Thus it is important to continue the study of the genetic and epigenetic modifications that underlie the CRC to discover new biomarkers. The main aim of future researches should be to perfect a noninvasive, cost-effective screening test with a high sensitivity and specificity that will allow the detection of a panel of biomarkers that can be employed in the clinical practice. Only through wide prospective studies on large series, it will be possible to validate the emerging biomarkers and standardize their practical use.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

## Authors' Contributions

Gaia Peluso and Paola Incollingo contributed equally to this work.

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*Review Article*

# Diagnostic, Predictive, Prognostic, and Therapeutic Molecular Biomarkers in Third Millennium: A Breakthrough in Gastric Cancer

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*Introduction*. Gastric cancer is the fifth most common cancer and the third cause of cancer death. The clinical outcomes of the patients are still not encouraging with a low rate of 5 years' survival. Often the disease is diagnosed at advanced stages and this obviously negatively affects patients outcomes. A deep understanding of molecular basis of gastric cancer can lead to the identification of diagnostic, predictive, prognostic, and therapeutic biomarkers. *Main Body*. This paper aims to give a global view on the molecular classification and mechanisms involved in the development of the tumour and on the biomarkers for gastric cancer. We discuss the role of E-cadherin, HER2, fibroblast growth factor receptor (FGFR), MET, human epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGFR), mammalian target of rapamycin (mTOR), microsatellite instability (MSI), PD-L1, and TP53. We have also considered in this manuscript new emerging biomarkers as matrix metalloproteases (MMPs), microRNAs, and long noncoding RNAs (lncRNAs). *Conclusions*. Identifying and validating diagnostic, prognostic, predictive, and therapeutic biomarkers will have a huge impact on patients outcomes as they will allow early detection of tumours and also guide the choice of a targeted therapy based on specific molecular features of the cancer.

## 1. Introduction

Gastric cancer is the fifth most common cancer after cancers of the lung, breast, colorectum, and prostate and it is the third cause of cancer death worldwide [1, 2]. The clinical outcomes of patient affected by gastric cancer are still not encouraging; indeed the 5 years' survival is less than 30% [3–5]. The incidence of gastric cancer is wildly different among the countries. Even though Japan has a higher incidence it also has a higher survival rate (52%) compared to other countries [4, 6, 7].

In 1965, Laurén classification of gastric cancer was introduced; it divides cancer into two types: intestinal and diffuse types which seem to have a different pathogenesis. The intestinal type is characterized by a cohesive and expansive growth pattern, it consists of neoplastic intestinal glands similar to the intestinal adenocarcinoma. The age of incidence of the intestinal type is higher than the diffuse type; it occurs more often in males and is more often located in the antrum; it predominates in high risk areas and is preceded by precancerous lesions. It is associated with *H. pylori* infection that leads to atrophic gastritis and intestinal metaplasia (precursor of intestinal type gastric cancer) [8–11]. The diffuse type is characterized by an infiltrative and noncohesive growth pattern with single neoplastic cell or small group of cells widely infiltrating the gastric wall. It occurs in younger patients, with no significant difference

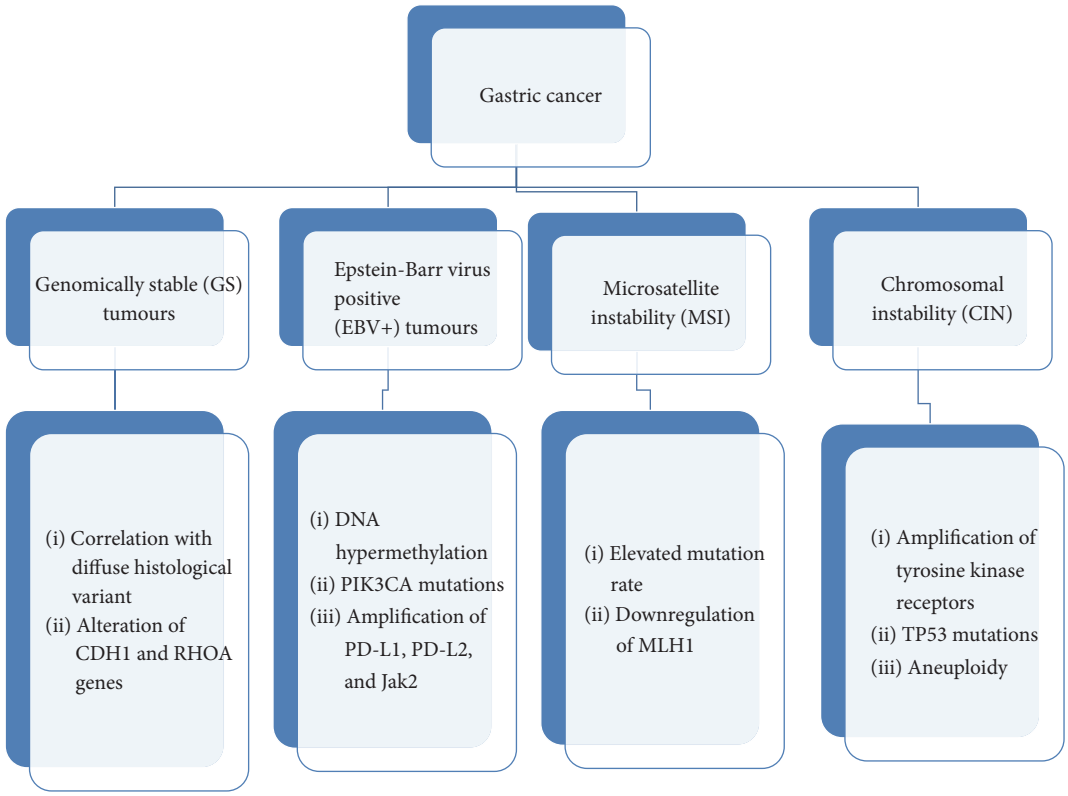


Figure 1: Classification of gastric cancers on the base of their molecular features (2014).

between men and women, and it is more often located in the gastric body [11, 12].

The TNM system is used for the staging of gastric cancer; however patients that belong to the same TNM stage often show very different clinical outcomes; this clearly manifests that there must be molecular factors responsible for those clinical differences.

A deep understanding of the molecular factors involved in the development of gastric cancer is needed in order to identify new biomarkers to diagnose GC in early stages and develop more effective therapeutic strategies.

In 2014 gastric cancer has been classified into four subtypes on the base of their molecular features: genomically stable (GS) tumours; tumours characterized by chromosomal instability (CIN); tumours positive for Epstein-Barr virus (EBV-positive); tumours characterized by microsatellite instability (MSI-positive). The GS tumours, nearly diploid tumours, are correlated to diffuse histological variant and alterations of genes CDH1 and RHOA. The CIN tumours are characterized by focal amplification of tyrosine kinase receptors, TP53 mutations, and aneuploidy. The Epstein-Barr positive tumours are associated with high levels of DNA hypermethylation, *PIK3CA* mutations, and amplification of *CD274* (also known as *PD-L1*) and *PDCD1LG2* (also known as *PD-L2*) and JAK2. The MSI tumours display elevated mutation rate and downregulation of the MLH1 gene that codifies MLH1 protein involved in the mismatch repair (MMR) [13] (Figure 1).

It is essential to discover new biomarkers of gastric cancer that could lead to an early detection of the tumour or give predictive information about the response to a therapy and finally improve the therapeutic outcomes [14].

A valid biomarker for malignant tumour needs to have specific characteristics: it has to be detectable in high level in patient affected by cancer and undetectable or present in low level in people not affected; it has to be easily quantifiable in clinical sample; it has to show functions related to the progression of the disease and it has to provide prognostic or diagnostic information about the cancer [15, 16].

Biomarkers can be classified into four types: diagnostic, prognostic, predictive, and therapeutic. A diagnostic biomarker allows the early detection of the cancer in a noninvasive way and thus the secondary prevention of the cancer. A predictive biomarker allows predicting the response of the patient to a targeted therapy and so defining subpopulations of patients that are likely going to benefit from a specific therapy. A prognostic biomarker is a clinical or biological characteristic that provides information on the likely course of the disease; it gives information about the outcome of the patient [14, 17, 18]. A therapeutic biomarker is generally a protein that could be used as target for a therapy [18].

This paper aims to give a global view on the biomarkers for gastric cancer; we discuss the role of E-cadherin, HER2, fibroblast growth factor receptor (FGFR), MET, human epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGFR), mammalian target of rapamycin (mTOR), microsatellite instability (MSI), PD-L1, and TP53.

We have also considered in this manuscript new emerging biomarkers as matrix metalloproteases (MMPs), microRNAs, and long noncoding RNAs (lncRNAs).

## 2. E-Cadherin

E-Cadherin is a transmembrane glycoprotein that is involved in the cellular calcium-mediated adhesion. It is codified by CDH1 located on the chromosome 16 (q22.1) [19, 20]. E-Cadherin plays a very important role in the adhesion and differentiation of the epithelial gastric cells and in the prevention of cancer onset [21]. CDH1 is one of the most important suppressor genes in gastric cancer; its inactivation increases tumour cells proliferation, invasion, and metastasis [18, 19, 22–25]. Several mechanisms can lead to loss of function of E-cadherin: mutations of the gene CDH1, loss of heterozygosis (LOH), silencing through suppressors that bind the promoter of CDH1 or through hypermethylation, and microRNAs that control the E-cadherin expression [20].

Analysing family affected, many germline mutations have been identified in hereditary diffuse gastric cancer [26]. Those mutations are spread in the 16 exons of the CDH1 gene; approximately 25% of them are missense mutations and 75% are truncating mutations [27, 28]. Only in a little percentage of family affected by gastric cancer (4%) have large deletions of CDH1 gene been identified [29, 30]. In the 70% of HDGC families germline there is a monoallelic mutation of the gene CDH1 that leads to a Loss of Heterozygosity (LOH) of the normal gene [31]. The cancer develops only when the second hit occurs, according to Knudson's model of the inactivation of tumour suppressor gene [31–34]. The second hit it is more often a hypermethylation of the gene's promoter and less often a second mutation or deletion occurring on the normal allele [21, 35–37]. The gastric cancer manifests when the complete inactivation of the CDH1 gene occurs, leading to a lack of the E-cadherin expression [27, 38]. Nowadays E-cadherin mutation cannot be considered a therapeutic biomarker as it would imply repairing E-cadherin expression through gene therapy [19]. Different E-cadherin alterations lead to various clinical manifestations and histotypes of gastric cancer so the presence of E-cadherin alteration is a weak prognostic biomarker [24]. A study showed a strong association between abnormal E-cadherin expression and tumour grade and metastases to regional lymph nodes [19]. Also another study showed the association between methylation of E-cadherin and dimension, stage of the cancer, and lymph nodes involvement [39]. Contrariwise another study did not find any association between E-cadherin mutation and gastric cancer stage and grade [40]. In most of the studies there is a correlation between E-cadherin abnormalities and worse clinical course, worse prognosis of the patient, and lower survival rate than patients negative for CDH1 mutations [25, 41].

E-Cadherin can also be considered as a predictive biomarker of the sensitivity to a specific therapy as its disablement reduces the response to both conventional and targeted therapy [24, 42]. Identifying CDH1 mutations at the moment of the diagnosis can predict if that cancer is going to be responsive to a therapy and so it could help in choosing the more suitable therapy for a specific patient [25] (Table 1).

It is important to highlight that a high percentage of families with HDGC have not got a mutation of E-cadherin gene; this obviously implies that there must be other molecular alterations that lead to the predisposition to gastric cancer and that still have not been identified [29, 43].

## 3. Microsatellite Instability

Microsatellites are short DNA repetitive sequences, in a non-random distribution along the human genome, that during DNA replication can lose out base-pairing mistakes [44, 45]. Those mistakes are normally repaired by the mismatch repair (MMR) proteins MLH1, MSH2, PMS2, and MSH6. Defects in the mismatch repair lead to a gathering of mutations that reflects the MSI and favour the onset of different types of cancer including the gastric one [46]. Several studies have reported an association between defects of mismatch repair and gastric cancer [47, 48]. MSI is observed in a percentage between 15 and 30 of all the gastric cancers and is more often due to hypermethylation of MLH1 promoter and the consequent lack of MLH1 expression [49–52]. MSI-positive gastric cancers show specific features: they usually have a later onset in life and are often located in distal part of the stomach and they usually have an intestinal histotype [45, 53–56]. The MSI in patients affected by gastric cancer seems to be a positive prognostic factor [57]. MSI-positive tumours show a better prognosis compared to MSI-negative as they have a lower local invasion capacity and have a lower prevalence of lymph nodes involvement; they also have a higher survival rate compared to MSI-negative gastric cancer at the same stage [50, 55–60] (Table 1).

## 4. PD-L1

PD-L1 and PD-L2 are ligands of Programmed Death-1 (PD-1) that is an important checkpoint receptor involved in the regulation of immunity and tolerance mechanism of T-cell. PD-L1 binding PD-1 is responsible for inducing and keeping the tolerance of peripheral T-cells [57]. PD-L1 is overexpressed in about the 40% of gastric cancer belonging to the EBV-positive type [13, 61]. Neoplastic cells use the PD-1/PD-L1 pathway to escape the immune surveillance of T-cells and the immune system reply to the cancer [62, 63].

A monoclonal antibody anti-PD-1, Pembrolizumab, has manifested efficacy in patients affected by advanced gastric cancer, showing a six-month OS of 69% [64]. The overexpression of PD-L1 can then be considered as a predictive biomarker of the response to a targeted therapy (Table 1). Targeting the PD1/PD-L1 pathway represents a promising strategy for the treatment of GC [57, 65].

## 5. TP53

p53 is a nuclear protein that works as a transcriptional factor whose duty is to keep the genomic stability. When a damage of the DNA occurs, p53 binds the DNA and activates the transcription of genes responsible for stopping the cellular

Table 1

| Biomarkers characteristics | Prognostic value | Predictive value |
|---|---|---|
| *E-Cadherin*<br>Transmembrane glycoprotein involved in calcium mediated adhesion, codified by gene CDH1 (chromosome 16q22.1).<br>Its alterations lead to increased cell proliferation, invasion, and metastasis. | It is associated with worse prognosis and lower survival rate | It is associated with reduced response to conventional and targeted therapy |
| *Microsatellite instability*<br>Microsatellites are short repetitive sequences that can lose out base paring during replication. Defects in mismatch repair lead to MSI. Tumours with MSI usually have<br>(i) later onset<br>(ii) distal location<br>(iii) intestinal histotype. | MSI-positive cancers are associated with<br>(i) a better prognosis than MSI-negative<br>(ii) higher survival rate<br>(iii) lower local invasion capacity<br>(iv) lower prevalence of lymph nodes involvement | — |
| *PD-L1*<br>It is the ligand of Programmed Death-1; it is responsible for inducing and keeping tolerance of peripheral T-cells. PD-1/PD-L1 pathway is used by neoplastic cell to escape immune surveillance. | — | PD-L1 overexpression is a predictive biomarker of response to Pembrolizumab |
| *TP53*<br>P53 is a nuclear protein that works as a transcriptional factor that activates apoptosis in case of DNA damage.<br>It is codified by TP53 (chromosome 17p13).<br>P53 alterations are often associated with CIN subtype. | There is a correlation between p53 overexpression and tumour size.<br>The association with lymph nodes metastasis and shorter survival is still controversial | — |
| *HER2*<br>It is a tyrosine receptor kinase (RTK) belonging to EGFR family, codified by ERBB2 (chromosome 17q21).<br>It is involved in cell survival and proliferation.<br>HER2+ tumours are often located at the gastroesophageal junction and often associated with intestinal histotype. | Still controversial: some studies report a more aggressive disease with worse prognosis but other studies do not confirm it | It is a predictive biomarker of the response to trastuzumab and lapatinib |
| *EGFR*<br>It belongs to the family of tyrosine kinase receptors. | It is associated with slightly differentiated, to high stage tumours and to a low survival | The use of anti-EGFR (cetuximab and panitumumab) associated with chemotherapy did not show any improvement in the clinical outcomes |
| *FGFR1-4*<br>The fibroblast growth factor receptors belong to RTK family. | Under evaluation | Under evaluation |
| *mTOR*<br>The activation of many RTK induces the activation of PIK3/mTOR pathway.<br>PIK3CA mutations frequently occur in EBV positive cancers. | PIK3CA mutation has been associated with<br>(i) worse prognosis<br>(ii) reduced survival rate<br>(iii) increased lymph nodes metastasis | Constitutive activation of PIK3/mTOR pathway is predictive of the response to Everolimus |
| *MET*<br>It is a RKT belonging to the family of Hepatocyte Growth Factor Receptors (HGFR); it binds HGF/SF.<br>Autophosphorylation of MET leads to the activation of downstream pathways responsible for cancer cells survival, proliferation, invasion, and metastasization. | It is associated with<br>(i) more aggressive disease<br>(ii) shorter survival | It is an important predictive biomarker of the response to rilotumumab |

cycle and causing apoptosis of the cell. p53 is encoded by the gene TP53 located on the chromosome 17p13.1 [11, 66, 67]. The mechanisms leading to the damage of TP53 function are usually LOH and mutations and less often methylation [68]. TP53 mutation is frequently mutated in gastric cancer and it is reported in association with CIN subtype [13, 68]. Heterogeneity of TP53 mutations in the same tumour is also reported as a result of multiple mutations of the gene [68]. Studies reported a higher prevalence of TP53 mutations in the intestinal type than in the diffuse type; another study instead reported a similar prevalence of TP53 mutations in the two types. Early and advanced intestinal type as well as advanced diffuse type show a high similar prevalence of TP53 mutations that are instead infrequent in early diffuse type of gastric

cancer [68–72]. A correlation between p53 overexpression and size of the gastric cancer has been reported [73]. The association between p53 overexpression with lymph nodes metastasis and shorter survival is still controversial because it has been reported in some studies but not in others; therefore, at this moment in time, p53 cannot be considered a trustworthy prognostic biomarker [57, 68] (Table 1).

## 6. HER2

HER2 is one of the four tyrosine receptor kinases (RTKs) belonging to the family of EGFR (EGFR or HER1, HER2, HER3, and HER4); it is codified by the protooncogene ERBB2 located on chromosome 17q21 and plays an important role in cell survival and proliferation [14, 74].

For signal transmission HER2 needs to heterodimerize with another member of the HER family, mainly with EGFR [75]. The amplification of ERBB2 gene produces an overexpression of HER2 protein that leads to cancer cells survival, growth, and proliferation through the PI3K-AKT and the MAPK pathways [76, 77]. Overexpression of HER2 receptor as a prognostic and predictive biomarker, identified before in breast cancer, is becoming noticeable even in gastric cancer [57]. HER 2 overexpression has a variable incidence ranging from 9% to 38% in most of the studies, depending on the location of the cancer and on its histology [76, 78–82]. The HER2 overexpression is more frequently observed in gastroesophageal junction tumours than in those with distal gastric location and it is more often associated with the intestinal type adenocarcinomas [83–90]. Cancers positive for HER2 overexpression are usually differentiated tumours [43, 80, 91]. ERBB2 gene mutation that leads to HER2 overexpression occurs in the early stage of carcinogenesis [92].

The role as a prognostic biomarker of HER2 is still doubtful; indeed some studies show an association of HER2 with a worse prognosis and a more aggressive disease; others contrariwise do not find a significant difference in prognosis between HER2 positive and HER2 negative cancers [76, 80–82, 91, 93–103].

Still controversial is also the correlation between HER2 overexpression and clinical features of the tumour. Some studies indeed suggest an association of ERBB2 amplification with tumour size, lymph node metastasis, local invasion, and cancer stage; other studies instead do not find any link between them [85, 87–90, 95, 100, 102].

HER2 overexpression has become a very important predictive biomarker that allows clinicians to identify patients that are going to have a survival benefit from a biological therapy with the monoclonal antibody (trastuzumab) [104–106].

The ToGa clinical phase 3 randomized controlled trial, conducted on patient affected by advanced gastric or gastroesophageal junction cancer, HER2-positive with an immunohistochemical 3+ score, compared the effectiveness of the association of trastuzumab and chemotherapy (cisplatin and a fluoropyrimidine) with the chemotherapy alone. The results of this study pointed out that patients treated with the association of trastuzumab and chemotherapy had a longer OS (13.8 months versus 11.1) and even their progression free survival (PFS) was heightened compared to that of the patient treated only with chemotherapy [104].

At the moment, trastuzumab is the only targeted therapy permitted for advanced gastric cancer [107]. Other ways of blocking the HER2 receptor are now being researched.

Lapatinib is a tyrosine kinase inhibitor that blocks both HER2 and EGFR. A randomized phase III TyTAN trial compared the efficacy of the association lapatinib and paclitaxel with paclitaxel alone, in patients affected by HER2-positive advanced gastric cancer. The OS was of 11.0 months in patient treated with the association of lapatinib and paclitaxel and 8.9 months in the ones treated with paclitaxel alone and also the response rate was increased with the associated therapy, yet there was no significant difference in PFS [108].

Other HER2 targeted drugs such as neratinib and pertuzumab, whose efficacy on HER2-positive breast cancer has already been proved, have not been assessed yet on advanced gastric cancer in randomized clinical trials [57].

Ado-trastuzumab emtansine (T-DM1) is a drug composed of the monoclonal antibody trastuzumab linked to a cytotoxic drug on microtubules DM1. This conjugate efficacy has been evaluated in the phase II/III Gatsby, whose results have not been released yet, but ImmunoGen has revealed that they are not encouraging [109].

HER2 can then be considered as an important predictive biomarker that can guide the choice of the best therapy for the single patient (Table 1).

## 7. EGFR

Even EGFR belongs to the family of tyrosine kinase receptors. It was found to be overexpressed in about the 27% of gastric cancer and the incidence of the amplification of the gene was from 3% to 8% depending on the detection method used [110–112].

EGFR overexpression has been related to cancer histology slightly differentiated, low survival, and high stage [111].

Unfortunately, the use of targeted therapy anti-EGFR (cetuximab or panitumumab) together with chemotherapy did not show any improvement in the outcomes of the patients affected by advanced gastric cancer [113, 114] (Table 1).

Even inhibitors of tyrosine kinase (TKIs) have been considered as therapy in patients affected by advanced gastric cancer resistant to chemotherapy [78].

Unluckily, none of the studies has demonstrated a significant improvement of results compared to conventional therapy. Considering premises already made, further investigations are needed to identify subgroups of patients that might benefit from anti- EGFR therapies.

## 8. FGFR

FGFR1, FGFR2, FGFR3, and FGFR4 are fibroblast growth factor receptors belonging to the RTK family [14]. In 2012, Deng et al. reported that FGFR2 copy number gain was detected in 9% of cancers [110]. Considering the high expression of this receptor in some tumours, phase II studies are evaluating

the efficacy of dovitinib (TKI258), a small FGFR2 inhibitor, on patients with FGFR2 amplification positive gastric cancer [110].

## 9. mTOR

The activation of many RTKs induces the activation of phosphatidylinositol-3-kinase (PIK3)/mTOR pathway. Mutations of the gene PIK3CA that codifies the alpha p110 catalytic subunit of PIK3 lead to constitutive activation of the PIK3/mTOR pathway [14, 115]. PIK3CA mutation has been associated with a worse prognosis with reduced survival and increased lymph node metastasis [14, 116] (Table 1). The frequency of mutations varies from 5 to 67% in different studies [117–120]. PIK3CA mutations frequently occur in EBV-positive gastric cancer [119].

A mTOR inhibitor, Everolimus, has displayed potential benefit in advanced gastric cancer in phase II trials; however in phase III trials it did not lead to any significant rising of OS [121–123].

## 10. MET

MET is a RKT belonging to the family of hepatocyte growth factor receptor (HGFR), it binds HGF/SF (hepatocyte growth factor/scatter factor). Autophosphorylation of MET leads to the activation of a number of downstream pathways (PIK3, Akt, and RAS-MAPK) responsible for cancer cell survival, proliferation, invasion, and metastasization [124].

It is overexpressed in about 50% of advanced gastric cancer [65, 125].

MET gene overexpression is related to a bad prognosis; it is associated with a more aggressive disease, a shorter OS, and disease free survival compared to MET-negative gastric cancers [126–129].

It is also an important predictive biomarker. Rilotumumab is a monoclonal antibody able to prevent the binding of MET receptor and its ligand HGF; this targeted therapy in association with the chemotherapy improves the OS to 11.1 months in patient affected by a high level MET amplification cancer compared with 5.7 months of the patient that received the chemotherapy alone [130] (Table 1).

MET importance on carcinogenesis is becoming so evident that, nowadays, multiple studies are evaluating the efficacy of TKIs (like crizotinib and foretinib) on cancers with MET overexpression [125, 131].

## 11. Promising Future Markers

*11.1. Matrix Metalloproteinase.* The matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinase whose function is to degrade the elements of the extracellular matrix [132]. Their work is regulated by the inhibitors of metalloproteinase (TIMPs) [133]. MMPs are involved in many physiological and also pathological processes [134]. MMPs have been found upregulated in gastric cancer and they have also been associated with specific pathological features of the cancer. Studies conducted on this subject prompt that MMPs and TIMP could be used as markers of peritoneal dissemination, depth of invasion, and metastasis [132].

Unfortunately, MMPs inhibitors have not demonstrated significant clinical benefit as therapy. In a clinical trial, conducted on patients affected by chemotherapy refractory advanced gastric cancer and gastroesophageal cancer, the MMP marimastat only determined a little difference in survival. The treatment was burdened by low tolerability and musculoskeletal pain [135]. Further studies about this subject are needed in order to identify the possible application of MMP in therapy of the advanced gastric cancer.

*11.2. MicroRNA.* MicroRNAs are 18 to 24 nucleotides noncoding RNA fragments whose function is to bind the 3′UTR region of their target gene and regulate its expression by impairing the translation [136–138]. MicroRNAs are involved in the regulation of several process of the cell: proliferation, differentiation, migration, and invasion [136]. Many genes can be regulated just by a microRNA [139]. MicroRNAs seem to play a very important role in the carcinogenesis of gastric cancer; they can increase the expression of oncogenes or reduce the expression of tumour suppressor genes [139, 140].

Several microRNAs have been identified and recognized to be implicated in gastric cancer [141, 142]. It is difficult to pick a miRNA as a cancer biomarker. Currently, there are no studies proving the effectiveness of miRNAs as predictive, prognostic or therapeutic biomarkers [57].

*11.3. Long Noncoding RNAs.* Long noncoding RNAs (lncRNAs) are sequences of nucleotides longer than 200 [143, 144]. Currently lncRNAs are catching researchers' attention because of an increasing amount of evidence suggesting that they play an important role in carcinogenesis and metastasis [139]. Nowadays about 135 lncRNAs have been recognized as altered in gastric cancer, so their potential role as diagnostic and prognostic markers has been speculated [143–145]. However, further studies about lncRNAs are needed in order to identify their possible clinical utilization.

## 12. Conclusions

Even if the incidence of gastric cancer reduced, it still remains the fifth most common cancer and it is characterized by negative prognosis and bad outcomes in response to chemotherapy. A deep understanding of molecular mechanisms of gastric carcinogenesis is essential to develop new therapeutic strategies and diagnostic, prognostic, and predictive biomarkers. The partition of gastric cancer into four molecular types (EBV-positive, MSI-positive, genomically stable, and chromosomal instability) allows dividing the patients on the basis of the molecular features of their cancer and identifying the best therapeutic approach [13]. A huge amount of studies has been conducted on molecular biomarkers; however the only predictive biomarker currently used is HER2 that allows identifying the patient that will benefit from a targeted therapy with trastuzumab. The majority of the patients still cannot be treated with a targeted therapy and nowadays still there are no diagnostic markers that can be used for secondary prevention. Most of

the biomarkers till now identified still need to be validated before they can actually be employed in clinical practice [14]. Further studies that will identify and validate diagnostic, prognostic, predictive, and therapeutic biomarkers will have a huge impact on the outcomes of the patients, as they will allow the early detection of the tumour and also guide the choice of a targeted therapy based on the specific molecular features of the cancer [146–149].

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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*Review Article*

# Clinical Applications of Immunotherapy Combination Methods and New Opportunities for the Future

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In the last decade, we have gained a deeper understanding of innate immune system. The mechanism of the continuous guarding of progressive mutations happening in a single cell was discovered and the production and the recognition of tumor associated antigens by the T-cells and elimination of numerous tumors by immune-editing were further understood. The new discoveries on immune mechanisms and its relation with carcinogenesis have led to development of a new class of drugs called immunotherapeutics. T lymphocyte-associated antigen 4, programmed cell death protein 1, and programmed cell death protein ligand 1 are the classes drugs based on immunologic manipulation and are collectively known as the "checkpoint inhibitors." Checkpoint inhibitors have shown remarkable antitumor efficacy in a broad spectrum of malignancies; however, the strongest and most durable immune responses do not last long and the more durable responses only occur in a small subset of patients. One of the solutions which have been put forth to overcome these challenges is combination strategies. Among the dual use of methods, a backbone with either PD-1 or PD-L1 antagonist drugs alongside with certain cytotoxic chemotherapies, radiation, targeted drugs, and novel checkpoint stimulators is the most promising approach and will be on stage in forthcoming years.

## 1. Introduction

*"Natural forces within us are the true healers of disease"* is a famous quote from Hippocrates which refers to the recent renaissance of cancer treatment. In the last decade, we have gained a deeper understanding of innate immune system and T-cell recognition. In particular, researchers found that the ignorance of self-proteins, which protect the body from autoimmune diseases (the *"natural forces"* in Hippocrates quote), acts as a key mechanism behind tumoral escape from destruction. Additionally, the mechanism of the continuous guarding of progressive mutations happening in a single cell (immune-surveillance) was discovered; the production of new cancer cell antigens (neoantigens), the recognition of both cancer specific and malignancy associated antigens by the T-cells, and elimination of numerous tumors by immunoediting were understood in detail. The new discoveries on immune mechanisms and its relation with carcinogenesis have led to development of a new class of drugs called immunotherapeutics (IT).

Cancer cells create an immunosuppressive microenvironment and grow inside it. In normal conditions, the immune system is capable of distinguishing the danger signal and capable of inducing an appropriate reaction towards tumor cells. The tumor associated antigens are recognised by T-cells, which leads to tumors being eradicated; however, tumoral cells escape from immunoediting by expressing programmed cell death ligand (PDL-1) and similar inhibitory gene products like IDO (indolamine 2,3 dioxygenase), TGF-$\beta$ (transforming growth factor-$\beta$), and Interleukin-10 (IL-10). One of the mechanisms of cancer evolution to escape from antitumor guarding of immune system is the deactivation or silencing the effector T-cells. T-cell exhaustion is mediated by inhibitory receptors such as programmed cell death protein-1 (PD-1), TIM-3 (mucin 3), and LAG-3 (Lymphocyte activation gene protein-3). One of the major cytokines released from T-cells, Interferon-$\gamma$ (IFN-$\gamma$), creates a vicious cycle of immunosuppression by increasing PD-1 expression.

Despite the promising developments, the strongest and most durable immune responses do not last long, as

resistance eventually develops and the more durable responses only occur in a small subset of patients. From the knowledge and experience of classical cytotoxic drugs, one of the solutions which have been put forth to overcome the challenges encountered in clinical practice is combination strategies.

## 2. The Rationale and Scientific Background of Combinatorial Immunotherapies

The idea that cancer treatment can occur via induction of immune response has been studied for more than a century [1, 2]. First hypothesis of immunotherapy relies on William Coley's, the father of immunotherapy, studies [3–5]. Coley experienced the beneficence of *Streptococcus* pyogenes infection in an inoperable sarcoma patient who obtained complete remission. Based on the foresight of an immunological efficacy of *Streptococcus* pyogenes infection against tumor cells, he treated nearly 100 mixed type cancer patients with a 10% overall response rate [4, 5]. While the mechanism of action was not known at that time, it is well known now that activation of immunologic response is based on leucocyte infiltration, clonal increase in T-cell population, and the increase in the release of inflammatory molecules which are the intermediary steps of the efficacy Coley's toxin [3, 4, 6, 7]. However, until very recently, definitive agents of immune manipulation have not been obtained apart from interleukin and interferon approaches in melanoma and metastatic renal cell cancer (mRCC) [8–10]. On the other hand, translational research on immunotherapy has given results, which brought the antagonists of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed cell death protein ligand 1 (PDL-1) to clinic [11, 12]. Two classes of most widely and effectively used drugs based on immunologic manipulation are collectively known as the "checkpoint inhibitors." CTLA molecule specifically inhibits T-cell activation and proliferation by binding to CD80 and CD86 and by suppressing costimulatory receptor CD28 and intracellular signaling [13]. PD-1 molecule is a transmembrane protein expressed mainly on T-cells, B cells, and natural killer (NK) cells which exhibits its inhibitory function by binding to specific receptors such as PD-L1 on tumor cells, various tissues, and PD-L2 on hematopoietic cells. The lock-and-key interaction leads to inhibition and T-cell exhaustion, which enables tumor cell to evade from active immune system guarding of cancer cells [14, 15].

CTLA-4 blockage was first tested in melanoma cases with an anti-CTLA-4 inhibitory molecule ipilimumab [16–18]. Ipilimumab was the first proven drug that demonstrated improved survival advantage in metastatic melanoma [16, 17]. Beyond the advantage of survival, complete responses have been obtained, and a plateau has been achieved in the survival curve which never occurred before in melanoma trials except for a limited number of patients [19]. The encouraging results of ipilimumab in melanoma have been supported by trials with a PD-1 antagonist, and overall advantage of survival in addition to improvement in objective response rate [20] and progression free survival (PFS) were shown in randomized controlled phase III trials of PD-1 antagonists Nivolumab and pembrolizumab [21–25]. Further trials with PD-1 inhibitory molecules have been run in melanoma and in other various tumors like RCC, nonsmall cell lung carcinoma (NSCLC), bladder cancer, and others [12, 25–30]. Checkpoint inhibitors have shown remarkable antitumor efficacy in a broad spectrum of malignancies and even in some refractory cases [31–33]. However, despite these promising results and the characteristic response durability, ipilimumab, nivolumab, pembrolizumab, and atezolizumab (a PD-L1 antagonist agent approved for advanced urothelial tumors by FDA in 2016) as single agents only have a range of 10–35% response rates. Only a small number of patients have benefited from immunotherapeutics.

The next challenge for scientists has been to enhance and broaden the overall benefits of IT. Predictive biomarkers like the PD-L1 expression on tumor tissue, mutational load of specific cancer type, and genetic signatures for inflammation have been put forward as a solution for issues with patient selection. Apart from patient selection, knowledge from cytotoxic drugs of cancer leads to the idea that the combination of immunotherapy drugs might allow blockage of different mechanisms of tumor development, overcome resistance, and improve response rates to increase the proportion of patients who benefit from the treatment. This review focuses on combination strategies of anti-CTLA-4, anti-PD1, and PD-L1 molecules with other coinhibitory molecules, costimulatory molecules, agents for molecules in tumor microenvironment, experimental cancer vaccines, cytotoxic chemotherapeutics, targeted agents, and radiation (Table 1).

## 3. Combination Strategies

*3.1. Combinatorial Immunotherapies with Checkpoint Inhibitors.* CTLA-4 inhibitors and PD1 blockers act differently by blocking parallel but distinct pathways on tumor cells. Although both of the molecules have similar negative input on T-cell activity, the timing of downregulation and the anatomic positions of action differ. These pathways operate on different stages of immune reaction. CTLA-4 is considered to be the chief of checkpoint inhibitor orchestra which has a role of stopping autoreactive T-cells in lymph node at initial priming stage [34, 35]. CTLA-4 molecule prevents T-cell activation and proliferation, and it blocks intracellular signaling by preventing the bonding of B7 ligands to T-cell costimulatory molecules via binding to CD80 and CD86 [13, 32, 35]. On the other hand, PD-1 is located on the T-cell surfaces and functions during the effector phase and the PD-1 pathway operates on later stages in peripheral tissues by regulating activated T-cells. Upon recognition of T-cell activation, it binds to PD-L1 and PD-L2 receptors, which results in T-cell exhaustion [14, 15, 36].

Preliminary results showed that the combined administration of ipilimumab and nivolumab results in an enhanced level of antimelanoma activity, compared to monotherapy with either agent with the cost of increased immune-related adverse events [24, 37, 38]. In the first dose escalation study of ipi-nivo combination in melanoma, 53 patients received concurrent treatment while 33 patients received sequential treatment. In the concurrent arm, 65% clinical benefit rate

Table 1: Selected Immunotherapeutics, mechanism of action, and major clinical therapeutic combinations.

| | Mechanism | Potential combination strategy | | | | |
|---|---|---|---|---|---|---|
| | | Co-in. | Co-st. | targt. | CT | RT |
| Ipilimumab | anti CTLA-4 | + | + | + | + | + |
| Tremelimumab | anti CTLA-4 | + | + | + | + | + |
| Nivolumab | anti PD-1 | + | | + | + | |
| Pembrolizumab | anti PD-1 | + | | + | + | + |
| Atezolizumab | anti PD-L1 | + | | + | | |
| Avelumab | anti PD-L1 | + | | + | | |
| Durvalumab | anti PD-L1 | + | | + | | |

Co-in.: coinhibitory, Cost.: costimulatory, targt.: targeted, CT: chemotherapy, and RT: radiotherapy.

was among the best results of a melanoma trial [37]. The response was remarkably durable, strong, and rapid. In the subsequent phase II trial of ipi-nivo combo, 59% clinical benefit rate was achieved with improved durable results [38]. Wolchok et al. has designed a phase III trial of ipi-nivo combo for treatment of naive melanoma patients and showed that overall response rate (ORR) was 57% with combination compared to only 19% response rate (RR) in ipi alone arm and 43% in Nivo-only arm. The updated results of CheckMate 067 trial showed that PFS 11.5 months was improved with a hazard ratio (HR) of 0.42 in combination arm (11.5 m) against the ipi-only arm (2.9 m) [39]. It is well described that combination strategy with dual checkpoint inhibition has remarkably improved the outcomes of patients. The CheckMate 204 trial was design to show efficacy of ipi + nivo combination especially in asymptomatic brain metastatic melanoma patients [40]. The primary endpoint was intracranial (IC) response rate and the results showed that IC response rate was 56%, and 19% of patients had a complete response. Not surprisingly, IC and extracranial responses were found to be largely concordant. Grade 3/4 AEs occurred in 48% of patients, 8% neurologic, including headache and syncope. Only 3 patients (4%) stopped treatment due to therapy related neurologic toxicity.

The second method of the in-group combination of checkpoint inhibitors is with pembrolizumab and ipilimumab. KEYNOTE-029 (NCT02089685) was a phase 1/2 study designed to assess the safety and efficacy of pembro + ipi in patients with advanced melanoma or RCC. According to the results of phase 1b of Keynote-029 trial pembrolizumab plus low-dose ipilimumab was tolerable and effective for patients with advanced melanoma, with an overall response rate (ORR) of 56%. Very recently, Matteo et al. presented the mature data of Keynote-029 trial which estimated 1-year PFS as 69% and 1-year OS as 89% [41]. As far as the safety concerned, immune-mediated AEs occurred in 90 (59%) patients; 25% were grade 3/4 and no treatment-related (TR) deaths occurred. In an Australia trial, the ABC trial, same strategy was tested for asymptomatic brain metastatic melanoma patients without previous cranial therapy [42]. PFS for 6 months was 50% in combo arm versus 29% in nivo alone arm; similarly 6-month OS was 76% versus 59%. Treatment-related grade 3/4 toxicity was reported as 68% versus 40%.

The encouraging results from the melanoma trials have led to the exploration of the use of this combination in other malignancies. Hammers et al. studied ipi-nivo combination in 2 different dose scale (Nivo 1 mg/kg versus 3 mg/kg + ipi 3 mg/kg versus 1 mg/kg) in mRCC. Similar results were observed as in melanoma trials, with up to 40% ORR. Furthermore, 65% of patients were progression-free at 24 weeks; however, grade 3 adverse events were in 62% of study population in nivolipi3 arm, and there was a 6/47 treatment discontinuation in nivolipi3 arm due to treatment-related adverse events (TAEs) [43]. The phase III trial of Ipi-nivo combination against sunitinib in previously untreated mRCC patients has recently been completed and results will be determined in 2019 (NCT02231749).

Nonsmall cell lung cancer is another malignancy where immunotherapy has reshaped the treatment landscape. Nivolumab is a FDA approved agent in both squamous and nonsquamous NSCLC that experience progression of disease on or after standard platinum-based chemotherapy (regardless of tumor PD-L1 protein expression). In CheckMate 057 trial, 3-month overall survival benefit was shown in nivo arm against docetaxel in second line (HR 0.72, 95% CI 0.60–0.88) in nonsquamous NSCLC [44]. In CheckMate 017 trial, 3-month overall survival benefit was again shown with HR of 0.59 (95% CI 0.44–0.79) [45]. An initial study of ipi-nivo combination showed some level of activity (16% ORR) with high grade of toxicity (35% treatment discontinuation) [46]. CheckMate 227 trial is a phase III study testing ipi-nivo combo in stage IV NSCLC and is currently recruiting participants.

Two other checkpoint inhibitor molecules, tremelimumab (Tre, CTLA-4 inh.) and durvalumab (Dur, PD-L1 inh.) have been studied in NSCLC both as single agents and in combination [47]. Ten different dose escalation cohorts were tested and higher TAEs were observed with increased tremelimumab doses. ORR were as high as 33% with manageable toxicity profile in lower dose cohorts of tremelimumab. As a result of promising ORR in NSCLC of this study, the 20 mg/kg durvalumab + 1 mg/kg tremelimumab combination was chosen as a result for further phase II and III studies. NCT02453282 study is a phase III study which is comparing tre-dur combination against tre monotherapy completed patient accrual and is estimated to be reported at 2018. In sum, combination strategies in NSCLC have yielded encouraging

result and will continue to be investigated further in various studies, highlighting the position of immunotherapy in the general landscape of NSCLC treatment.

The use of CTLA and PD-1 combination is not limited to melanoma, RCC, and NSCLC. A growing body of literature shows that this combination is effective in other malignancies as well. Antonia et al. have showed that ipi-nivo combo can have durable antitumoral response with manageable toxicity in previously treated platin-resistant small cell lung cancer (SCLC) [48]. Furthermore, several clinical trials are already recruiting patients in various tumor types including gastric cancer, head and neck cancer, sarcoma, and endometrial carcinoma, and combinations are being tested in basket trials (NCT02872116, NCT02982486, NCT01658878, and NCT02304458).

Lymphocyte activation gene-3 (LAG-3) is a coinhibitory molecule which enhances regulatory T-cell activity and inhibits T-cell proliferation and effector function [49, 50]. T-cell immunoglobulin and mucin domain 3 (TIM-3) is an inhibitory receptor under the control of helper T-cells and cytotoxic T-cells via IFN-$\gamma$ [51]. Higher expression of TIM-3 was found to be associated with T-cell exhaustion [52]. In preclinical models, monotherapy with LAG-3 or TIM-3 blockade resulted in antitumoral activity and synergistic effect with PD-1 and PD-L1 blockade [53, 54]. There are ongoing preclinical and clinical trials to study the synergism of LAG-3 and TIM-3 inhibition with checkpoint inhibitors.

In sum, single agent durable responses of CTLA-4 inhibitors, PD-1 antagonists, and ORR in almost 25% of patients provide a strong rationale for checkpoint inhibitors being used as backbone in combination immunotherapy regimens.

*3.2. Combinatorial Immunotherapies with Checkpoint Stimulators.* Apart from the targeting invisibility of tumor cells by the immune system, another target for developing immunotherapy are the activator pathways of innate immunity. In murine models, 3 molecules were found to be effective as treatment strategy goals: OX40 (tumor necrosis factor receptor superfamily member 4), GITR (glucocorticoid-induced tumor necrosis factor receptor-related protein), and 4-1BB (CD137). OX40 is a secondary stimulatory molecule expressed by activated T-cells and is responsible for T-cell expansion, activator signal expression, and inhibition of regulatory T-cells [55–57]. OX40 agonism via selectively designed antibodies has showed antitumor response and has been tested in combination with PD-1 antagonists, which yielded promising results [58, 59].

GITR is another stimulatory surface protein responsible for regulatory T-cell suppression and creates resistance by regulatory T-cell inhibition. Both preclinical and in vivo models have showed that GTIR agonism results in reduction in the regulatory T-cell accumulation within tumoral tissue [60, 61]. Dual therapy with GTIR agonism and anti PD-1 inhibition was tested in murine models [62] and resulted in clinical activity as dual therapy, which lead to development of further clinical trials (NCT02221960, NCT01239134) [60, 61].

The surface protein 4-1BB is a multistimulatory receptor protein primarily expressed in T-cells, NK cells, and regulatory T-cells [63]. 4-1BB stimulation leads to an enhancement of the activity of cytotoxic T-cells and increase in survival rates [64]. Murine models showed that 4-1BB is a targetable agent that leads to immune activation and clinical response [65]. A 4-1BB agonist antibody Urelumab was tested in Phase I basket trial, where melanoma patients showed clinical response but at the cost of significant liver toxicity [66].

The strategies of inhibition of the checkpoint with PD-1 and the activation of costimulatory molecules with specific agonistic antibodies are complementary to each other and showed synergistic effects in previous trials, thus providing a compelling rationale for further combination trials.

*3.3. Combination of Immunotherapeutics with Cancer Vaccines and Oncolytic Viruses.* Oncolytic viruses offer synergistic effects with checkpoint blockade by inducing immunogenic cell death and inflammatory tumor response. The use of immune-based treatment approaches is expected to rise, with an increase in variety of the approaches. In preclinical models, Newcastle disease virus (NDW) injections have resulted in systemic responses, and together with anti CTLA-4 therapy, the overall response rates in NDW injections have increased [67, 68].

Talimogene laherparepvec (T-VEC) is an oncolytic virus therapy generated from herpes simplex virus-1. OPTIM study, when compared to T-VEC with a nonstandard treatment arm (GM-CSF), has had durable response rates [69]. After that, T-VEC and ipilimumab combination was tested in phase Ib trial, in which 56% RR was observed [70]. Further studies are needed to conclude on the benefit of combination treatment approach of T-VEC with other IT.

*3.4. Combinatorial Immunotherapies with Cytotoxic Chemotherapy.* Over the past decades, substantial evidence has been found supporting the idea that cytotoxic chemotherapy agents may have potential immune modulatory actions besides being active during cell division and inducing apoptosis. The interaction between the chemotherapeutics and immune system resembles a commensalism. The presence of tumor infiltrating lymphocytes is associated with increased response of CT, whereas some agents like gemcitabine, paclitaxel, cyclophosphamide, and 5-fluorouracil may improve immunity by suppressive T-cell depletion and cytotoxic T-cell activation [71–74]. The findings are consistent with the outcomes from clinical trials. In the first line treatment with NSCLC and SCLC, ipilimumab was used with carboplatin/paclitaxel [75, 76]. Although the response rates were shown to be similar to historical controls, durable responses might occur, which might warrant further clinical trials.

One of the most popular topics of cytotoxic drug and immunotherapy combo trials is gastrointestinal system and especially colorectal cancer (CRC). When considering genomic instability across tumor types, CRC stay in the middle of row in terms of mutational load; however there is heterogeneity. A subset of CRC possesses markedly elevated mutational burden; predominantly these types of CRC are characterized by high microsatellite instability [77, 78]. Nowadays there is an ongoing effort to classify the

colorectal cancers according to genomic profiles [79]. Four consensus molecular subtypes (CMS) of CRC were defined upon agreement [80]. The CMS 1 subtype is characterized by hypermutation, microsatellite instability, and strong immune activation especially [80]. CMS 2 and CMS 3 tumors show low inflammatory and immune characteristics and CMS 4 tumors demonstrate inflammatory and immunosuppressive signatures. Hence, different strategies and different catalyzers and combinations may be required for the success of immunotherapy in subtypes. Microsatellite instability may be a biomarker for immune response to chemotherapy and immunotherapeutic synergy [77]; however, optimal dosing, optimal timing, and necessary precautions to avoid adverse events need to be investigated.

Classical cytotoxic drugs act on tumor microenvironment and create immunogenicity via therapy-induced cell death. Both 5-FU and oxaliplatin have been thought to have a beneficial effect [81]. Based on this hypothesis, FOLFOX is being combined with pembrolizumab in two studies, targeting GI cancers or colon cancer, respectively (NCT02268825, NCT02375672). In a study of atezolizumab in combination with VEGF inhibition with or without chemotherapy, 7% of refractory patients showed response, 14% had stable disease for more than 24 weeks, and a total of 64% patients had stable response. Final data of this trial (NCT01633970) is estimated to announced at the end of 2018. In a very recent analysis, pembrolizumab in combination with mFOLFOX6 had shown efficacy in a phase II trial [82]. Of total 30 patients enrolled, one complete response had occurred in MSH tumor harboring patient and 53% of patients had partial response. The rate of grade 3/4 toxicity was 36.7% in combo arm versus 13.2% in chemotherapy only arm. There was no treatment associated death. Clinical activity was seen in patients with untreated advanced CRC including those with proficient MMR.

Another role of cytotoxic drugs on immune system is in metronomic schedules. Metronomic chemotherapy refers to the administration of chemotherapeutic agents at relatively low, minimally toxic doses, without a prolonged drug-free lag period. It allows for continued, low toxicity and more tolerable drug dosage applications for patients and had shown efficacy [71, 83]. Metronomic therapy was thought to primarily alter endothelial cells and acts via inhibition of angiogenesis [84]. Additionally, there is preclinical and clinical data that support the immune modulatory role of metronomic treatment [71, 83, 85, 86]. Metronomic cyclophosphamide was shown to be effective immunologically and decreasing circulating suppressor T-cell population in low doses whereas high dose applications resulted in depletion of whole lymphocyte population [83].

*3.5. Combination of Immunotherapeutics with Targeted Agents.* Cancer medications have developed in two parallel arms of science. Firstly, a deeper understanding of cancer biology, genetic drivers of carcinogenesis, and signal transduction pathways has led to the development of targeted agents for genetically chosen patients and has resulted in profound and rapid, albeit short-lasting, responses. Secondly, we have come to understand the different ways of tumoral escape from natural protective mechanisms of the body and have obtained immunotherapeutic drugs achieving more durable responses in various types of malignancies. Additional insights of targeted therapies and their effects on immunologic microenvironment of malignancies have served as a foundation for their combinational use.

The Mitogen activated protein (MAP) kinases (MAPKs) comprise part of the intracellular signaling cascade which is essential for signal transduction. Activity of MAPKs plays a crucial role in immune system activity in various steps. First of all, by taking part in cytokine production upon getting signal form toll like receptors, MAPKs are involved in the initial step of innate immunity. Secondly, MAPKs are important for differentiation of T lymphocytes in response to cytokines via binding to appropriate receptors. Additionally, T lymphocyte dependent cytotoxicity is correlated with MAPKs signaled apoptosis and enables the removal of damaged or transformed cells. Hence, the function and appropriate signaling by MAPKs are important for efficacy of immune system and serves as a promising therapeutic role.

Mitogen activated protein kinase pathway is also crucial for various melanoma cases for tumorigenesis. Inhibition of mutated BRAF and MEK has been investigated in many clinical trials and is now one of the most preferred treatments of BRAF mutated melanoma. Aside from clinical efficacy, BRAF and MEK inhibition leads to increased melanoma neoantigen expression, paradoxical activation of MAPK signaling on T lymphocytes, PD-L1 expression upregulation, and inhibition of suppressive cytokines [10, 38, 87, 88]. As the tumor progresses, neoantigen expression diminishes and immunogenic recognition also decreases. BRAF and MEK inhibition results in the reversal of recognition. Furthermore, in the early phases of BRAF/MEK inhibition, there is increased cytotoxic T-cell infiltration in tumor samples [87–89]. Clinical resistance to BRAF inhibitors has been found to be associated with increased PD-L1 expression on melanoma cells [90]. First of all, the combination of BRAF inhibitors with anti-CTLA-4 agents has been tested. A phase II study of sequential therapy with ipilimumab after vemurafenib showed that ORR was 30% with median OS 20 months [91]. However, it is also important to note that the Phase I trial of concurrent administration of ipilimumab and Dabrafenib (Dabra) was terminated early due to hepatotoxicity [92]. A second phase I/II study investigated the safety of Dabra + ipi doublet and Dabra + ipi + trametinib triplet therapy [93]. Severe colitis and intestinal perforation in triplet arm led to the early closure of this cohort. Anti-PD-1 and PD-L1 strategies with BRAF inhibition is also a popular combination for trials. Vemurafenib in combination with anti-PD-L1 agent atezolizumab (Atezo) was tested in the treatment of naive BRAF mutant melanoma cases, which yielded promising early results in RRs [94]. Triplet regimen with vemurafenib + cobimetinib + atezolizumab was tested, which yielded a 83% RR with cumulative 40% grade 3-4 adverse events. Based on these findings, a number of phase III trials have been designed and are currently underway (NCT02908672, NCT02902029). A randomized phase II study with Dabra + trametinib combination with pembrolizumab/placebo is now recruiting patients as a part of KEYNOTE-022 trial (NCT02130466).

Another target for combination strategies in melanoma is c-KIT. Preclinical data and murine models supported that c-kit inhibition results in augmentation of antitumor immunogenicity. A phase I dose escalation study confirmed a clinical response in a small subset of patients with imatinib and ipilimumab (NCT01738139) [95]. The clinical evidence obtained so far supports the clinical use of BRAF, MEK and c-KIT inhibitors with immune checkpoints with manageable toxicity.

Tumor vasculature not only is important for tumor growth and metastasis but also has a crucial role in tumor-immune cell interaction. Vascular endothelial growth factor (VEGF) modulates T-cell response and inhibits APC maturation and the migration of immune cells via endothelia [96–98]. Hodi et al. demonstrated the histologically proven augmentation of immune war with cytotoxic T cells and dendritic macrophages against tumor cells after ipi + Bevacizumab treatment [99]. Consequently, VEGF inhibition with checkpoint inhibitors may be an effective option for advanced tumors in order to increase the monotherapy response rates. Several clinical trials are currently investigating the clinical efficacy of this combination in mRCC, melanoma, glioblastoma, and NSCLC (NCT02210117, NCT02017717, NCT02210117, and NCT00790010).

Sunitinib is the VEGF receptor tyrosine kinase inhibitor that regulates signaling in tumor cells and vasculature. In an early phase I trial, Sunitinib/Pazopanib with Nivolumab was tested and demonstrated better antitumor efficacy compared to the use of single agent mRCC (NCT01472081). Furthermore, targeting agents of WNT pathway, AKT-mTOR signaling, and epidermal growth factor and its receptor inhibition may also be promising strategies for use in combination with IT.

Src family kinases (SFKs) promote cancer progression and are commonly expressed in nonsmall cell lung cancer (NSCLC). Johnson et al. investigated the efficacy of dasatinib in patients with advances NSCLC and showed that it had modest clinical activity [100]. Besides, dasatinib was shown to have immune boosting activity [101–103]. Recently, dasatinib is tested with nivolumab in "An Investigational Immunotherapy Study to Test Combination Treatments in Patients With Advanced Non-Small Cell Lung Cancer" (FRACTION-Lung) trial (NCT02750514).

*3.6. Combination of Immunotherapeutics with Radiation.* Radiation therapy aids immune system in two ways. First of all, it does so via direct toxicity and killing of tumor cells, where antigens are released. Secondly, radiation works as an immune-adjuvant and the inflammatory microenvironment leads to the induction of immune response. Moreover, in murine models, researchers demonstrated that when radiation tumor infiltrating lymphocyte count is upregulated, suppressive CD 8 positive T-cells were abrogated [104]. The abscopal effect defined by Mole et al. refers to the tumor regression distant from the primary radiation field, which is clearly explained by the systemic immune stimulation effect of radiotherapy (RT) [105].

In various tumor models, experiments have demonstrated clinical efficacy of combination with IT [106–108]. CTLA-4 blockade showed synergy with RT [104, 109]. One of the mechanisms of resistance against radiation and anti-CTLA-4 agents was found to be related to T-cell exhaustion due to increased PD-L1 upregulation [110]. Therefore, the blockade of PD-L1 was tested in murine experiments, which yielded evidence supporting the use of combination of PD-L1 and RT [111]. In an analysis of melanoma patients who received RT after progression on ipilimumab, 62% of the patients had a abscopal type of response with 43% ORR [112]. Researchers tested pembrolizumab for head and neck cancer in the concomitant chemoradiation method with cisplatin at a fixed dose of 200 mg IV in every 3 weeks and reported that pembrolizumab with cisplatin is safe and has no deleterious effect on radiation or chemotherapy [113].

## 4. Conclusion

Novel developments in immunotherapy have led to a new era in cancer treatment. Immunotherapeutics, specifically PD-1 and anti PD-L1 antagonists, have shown to elicit important, durable, and safe responses in many tumor types that were once considered among the most desperate malignancies. However, the response rates for immunotherapies still remain modest and the most durable responses are observed only in a small subset of patients.

One of the key limitations of achieving broader responses in clinical trials is the complexity of the host immune system and its interactions with tumor cells. Besides, a more in-depth understanding of tumoral antigen production and recognition, as well as of the escape mechanisms from host immunity and the antitumoral death responses, is essential to overcome the major problems of immune-related drug development. Increased efforts in translational research will further shed light on anticancer drug developments, especially in the immunotherapy area, which will lead to a better understanding of the dynamic interactions between the host immunity and tumor cells.

In order to overcome restricted response rates and increase the number of patients who benefit from the treatment, approaches from precision medicine have been investigated, and predictive biomarker studies have been conducted. Besides these approaches, combinational applications of IT have been hypothesized as solutions for broader range benefits for patients and improved response rates. Among the combination strategies, a backbone with either PD-1 or PD-L1 antagonist drugs along with certain cytotoxic chemotherapies, radiation, targeted drugs, and novel checkpoint stimulators will be the most promising approaches in the future.

## Conflicts of Interest

The author declares that there are no conflicts of interest regarding the publication of this paper.

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*Review Article*

# Immunotherapeutic Strategies for Gastric Carcinoma: A Review of Preclinical and Clinical Recent Development

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Gastric carcinoma (GC) is the 2nd most common cause of cancer-related death. Despite advances in conventional treatment and surgical interventions, a high percentage of GC patients still have poor survival. Recently, immunotherapy has become a promising approach to treat GC. Here, we present preclinical and clinical studies encouraging the use of vaccination, adoptive T-cell therapy (ACT), and immune checkpoint inhibitors, such as programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). The ongoing immunotherapy clinical trials have shown promising results in safety and tolerability even in late-stage GC patients. Moreover, we highlight that the combination of ACT with chemotherapy could be the best choice to treat GC.

## 1. Introduction

GC is the fourth most common cancer in the world and the second most common cause of cancer-related death [1]. Radical surgery remains the first curative choice, while perioperative chemotherapy is a standard treatment in early GC [2, 3]. However, 50% of advanced GC patients suffer from local or systemic recurrence even after standard adjuvant treatment, and only 10–15% of all GC patients achieve 5-year overall survival (OS) [4, 5].

Today, immunotherapy has important clinical applications with potential favorable outcomes and limitations. Common obstacles are the generation of immune effectors, safety, and applicability to a large number of patients. In this regard, it is critical to understand how cancer cells behave and interact with surrounding components in the tumor microenvironment such as parenchymal cells and inflammatory cells including lymphocytes and extracellular matrix (ECM) [6, 7] and the role these elements have in tumor survival, proliferation, and metastasis [6]. In tumor microenvironment, cancer cells release cytokines that modify the microenvironment contexture, while noncancer cells secrete cytokines and growth factors that affect both tumor growth and behavior, such as invasion and metastasis [7]. In this dynamic microenvironment, cells interact, which leads to tumor progression.

GC microenvironment is infiltrated with tumor infiltrating lymphocytes (TILs), which have a more pronounced cytolytic activity than stromal T-cells in chronic gastritis, and the high levels of TILs could be considered a good prognostic factor [8].

The oncogenic bacteria *Helicobacter pylori* (*H. pylori*) promote gastric chronic inflammation that contributes to intestinal metaplasia development and oncogenic mutations in GC by downregulating immune reactions through interference with antigen presentation, inactivation of T-cell proliferation, and fostering of T-cell apoptosis partially via human interaction domain 2 (VacA) [8, 9]. Accordingly, in vivo studies have proposed that type 1 T helper cells (Th1) have a main role in controlling *H. pylori* through cytokine release, B-cell activation, and production of antibodies [9]. Therefore, in the absence of Th1 cytokines, such as interferon-gamma (IFN-$\gamma$), both gastric atrophic changes and prolonged inflammatory response are abrogated [9].

Here, we will review current research and application of immunotherapy in GC, also focusing on novel therapies

with immune checkpoint inhibitors such as the monoclonal antibodies (mAbs) to PD-1/PDL1 or CTLA-4.

## 2. Immunotherapy in GC

Malignant cells can express many different proteins that are potentially recognizable by the immune system; nonetheless, tumors develop immune regulatory circuits with immunosuppressive effects on the cancer environment which interfere with the antitumor response [10]. Immunotherapy represents a therapeutic opportunity capable of modulating the host immune system to fight cancer with less toxicity than conventional chemotherapy [10]. Recently, immunotherapy has shown satisfactory clinical results in patients with advanced cancers treated with vaccination, ACT, and/or checkpoint inhibitor mAbs.

## 3. Vaccination in GC

The main role of cancer vaccines is to activate and expand tumor associated antigen- (TAA-) specific T-cells, thus enhancing the antitumor immune response through activation of preexisting immunity, initiation of unprecedented immunity, or strengthening of the current immune response. Several vaccination studies have been performed to enhance immune responses against GC. Dendritic cells (DCs) are antigen presenting cells (APCs) that can activate natural killer (NK) cells, B-cells, and naïve and memory T-cells [11, 12]. Despite having a promising role in cancer vaccination, the use of DCs is limited in clinical trials due to their short life span. Some studies in GC patients have demonstrated the correlation between DC numbers and clinicopathological status and prognosis, where patients with more DC infiltration had less lymph node (LN) involvement and better OS [13–15]. A study where DCs from advanced gastrointestinal tumor patients were pulsed ex vivo with melanoma-associated antigen (MAGE) A3 peptides (expressed also in GC-56-REF) showed an improvement in performance status in 4 patients, while 3 additional patients had minor tumor regression without direct correlation between outcome and immune response [16]. In a phase I clinical trial, 9 advanced or recurrent GC patients with tumors overexpressing the human epidermal growth factor receptor-2 (HER2)/neu received a regimen of DCs pulsed with HER2($_{p369}$) peptide. Vaccine was well tolerated and induced tumor specific T-cell response, with partial clinical response and decrease in carcinoembryonic antigen (CEA) marker in one patient and stable disease for 3 months in another patient [17]. Regimens of cancer vaccines associated with chemotherapy showed promising results in GC patients. In radically resected stage III/IV GC, a combination of adjuvant Bacille Calmette-Guérin (BCG) vaccine with chemotherapy resulted in a prolonged 10-year OS (47.1%) as compared to monochemotherapy (30%) or surgery alone (15.2%) [18]. In a phase II clinical trial involving patients with advanced GC and gastroesophageal junction (GEJ) adenocarcinoma, the gastrin-17 diphtheria toxoid (G17DT) vaccine targeting gastrin peptide in association with cisplatin and fluorouracil (5-FU) chemotherapy led to a longer time-to-progression (TTP in 69% of patients considered immune responders and a better OS compared to the nonimmune responder patients) [19]. Recently, a phase I clinical trial by Higashihara et al. demonstrated the safety of HLA-A∗2402-restricted URLC10-A24-177 and vascular epidermal growth factor receptor (VEGFR1-A12-9 1084) epitope peptide cancer vaccines in 14 chemotherapy-resistant advanced GC patients. Specific cytotoxic T-lymphocytes (CTLs) positive responses were determined in 62.5% and 50% of patients for URLC10 and VEGFR1, respectively [20].

## 4. Preclinical Studies of ACT in GC

GC has different precursor events such as *H. pylori*, atrophic gastritis, and intestinal metaplasia and dysplasia [21] with a multistep carcinogenesis including genetic variants and molecular abnormalities that lead to a malignant transformation of the gastric mucosa [22–24]. The cofactors involved in GC pathogenesis are still unknown and the detailed mechanism of cancer development is uncertain [25].

GC adenocarcinomas are histologically classified according to the 2010 WHO classification [24] into four major subtypes: tubular, mucinous, papillary, and poorly cohesive and uncommon variants.

Each GC subtype has its featured genetic profile and molecular diversities. Targeting the specific molecular abnormalities could prevent tumor cells from skipping the host immune system and also predict the prognosis. Hence, genetic and molecular studies are needed to understand different pathognomic molecular expressions in GC cells and distinguish which subtype will benefit from immunotherapy [22, 26].

NK cells have cytotoxic activity against solid tumors including both allogeneic and autologous derived GC cells lines [27] and could prevent cancer metastatic dissemination [28]. A high NK cell level, demonstrated by the expression of CD57 antibody in 146 GC tissue sample, was associated with smaller tumors, less LN involvement, a higher rate of surgical care, and a better 5-year OS [29], indicating a possible prognostic role of these cells in GC. Nie et al. used different HLA-A matched allogeneic GC cells to stimulate peripheral blood lymphocytes from GC patients or from healthy donors and assessed them against different cell lines. Induced CTLs had antitumor effects against HLA-A2 and HLA-A24 GC cell lines with no effect against HLA-A2 negative GC cells or any other cancer cells [30]. When TILs and specific T-cells from peripheral blood of GC patients are expanded in vitro, they show specific type 1 T-cells response to GC antigens. This would reduce tumor growth; however, Th1/Tc1 response would be enhanced by vaccination with the appropriate cancer peptides or by injection of the autologous tumor peptide-specific T-cells expanded in vitro [31].

In addition, Kono et al. isolated major histocompatibility complex-1 (MHC-1) restricted T-cells specifically binding to GC antigens from primary tumors, metastatic LNs, and ascites of autologous GC, which showed different recognition patterns towards GC antigens [32]. Fujie et al. succeeded in using splenic MAGE-specific CTLs targeting HLA-A2

cancer cells, an antigen expressed in testis and several cancers including GC, pointing out the role of spleen in ACT in GC [33]. Cytokine induced killer cells (CIK), as well as other interesting immune competent cells, are considered a good choice in ACT in different tumors [34–37]. CIK cells are a heterogeneous population of immune effector cells generated after culturing lymphocytes with an anti-CD3 antibody and other cytokines such as IFN-$\gamma$ and interleukin-2 (IL-2) in vitro with a high proliferative activity and antitumor cytotoxic effect [38]. CIK cells have antiproliferative and antiapoptotic activity against the MGC-803 GC cell line [39] and the MKN74 human GC cell line, mainly releasing IFN-$\gamma$ and tumor necrosis factor-alpha (TNF-$\alpha$). MKN74 tumor bearing nude mice injected with 3 million and 10 million CIK cells showed 58% and 78% tumor reduction, respectively [40].

ACT is recommended in combination with chemotherapy due to difficulty in GC stroma infiltration as shown in in vivo studies [41, 42].

Besides its cytotoxic effect through inhibition of DNA synthesis and transcription, oxaliplatin can also induce an immunogenic cancer cell death (ICD) triggering the high-mobility group box 1 protein to induce T-cells against tumor cells [43]. Therefore, the combination of CIK cells with oxaliplatin against drug resistant GC in in vitro and in vivo experiments resulted in a release of large amounts of cytokines, such as IL-2, with a significant antitumor effect compared to monotherapy with chemotherapy or CIK cells only [44].

T-cell depleting chemotherapy would improve ACT efficacy as host immunosuppression status prolongs the persistence of endogenous T-cells in circulation, while reducing autoimmune reactions on normal tissue. However, patients have severe toxicities due to infectious complications [45]. Thus, Kobold et al. improved ACT efficacy in a GC mouse model without depleting T-cells by addressing T-cell recruitment to tumors. Simian virus 40 (SV40) T antigen-specific T-cells were transduced with a truncated human epidermal growth factor receptor (EGFR) as a marker protein. The combination of ACT with an anti-EGFR, antiepithelial cell adhesion molecule (EpCAM) bispecific antibody (BiAb) that selectively recognizes transduced T-cells increased T-cell infiltration of tumors, reduced tumor growth, and prolonged survival when compared to ACT only or control antibody [46].

Du et al. studied the biodistribution and antitumor effects of CIK cells via peritumoral, intravenous (I.V.), and intraperitoneal routes in GC mice model. Only a limited number of CIK cells succeeded in reaching the tumor via I.V. and intraperitoneal routes, while peritumoral injection showed high accumulation of CIK cells in the tumor site for 48 hours with a better antitumor response. This indicates that peritumoral injection of effector cells represents an effective delivery method of ACT with a minimally invasive surgical procedure [47].

## 5. Clinical Studies of ACT in GC

Activated T-lymphocytes showed promising results against several malignancies in several clinical trials [48]. Some clinical trials evaluated the efficacy of ACT when combined with chemotherapy in advanced GC patients.

Zhang et al. evaluated the prognostic role of expanded activated autologous lymphocytes (EAALS) stimulated by anti-CD3 mAb (OKT3) and IL-2 in GC patients. 42 GC patients who received EAALS had a better OS than the control group that received conventional treatment only ($p = 0.028$) [49]. In a randomized clinical trial, T-activated lymphocytes (TALs), extracted from patients, expanded in vitro with IL-2, and stimulated with autologous tumor, were administered either intraperitoneally or intravenously to 44 advanced GC patients in combination with chemotherapy (low-dose cisplatin and 5-FU) to evaluate the survival benefit. Patients receiving the combined treatment showed a marked improvement in OS compared to those who received chemotherapy only ($p < 0.05$) [50].

Jiang et al. evaluated the combined regimen of CIK cells with chemotherapy (FOLFOX4) in 32 advanced GC patients after palliative gastrectomy. In comparison with the control group (FOLFOX4 only), the combined regimen had a marked reduction of tumor markers, higher total remission rate (56.3% against 48%), and better quality of life (QoL) but no differences in 2-year OS [51]. To evaluate the possible toxicities of combining ACT and chemotherapy in GC elderly patients, Jäkel et al. assessed a regimen of chemotherapy (FOLFOX) followed by autologous CIK cells. Side effects were not severe and were reversible, and patients had a better total remission rate [52]. These results motivate more studies on combining CIK cells with chemotherapy in advanced GC to confirm the effects on OS.

In a clinical trial, GC patients received a combination of autologous NK cells, $\gamma\,\delta$ T-cells, and CIK cells with chemotherapy. Two-year progression free survival (PFS) improved significantly and the regimen was well tolerated with better QoL but with no statistically significant difference in 2-year OS [53]. Wada et al. performed a pilot study, where 7 patients received gamma delta T-cell type (V$\gamma$9V$\delta$2) with zoledronate intraperitoneally as a local treatment for malignant ascites in advanced GC; a marked reduction in the number of peritoneal malignant cells and ascetic volume was observed with no marked or irreversible side effects [54]. In another trial, a regimen of capecitabine and oxaliplatin in combination with CIK cells administered intraperitoneally in GC malignant ascites showed a marked improvement of malignant ascetic volume and OS with low side effects [52].

Other clinical trials were performed to evaluate the ACT/chemotherapy combination in R0 postsurgically resected GC patients. A combination of CIK cells and chemotherapy was used in stage II/III GC after radical gastrectomy. A marked benefit was noticed with significant difference in 5-year OS compared to the control group that received chemotherapy alone (56.6% versus 26.8%, $p = 0.014$) and no marked side effects were noted [55]. Shi et al. conducted a clinical trial evaluating autologous CIK cells with chemotherapy (5-FU backbone) in 151 stage III/IV (M0) GC patients after (R0/D2) gastrectomy. Results showed a significant improvement in both 5-year OS (32.4%, $p = 0.071$) and 5-year disease-free survival (DFS) (28.3%, $p = 0.044$) compared to the monochemotherapy control group [56].

A clinical trial evaluated the possible toxicities of ACT/chemotherapy regimens in GC patients. After R0/D2 gastrectomy, 89 stage II/III GC patients received autologous CIK cells plus 5-FU or capecitabine backbone chemotherapy. Only 23.6% of patients had grade I/II side effects such as fever, fatigue, rash, and diarrhea, while none suffered from grade III/IV side effects or an autoimmune response. In addition, the regimen showed improvement in DFS ($p = 0.006$) and OS ($p = 0.028$) [57].

## 6. Ongoing Clinical Trials of ACT in GC

Currently, several ongoing clinical trials use ACT in different advanced solid tumors including GC. A regimen of preconditioning chemotherapy (cyclophosphamide/fludarabine) and anti-PD-1 mAb is administered followed by I.V. infusion of in vitro expanded autologous TILs and IL-2 [58]. In a current clinical trial, chimeric antigen receptor (CAR) T-cells specific for EpCAM were infused into relapsed/refractory GC patients evaluating CAR T-cell safety and efficacy [59].

Currently, a phase I/II clinical trial is investigating the cytotoxic activity of engineered pluripotent stem cells (iPIK) and T-cells, which specifically bind to HER2 of GC in patients with liver metastasis [60]. In a current clinical trial also targeting HER2 in GC, the safety and efficacy of therapy with trastuzumab and NK cells are being evaluated. Patients receive both trastuzumab and NK cells in the first cycle and then trastuzumab for another 3 cycles, except for patients with a tumor response after 2 cycles who then receive NK cells in the fourth cycle [61]. Another clinical phase I trial assesses the safety of bispecific antibody armed autologous T-cells (HER2Bi-Armed T-cells) in GC and esophageal cancers [62].

Currently, a phase I/II clinical trial assesses CAR T-cells specifically targeting mucin 1 (MUC1) in solid tumors including GC, as its overexpression interferes with chemotherapy leading to refractory cancers [63].

In a current phase I/II clinical trial, advanced metastatic GC and GEJ cancer patients receive a combination of S-1 (5-FU prodrugs tegafur, gimeracil, and oteracil) and dendritic cell activated CIK (DC-CIK) [64].

A current phase I/II clinical trial is assessing adoptive $\gamma\ \delta$ T-cell and CIK cell therapy by monitoring drug related toxicity in stages II-IV GC patients [65]. In a current phase 1b clinical trial, anti-CEA CAR T-cells are injected into the hepatic artery targeting hepatic metastasis from GC expressing CEA as TAA [66].

Other clinical trials are evaluating regimens of ACT and chemotherapy after oncosurgical intervention in advanced GC patients [67]. In one such phase II trial, a regimen of autologous tumor lysate-pulsed dendritic and CIK cells (Ag-D-CIK) and chemotherapy is currently being evaluated in stages I-III GC after radical gastrectomy [68].

## 7. Preclinical Studies of Checkpoint Inhibitors

CTLA-4 and PD-1 are T-cell inhibitory receptors known as checkpoint molecules that play a critical role in immune inhibition. Due to its higher affinity, CTLA-4 competes with CD28 on T-cells for receptors CD80 and CD86 on APCs interfering with T-cell activation downregulating the immune response [69–71]. PD-1 is expressed on activated T-cells, NK cells, and B-cells, while the transmembrane protein PD-L1 is expressed on several immune cells and tumor cells in the presence of inflammatory mediators. PD-1/PD-L1 axis is dynamically active in peripheral tissue to control inflammatory reactions [72], while, in malignancy, PD-1 on activated T-cells binds to PD-L1 on tumors providing tumor escape and subsequent tumor progression [73, 74]. PD-1/PD-L1 overexpression has been observed in numerous malignancies including GC, and restoration of antitumor T-cell activity by targeting checkpoint molecules has been demonstrated in several studies [75]. Currently, different studies are trying to better understand the genetic and molecular pathways of checkpoint molecules to develop targeted mAbs in GC, which is considered a good candidate for this field of study [76, 77].

## 8. Genetic Studies of Checkpoint Inhibitors

Aberrant PD-1 expression was determined in GC, provoking its role in tumor skipping from the immune system. Several studies have demonstrated a possible connection between PD-1 or CTLA-4 polymorphism and GC development [78–82].

Savabkar et al. analyzed DNA of 122 GC using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay, showing high frequencies of PD-1.5CT genotypes in GC ($p = 0.026$) [78]. Tang et al. extracted DNA from lymphocytes and used ligation detection reaction (LDR) to detect polymorphisms. The study, which involved analysis of three single nucleotide polymorphisms (SNPs) in newly diagnosed 330 gastric cardia adenocarcinoma (GCA) patients, revealed a possible correlation between GCA and PD-L1 SNPs (PD-1 rs2227982 C>T type) [79]. Hayakawa et al. reported a patient with an autosomal dominant immune dysregulation syndrome developed from CTLA-4 haploinsufficiency. When the patient was 34 years old, he developed multifocal poorly differentiated GC with atrophic gastritis, the same condition observed in at least 2 other patients, suggesting a role of autosomal dominant immune dysregulation syndrome due to CTLA-4 haploinsufficiency in GC development [83]. In 2014, Kordi-Tamandani and his group pointed out the role of CTLA-4 gene promoter hypermethylation as a risk factor in developing GC. CTLA-4 gene methylation was markedly correlated with GC when compared to the unmethylated gene (OR = 4.829; 95% CI: 2.46–9.48; $p < 0.001$) and the CTLA-4 expression profile was markedly higher in GC tissue samples than in normal tissue on the tumor margins [84].

## 9. PD-1/PD-L1 and CTLA-4 Expression and Prognostic Role

Several studies revealed high PD-L1 expression on GC, suggesting a possible response to a PD-L1 mAb therapy. PD-L1 is 50% expressed in Epstein-Barr virus $(EBV)^+$ GC

tumor cells and 94% in immune cells, while in EBV$^-$ GC the PD-L1 expression was positive only when associated with microsatellite instability (MSI), suggesting that patients with EBV$^+$ and MSI GC may have better response to PD-1 blocking therapy [85]. Furthermore, Saito et al. confirmed that PD-1 expression on CD8$^+$ and CD4$^+$ T-cells in GC is higher compared to normal gastric mucosa [86].

CD8$^+$ T-cells, isolated from GC tissue samples and peripheral blood mononuclear cells (PBMCs), markedly expressed PD-1 in GC patients compared to healthy donors. Studies that evaluated PD-1/PD-L1 role as a prognostic factor and its correlation with clinicopathological status showed controversial results. Although some studies revealed PD-L1 expression as a predictive marker for a PD-L1 mAb therapy, other studies revealed a tumor response to PD-L1 therapies with no PD-L1 expressing malignant cells [87, 88]. Sun et al. detected PD-L1 expression in 42.2% of GC tissues with no expression in normal gastric and gastric adenoma samples. PD-L1 expressing GC was associated with an increase in tumor size ($p < 0.05$), LN involvement ($p < 0.01$), and deep invasion ($p < 0.01$). PD-L1 was expressed in fresh isolated T-cells while it was less expressed in B-cells and DCs [89] and one of these mAbs dampened PD-L1 inducing T-cell apoptosis [89]. Schlößer et al. evaluated PD-1 and PD-L1 expression in GC tumor microenvironment and regional LNs [90]. Nearly half of GC patients (44.9%) expressed PD-L1 in tumor microenvironment which contained high numbers of TILs. PD-L1$^+$ primary tumors were associated with 100% regional LN involvement. Additionally, mean OS in PD-L1$^+$ was markedly lower than in PD-L1$^-$ patients (39.1 months versus 54.2 months, $p = 0.011$), indicating the role of PD-L1 as an independent worse prognostic factor in GC ($p = 0.024$) [90]. In 34 newly surgically resected GC and GEJ adenocarcinoma samples, PD-L1 was expressed in 12% of malignant cells and in 44% of tumor microenvironment nonmalignant cells. Samples dense with CD8$^+$ T-cells showed higher PD-L1 expression in both malignant and nonmalignant stromal cells with a decrease in PFS and OS [91]. No correlation was found between PD-L1 expression and staging, indicating that inhibition may occur in early stages as well as late stages of disease [91]. The study by Chang et al. revealed a marked correlation between PD-1/PD-L1 expression in tumor cells and TILs of GC and clinical progression, namely, advanced tumors ($p < 0.001$), LN involvement ($p < 0.001$), and perineural invasion ($p < 0.001$). In TILs, CD8$^+$ T-cells with high PD-L1 expression had a lower 5-year OS ($p < 0.001$); thus, their expression as an independent prognostic factor in 5-year OS is still controversial [92].

Another study considered PD-L1$^+$ T-cell increase as a poor prognostic factor in GC. Immunohistochemistry (IHC) analysis performed in 132 stage II/III GC after surgical resection showed PD-L1$^+$ expression in 50.8% of samples, especially in tumors larger than 5 cm ($p = 0.036$) with low 5-year OS ($p < 0.001$) [93]. An IHC study correlated PD-L1 expression to a poor 3-year DFS ($p < 0.05$), enlarged tumors ($p = 0.046$), and lymphatic invasion ($p = 0.007$) [94].

In addition, PD-L1 expression was correlated with tumor invasion ($p = 0.004$) and poor survival ($p = 0.017$) in GC patients. In this study, tumor invasion was determined using the contrast enhanced ultrasonography (CEUS). CEUS has several advantages; it is a well-tolerated noninvasive technique in contrast to the standard invasive upper gastrointestinal endoscopy and has a smaller ionizing burden than a computed tomography (CT) scan. This study pointed out the promising role of this imaging technique in predicting PD-L1 expression ($p = 0.0003$) [95]. A recent meta-analysis comprised 10 studies with 1901 GC patients assessing PD-L1 expression, low OS ($p = 0.01$), and poor clinicopathological status [96]. In contrast to previous studies, more recent studies showed that PD-L1 expression in GC may be a good prognostic factor. Böger et al. studied PD-1 and PD-L1 expression in 465 GC and 15 hepatic metastasis tissue samples. Results correlated with the high PD-L1 expression in tumor and immune cells and the better OS [73]. In another study, the high circulating PD-L1 expression in 80 advanced GC patients showed a marked correlation with LN involvement ($p = 0.041$) and a statistically significant better 5-year OS ($p = 0.028$) [97]. In addition, Kim et al. involved 243 GC patients who underwent radical oncosurgical resection, revealing a favorable role of PD-L1 expression as a prognostic factor [98]. In the above-mentioned study by Schlößer et al., CTLA-4 expression was also evaluated in tumor microenvironment and regional LNs in 127 GC patients. Positive CTLA-4 expression was revealed in the tumor microenvironment in 86% of patients; it had low expression in TILs but a strong correlation between its positive expression and poor OS ($p = 0.018$) and between its negative expression and the high grading and diffuse type malignant cell occupation ($p = 0.012$ and $p = 0.006$, resp.). Also, CTLA-4$^+$ primary tumors are associated, in most cases, with positive LN involvement. Yet, the CTLA-4 expression is not considered as an independent prognostic factor ($p = 0.062$) [90].

## 10. Clinical Trials of Checkpoint Inhibitors

Up to now, most GC clinical trials involving checkpoint inhibitors are phase I and II trials. Takaya et al. evaluated PD-1$^+$ T-cells levels before and after gastric resection in 33 GC patients, showing higher PD-1$^+$ T-cell expression after surgical resection [77]. Therefore, according to this study, the use of checkpoint inhibitors as adjuvant chemotherapy after gastric resection is recommended in more trials as the surgical stress could upregulate PD-1$^+$ T-cell levels inhibiting the immune response. A multicenter study evaluated anti-PD-L1 adverse effects in a phase I clinical trial when applied to patients with different solid tumors, including 7 GC patients. The majority of patients (61%) suffered from side effects, mostly low grade, such as fatigue, nausea, diarrhea, and headache, while only 9% of patients suffered from grade III/IV side effects. However, 39% of patients had related immune toxicity, including hypothyroidism and hepatitis [99]. A phase II clinical trial by Ralph et al. showed a low objective response rate when anti-CTLA-4 mAb tremelimumab was administered in 18 locally advanced/metastatic GC and esophageal cancer patients as a second-line treatment after failure of cisplatin backbone chemotherapy. Patients

received varying numbers of tremelimumab cycles every 3 months. Drug was tolerable with mild toxicities and only a single death due to intestinal perforation resulting from autoimmune colitis. Antitumor response was evaluated in four patients who had stable disease and one patient who achieved partial response in the period between 25.4 months and 32.7 months after the beginning of treatment [100]. In a case study, a 64-year-old stage IIA GC patient underwent subtotal gastrectomy, had a recurrence, and subsequently received conventional chemotherapy with trastuzumab and pertuzumab. He had no clinical response. With pembrolizumab every 3 weeks, he achieved partial response with no drug related toxicity and a marked decrease in CEA levels. In this patient, IHC and PCR studies showed PD-L1$^+$ and proficient mismatch repair (pMMR)$^+$. This is the first study showing pMMR/microsatellite stability response to anti-PD-L1 mAbs in GC patients [101].

## 11. Ongoing Clinical Trials of Checkpoint Inhibitors

Recently, ongoing phase I/II clinical trials use the combination of checkpoint inhibitors nivolumab and ipilimumab or monotherapy with nivolumab in advanced GC and GEJ cancer patients; MEDI4734 and tremelimumab are being used in another trial [102, 103]. Up to date, results of the first trial showed nivolumab to be a well-tolerated drug with antitumor efficacy in advanced GC and GEJ adenocarcinoma [104]. Another ongoing phase III study compares the combination of nivolumab and ipilimumab with the combination of nivolumab and chemotherapy in advanced GC and GEJ adenocarcinoma patients [105]. In other studies, anti-PD-L1 mAbs are being evaluated as a monotherapy and compared with conventional chemotherapy in GC. Monotherapy nivolumab is currently being assessed in a phase III clinical trial in advanced GC and GEJ cancer patients and atezolizumab is currently being assessed in a phase I clinical trial [106, 107]. Currently, nivolumab is the first immunotherapy treatment for advanced GC and GEJ cancer patients in phase III trial, achieving marked improvement in OS ($p < 0.0001$) and PFS ($p < 0.0001$) [108].

Nivolumab is also being investigated as an adjuvant monotherapy in resectable GEJ cancer patients [109]. Anti-PD-L1 avelumab is currently being investigated in a phase I clinical trial against different advanced solid tumors including GC and GEJ cancer, and the preliminary results show a safe and tolerable drug in treated patients [110, 111]. An ongoing phase III clinical trial currently compares pembrolizumab (MK-3475) and paclitaxel as a second-line treatment in advanced GC and GEJ cancer after a first-line failure with platinum or 5-FU [112]. Another ongoing phase 1b trial is assessing the antitumor effect and safety of pembrolizumab in different solid tumors including PD-L1$^+$ GC, and preliminary results reveal its controllable toxicity and effective cytotoxicity against advanced GC patients [113, 114]. Anti-PD-L1 (avelumab) is compared with conventional chemotherapy as a first- and third-line treatment in advanced GC and GEJ cancers in phase III trials [115, 116].

In a phase II clinical trial, ONO-4538 (nivolumab) combined with chemotherapy is assessed in advanced and recurrent GC [117]. In another phase I/II study, nivolumab was evaluated as monotherapy and in combination with chemotherapy against EBV$^+$ GC [118]. In a phase I/II clinical trial, pembrolizumab is involved in a neoadjuvant treatment plan, which includes chemotherapy and radiotherapy in resectable GCA and GEJ (cancer stages IB-IIIB) [119]. Pembrolizumab combined with trastuzumab and chemotherapy in HER2$^+$ GC patients is being evaluated in another phase I/II clinical trial [120]. Pembrolizumab (MK-3475)/chemotherapy or monotherapy pembrolizumab is currently being assessed in clinical trials phases II and III in advanced GC and GEJ cancers [121–123]. Maintenance therapy using anti-PD-L1 (MEDI4736) in locally advanced and metastatic GEJ adenocarcinoma after the standard first-line treatment is currently being investigated in a phase II trial [124].

Ongoing clinical trials of checkpoint inhibitors are summarized in Table 1.

## 12. Conclusion

GC is a common malignancy with poor prognosis despite advances in surgical interventions and chemotherapy and radiotherapy techniques. Therefore, seeking novel treatment approaches is necessary. In this paper, we reviewed the recent studies on vaccination, on ACT, and on the use of checkpoint inhibitors in GC.

Vaccination is safe and tolerable and showed improvement in PFS and OS, especially when combined with chemotherapy. GC microenvironment is highly infiltrated with high cytolytic TILs with different recognition patterns towards GC antigens depending on their presentation in primary site, involved LNs, or metastatic sites. ACT in GC showed promising results in preclinical studies; it demonstrated tolerable side effects and antitumor cytotoxic efficacy against GC in both primary and metastatic sites. In clinical studies, ACT has a tolerable toxic profile, even in elderly patients, tumor reduction when administered either systemically or locally (intraperitoneal injection), and improved QoL and OS, especially when combined with conventional chemotherapy in both radically resected and advanced GC patients. However, more genetic and molecular studies are still needed to understand different pathognomic molecular expressions and distinguish which subtype of GC could be more sensitive to ACT. The PD-1/PD-L1 expression could be a prognostic factor in GC; however, results are controversial and it remains to be seen whether to consider high expression as a good prognostic factor or a poor one. Although clinical trials targeting PD-1/PD-L1 or CTLA-4 are, in most of cases, in phase I or II but with too few patients to make any conclusions, some updated results of ongoing clinical trials show promising results. Nevertheless, checkpoint inhibitor therapy provides a good safety profile in most cases, with modest antitumor response when combined with chemotherapy in advanced chemoresistant GC.

Table 1: Ongoing clinical trials using the immune checkpoint inhibitors in GC.

| Agent | Trial name/number | Phase | Trial population | Primary end points | Estimated study completion date |
|---|---|---|---|---|---|
| Nivolumab | A Study of Nivolumab by Itself or Nivolumab Combined With Ipilimumab in Patients With Advanced or Metastatic Solid Tumors/NCT01928394 | I/II | Advanced solid tumors including GC | Overall response rate (ORR) | Dec-18 |
| MEDI4736 Tremelimumab | A Phase 1b/2 Study of MEDI4736 With Tremelimumab, MEDI4736 or Tremelimumab Monotherapy in Gastric or GEJ Adenocarcinoma/NCT02340975 | I-II | GC or GEJ Adenocarcinoma | ORR, PFS, and safety | 17-Aug-18 |
| Nivolumab/Ipilimumab | Efficacy Study of Nivolumab Plus Ipilimumab or Nivolumab Plus Chemotherapy Against Chemotherapy in Stomach Cancer or Stomach/Esophagus Junction Cancer (CheckMate649)/NCT02872116 | III | GC or GEJ Adenocarcinoma | OS | 11-Oct-20 |
| ONO-4538 (Nivolumab) | Study of ONO-4538 in Unresectable Advanced or Recurrent Gastric Cancer/NCT02267343 | III | Unresectable advanced or recurrent GC and GEJ adenocarcinoma | OS | Aug-17 |
| MPDL3280A (Atezolizumab) | A Phase 1 Study of Atezolizumab (an Engineered Anti-Programmed Death-Ligand 1 [PDL1] Antibody) to Evaluate Safety, Tolerability and Pharmacokinetics in Participants With Locally Advanced or Metastatic Solid Tumors/NCT01375842 | I | Locally advanced/metastatic solid tumor including GC | Dose limited toxicity | 31-May-18 |
| Nivolumab | An Investigational Immuno-therapy Study of Nivolumab or Placebo in Patients With Resected Esophageal or Gastroesophageal Junction Cancer (CheckMate 577)/NCT02743494 | III | Resected esophageal and GEJ cancer | DFS/OS | 1-Apr-21 |
| Avelumab | Avelumab in Metastatic or Locally Advanced Solid Tumors (JAVELIN Solid Tumor)/NCT01772004 | I | Metastatic or locally advanced solid tumors including GC and GEJ adenocarcinoma | Dose limiting toxicity/best overall response | 31-May-18 |
| Pembrolizumab (MK-3475) | A Study of Pembrolizumab (MK-3475) Versus Paclitaxel for Participants With Advanced Gastric/Gastroesophageal Junction Adenocarcinoma That Progressed After Therapy With Platinum and Fluoropyrimidine (MK-3475-061/KEYNOTE-061)/NCT02370498 | III | Advanced GC and GEJ adenocarcinoma | PFS/OS | Aug-17 |
| Pembrolizumab (MK-3475) | Study of Pembrolizumab (MK-3475) in Participants With Advanced Solid Tumors (MK-3475-012/KEYNOTE-012)/NCT01848834 | I | Advanced solid tumors including GC | Adverse events | May-17 |

Table 1: Continued.

| Agent | Trial name/number | Phase | Trial population | Primary end points | Estimated study completion date |
|---|---|---|---|---|---|
| Avelumab | Avelumab in First-Line Maintenance Gastric Cancer (JAVELIN Gastric 100)/NCT02625610 | III | Unresectable locally advanced/metastatic GC and GEC adenocarcinoma | OS | 31-Mar-24 |
| Avelumab | Avelumab in Third-Line Gastric Cancer (JAVELIN Gastric 300)/NCT02625623 | III | Unresectable, recurrent, locally advanced, or metastatic GC and GEH adenocarcinoma | OS | 30-Sep-22 |
| ONO-4538 (Nivolumab) | Study of ONO-4538 in Gastric Cancer/NCT02746796 | II | Unresectable advanced or recurrent GC and GEJ adenocarcinoma | ORR | Aug-20 |
| Nivolumab/Ipilimumab | An Investigational Immuno-therapy Study to Investigate the Safety and Effectiveness of Nivolumab, and Nivolumab Combination Therapy in Virus-associated Tumors (CheckMate358)/NCT02488759 | I/II | Virus-associated tumors including EBV GC | Drug related toxicity, ORR, and rate of surgery delay | Dec-19 |
| Pembrolizumab | Pembrolizumab, Combination Chemotherapy, and Radiation Therapy Before Surgery in Treating Adult Patients With Locally Advanced Gastroesophageal Junction or Gastric Cardia Cancer That Can Be Removed by Surgery/NCT02730546 | I/II | Unresectable locally advanced GC and GEJ adenocarcinoma | Pathological complete remission/PFS | Apr-18 |
| Pembrolizumab | Pembrolizumab, Trastuzumab, HER2 Positive Gastric Cancer/NCT02901301 | I/II | HER2 positive GC | ORR | Mar-18 |
| Pembrolizumab (MK-3475) | Study of Pembrolizumab (MK-3475) as First-Line Monotherapy and Combination Therapy for Treatment of Advanced Gastric or Gastroesophageal Junction Adenocarcinoma (MK-3475-062/KEYNOTE-062)/NCT02494583 | III | Advanced GC and GEJ adenocarcinoma | PFS/OS | 6-Jun-20 |
| Pembrolizumab (MK-3475) | Study of Pembrolizumab (MK-3475) Versus Investigator's Choice Standard Therapy for Participants With Advanced Esophageal/Esophagogastric Junction Carcinoma That Progressed After First-Line Therapy (MK-3475-181/KEYNOTE-181)/NCT02564263 | III | EGJ adenocarcinoma | PFS/OS | 31-Aug-18 |
| Pembrolizumab (MK-3475) | A Study of Pembrolizumab (MK-3475) in Participants With Recurrent or Metastatic Gastric or Gastroesophageal Junction Adenocarcinoma (MK-3475-059/KEYNOTE-059)/NCT02335411 | II | Advanced GC and GEJ adenocarcinoma | Drug related toxicity/ORR | Jun-18 |

Table 1: Continued.

| Agent | Trial name/number | Phase | Trial population | Primary end points | Estimated study completion date |
|---|---|---|---|---|---|
| MEDI4736 | Planning Treatment for Oesophago-gastric Cancer: a Maintenance Therapy Trial (PLATFORM)/NCT02678182 | II | Locally advanced or metastatic HER2 positive or HER2 negative oesophagogastric adenocarcinomas | PFS | Feb-20 |

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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*Review Article*

# Mismatch Repair Deficiency as a Predictive Biomarker for Immunotherapy Efficacy

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Immunotherapy has revolutionized cancer treatment. Immune-checkpoint inhibitors, on balance, showed a favorable efficacy/toxicity profile with durable response in different cancer types. No predictive biomarker has been validated thus far to select patients who would benefit from therapy. Among the candidate predictive biomarkers, mismatch repair status of the tumor is currently one of the most promising. Indeed, tumors displaying mismatch repair deficiency or microsatellite instability showed remarkable response to immunotherapy in clinical trials. This correlation has been first reported in colorectal cancers, but similar results have been observed also in other cancer types. The possible mechanism behind this correlation may be the higher mutational load observed in mismatch repair deficient tumors, leading to neoantigens formation, recruitment of immune cells, and release of proinflammatory factors in the microenvironment. These results support an approach to treatment based on assessment of the genomic stability of the tumor besides its biologic characteristics and may change our therapeutic decision making process. However, due to the small percentage of patients with tumors displaying mismatch repair deficiency, data from clinical trials should not be considered definitive and need further confirmation.

## 1. Introduction

*1.1. Immunotherapy and Immune-Checkpoint Inhibitors.* The immune system manipulation has been increasingly acquiring a central role in cancer treatment; thanks to a deeper understanding of immune system function in terms of antitumor activity, several strategies targeting immune cells and the microenvironment are under development. Undoubtedly, immune-checkpoint molecules are some of the best-characterized and studied mechanism of interaction between immune system and cancer.

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) has been the first immune-checkpoint molecule to be clinically targeted. Its main role is to regulate T cells activation at the time of their initial response to the antigen, counterbalancing the effect of T cell receptor (TCR)/CD3 activating and CD28 costimulation signals. CTLA4 function is exerted by binding to its ligand, CD80/CD86 (mainly expressed by the antigen presenting cells, APCs), thus blocking the costimulation signals of T cells and dampening the amplitude of the response, resulting in immune suppression [1].

Similarly, a well-characterized immune-checkpoint molecule is the programmed cell death protein 1 (PD-1), expressed by activated T cells, B cells, and natural killer (NK); PD-1 regulates the inflammatory responses mainly in the peripheral tissues, limiting collateral tissue damage in inflammatory process resolution and autoimmunity phenomena [1, 2]. PD-1 activity is modulated by a specific set of ligands, the programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2).

Inflammatory signals (i.e., interferon-$\gamma$, IFN$\gamma$) in the microenvironment induce expression of PD-L1 and PD-L2. PD-L1 is the most characterized PD-1 partner; it is commonly expressed on T helper cells, myeloid derived suppressor cells in the tumor microenvironment, and cancer cells, too.

The activity of PD-1/PD-L1 axis is immunosuppressive: in particular, excessive induction of PD-1 pathway in the setting of chronic antigen exposure as well as in cancer has been

shown to induce an exhausted or anergic phenotype in T cells [1, 2], thus impairing the antitumor activity of the immune system.

Reverting immunosuppression promoted by immune-checkpoint molecules like CTLA4, PD-1, and PD-L1 was demonstrated to be an effective anticancer therapeutic strategy. Immune-checkpoint inhibitors have shown a remarkable clinical efficacy and durable response with a favorable toxicity profile in a large number of solid and hematologic malignancies [3], such as melanoma [4, 5], lung cancer [NSCLC, [6, 7]], bladder cancer [8], and renal cancer [RCC, [9]].

In particular, Ipilimumab, a CTLA4 blocking monoclonal antibody, has been FDA-approved for the treatment of metastatic melanoma (MM), after showing an overall survival advantage with a favorable toxicity profile. Another anti-CTLA4, Tremelimumab, has been more recently developed and received orphan drug status for the treatment of malignant mesothelioma. Similarly, FDA granted accelerated approval of PD-1 inhibitors Nivolumab and Pembrolizumab for the treatment of different tumors (as MM, advanced NSCLC, head and neck squamous cell carcinoma, and classical Hodgkin's lymphoma). On the other hand, anti-PD-L1 antibodies Atezolizumab, Avelumab, and Durvalumab obtained as well FDA accelerated approval in different solid tumors, as advanced urothelial bladder cancer and Merkel cells carcinoma. Many other anti-PD-1/PD-L1 molecules are being developed with promising results in clinical trials.

*1.2. Predictive Biomarkers.* No predictive biomarkers have been validated thus far to select patients who would mostly benefit from immunotherapy, sparing nonresponders from the risk of severe adverse events and saving costs.

PD-L1 protein expression by tumor and immune cells has been investigated as a potential predictive biomarker [10], but its correlation with immunotherapy efficacy is still debated [11–13] and technical issues prevent its routine use in clinical practice [6, 8]. In addition, PD-L1 expression varies widely between tumor types and presents a significant intrapatient heterogeneity with a frequent discordance between primary tumors and metastases [14, 15]. Probably, PD-1/PD-L1 expression reflects a dynamic process influenced by multifactorial events like concomitant treatments, mainly targeted therapies [16]. Other promising candidate predictive biomarkers are currently under investigation [17], particularly cells or molecules related to immune response in tumor microenvironment such as tumor infiltrating lymphocytes (TILs) [18], indoleamine 2,3-dioxygenase (IDO) [19], BCL-2 interacting mediator of cell death-Bim [20], and interferon-gamma [21].

A different possible approach to predict immunotherapy efficacy is to analyze the somatic mutational landscape of the tumor, since a high mutational burden has been shown to correlate with benefit from immunotherapy [22, 23]. However, whole exome sequencing is time and cost consuming and currently not feasible routinely [24].

An increased rate of somatic mutations has been observed particularly in mismatch repair (MMR) deficient tumors that indeed have shown responsiveness to immunotherapy independently of histologic and anatomic defined subtypes [25]. Thus, MMR status of the tumor may represent a potentially feasible and useful predictive biomarker; besides, it has a well-known prognostic role. Although MMR deficient cancers frequently show aggressive histological features like high nuclear grade at microscopy, they have a paradoxically favorable outcome. In a large series of young colorectal cancer patients, microsatellite instability was associated with a significant survival advantage independently of all standard prognostic factors, including tumor stage [26].

## 2. Mismatch Repair: Role and Implications

MMR system is a DNA integrity maintenance system. The main role of MMR proteins is the correction of single base nucleotide mismatches (insertions or deletions) generated during DNA replication and recombination, thus maintaining the genomic stability [27]. These proteins are responsible for the corrections of mismatches that occurred during meiosis and mitosis [28] and might have a potential role in oxidative DNA damage repair [29] as well as in antibody class-switch recombination [30].

The mechanism of MMR involves at least three different processes: recognition, excision, and resynthesis. Recognition of single base replication errors is performed by the MutS$\alpha$ (MSH2-MSH6 heteroduplex) or MutS$\beta$ (MSH2-MSH3 heteroduplex), excision of the lagging strand from the mismatch by one of the MutL complexes (mainly MutL$\alpha$ formed by MLH1/PMS2) recruited by MutS protein, and resynthesis of the excised-DNA and ligation by DNA polymerase delta and DNA ligase I [31].

Loss of expression of one of the MMR proteins may result from inherited germline defects (usually mutations) in one of the mismatch repair genes; rarely both of inherited alleles are mutated as in constitutional MMR deficiency syndrome leading to cancer in early childhood called constitutional mismatch repair deficiency [32]. More frequently, only one mutated allele is inherited and loss of the other allele occurs somatically, as in Lynch syndrome (LS), an autosomal dominant condition that predisposes to cancer development (particularly colorectal cancer (CRCs) and ovarian and endometrial cancer) [28]. Alternatively, MMR deficiency may be derived by either somatic mutation or methylation of one of the MMR genes: sporadic MMR deficient tumors are often the result of epigenetic silencing of MLH1 promoter due to a hypermethylation mechanism [33, 34].

Due to its role in genomic stability, MMR deficiency leads to accumulation of somatic mutations [31]. Microsatellites—repetitive short (1–6 base pairs) tandem DNA sequences scattered throughout the whole genome—are particularly subject to copying errors when mismatch repair is compromised. Therefore, it is possible to trace the MMR deficiency by studying the microsatellites: when they are demonstrated to be hypermutated (instable), MMR may be deducted.

Recent evidence showed that tumors with microsatellite instability due to MMR deficiency have different phenotype

and histologic characteristics—and in some cases even a different prognosis [35]—as compared to MMR proficient tumors [36–38].

MMR status of the tumor may be assessed either by immunohistochemistry (IHC) that tests loss of a MMR protein or by PCR based assays for microsatellite instability [39]. IHC and MSI testing are complementary as both have a false negative rate of approximately 5–10%.

## 3. MMR Status as a Predictor of Immunotherapy Efficacy: Clinical Data

The correlation between tumor MMR status and the outcome in patients treated with immunotherapy has been initially observed in CRCs treated with PD-1 blocker: only 1 of 33 patients with CRCs showed a response to immune-treatment, despite remarkable efficacy of these anticancer agents in other tumor subtypes [40, 41]. Since both MMR deficiency and immunotherapy benefit are expected in a very small fraction of CRCs patients, a possible correlation between the two has been hypothesized and confirmed in a recent phase II study [25]. A total of 41 patients with treatment refractory progressive MMR deficient and proficient metastatic CRCs were recruited, as well as a small proportion of patients with MMR deficient cancers of other types (cholangiocarcinoma, endometrial, small bowel, and gastric cancer). The three different cohorts, consisting of 11, 21, and 9 patients, respectively, were treated with Pembrolizumab. An immune related objective response rate (ORR) of 40% was observed in MMR deficient CRCs compared to a total lack of response in MMR proficient CRCs (ORR 0%), with a similar difference in progression free survival (PFS) rate at 20 weeks between the two groups (78% versus 11%). Likewise, MMR deficient tumors other than CRCs showed an ORR of 71% with a PFS of 67%. The difference in survival of patients with MMR deficient and proficient CCRs is independent from other prognostic factors, since no significant differences in PFS between the two groups were observed while receiving previous chemotherapy regimens. Interestingly, all the six patients with MMR deficient tumors not associated with Lynch syndrome had an objective response, whereas only 27% of patients with Lynch syndrome had a response. However—due to the small sample size of the study population—these results require further confirmation.

Numerous studies demonstrated that MMR status correlates also with chemotherapy resistance, with MMR deficient tumors being commonly resistant to methylating agents, platinum compounds and fluoropyrimidines [42, 43]. A possible explanation may involve DNA damage response proteins (i.e., ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR)), recruited by MMR proteins during treatment with DNA-damaging agents. ATM/ATR, in turn, lead to cell cycle arrest, DNA repair, or apoptosis through DNA damage checkpoint proteins activation [43, 44]. MMR deficiency might alter this mechanism and confer resistance to many chemotherapies [45].

## 4. Exploiting Mismatch Repair Deficiency as a Predictor of Immunotherapy Efficacy: Biologic Rationale

Multiple possible mechanisms have been proposed to explain the correlation between MMR deficiency and immune response in some cancer types. It has been observed that MMR deficiency is associated with a 10–100-fold-increased rate of somatic mutations [46]. The genomic analysis of whole exome sequences of primary tumor samples from 15 patients included in the study by Le and colleagues [25] revealed a mean of 1782 somatic mutations per tumor in MMR deficient neoplasms, compared to 73 mutations per tumor in MMR proficient ones.

MMR deficiency may provide an upregulation of a large number of genes involved in the immune response, as proinflammatory cytokines and cytotoxic mediators through a genome expression dysregulation, thus resulting in an increased secretion of soluble mediators in the tumor microenvironment with the subsequent activation of the PD-1 pathway. This might justify the observation that MMR deficient tumors are immunogenic [47].

In addition, somatic mutations may lead to the expression of a high number of tumor neoantigens that could promote the release of proinflammatory cytokines and elicit the recruitment and activity of cytotoxic T cells [48, 49]. Indeed, it has been described that MMR deficient tumors have a dense infiltration of intraepithelial CD8+ T lymphocytes and activated T helper cells.

Nevertheless, a recent study reported that the active anti-tumor immune microenvironment may be counterbalanced by the presence of immune-checkpoint ligands (i.e., PD-1/PD-L1, CTLA4, LAG3, IDO1, TIM3, GITR, and TIGIT) that favor immune escape, thus suggesting that TILs are mainly directed at neoantigens [50]. This hypothesis appears to be confirmed by clinical data, as NSCLC and MM—cancers known to have a high mutational load as a result to exposure to cigarette smoking and UVA radiation, respectively—are among the tumor types most responsive to PD-1 blockade. Moreover, patients affected by NSCLC with a high number of somatic mutations have significantly better clinical outcomes compared to patients with less mutated tumors [51]. A similar correlation has been observed for MM patients treated with anti-CTLA-4 therapy [52]. Since neoantigens are frequently different between patients and a single mutation cannot predict response to immunotherapy, the candidate predictive factor is the presence of high mutational load and the consequent recruitment of T cells in the microenvironment [53].

A recent study evaluated PD-L1 expression in MMR deficient endometrial tumors, either Lynch syndrome associated or sporadic tumors (with MLH1 hypermethylation), and showed a significant higher PD-L1 expression compared to the MMR proficient counterpart [54]. Likewise, a case of MMR deficient sporadic high-grade urothelial carcinoma of the renal pelvis treated with immunotherapy was reported: the patient experienced a prolonged complete remission in two months [55]. Besides sporadic case reports, the landscape of microsatellite instability across different cancer subtypes

Table 1: Ongoing clinical trial with immune-checkpoint inhibitors alone or in a combination regimen according to mismatch repair status for different solid tumors. Last updated, April 2017.

| Experimental arm | Active comparator regimen | Disease | Setting | Phase | Comments | ClinicalTrials.gov Identifier |
|---|---|---|---|---|---|---|
| Atezolizumab + FOLFOX | FOLFOX | CRC | Adjuvant, stage III | 3 | CT plus IO up to 25 courses | NCT02912559 |
| Pembrolizumab | FOLFOX<br>FOLFIRI<br>Bevacizumab<br>Cetuximab | CRC | IV | 3 | KEYNOTE-177<br>IO for up to 35 treatments | NCT02563002 |
| *GVAX*°<br>Pembrolizumab<br>Cyclophosphamide | Single arm | CRC | Advanced | 2 | MMRp | NCT02981524 |
| *AZD9150*§<br>Durvalumab | Single arm | Pancreatic, NSCLC, and MMRd CRC | Advanced | 2 | — | NCT02983578 |
| Pembrolizumab<br>Poly-ICLC+ | Single arm | MMRp CRC | IV | 1/2 | IO for 1 year | NCT02834052 |
| DS-8273^<br>Nivolumab | NA | MMRp CRC | IV | 1 | — | NCT02991196 |
| Nivolumab | Single arm | Hypermutated malignancies | Recurrent or refractory disease | 1/2 | Pediatric patients (12 months to 18 years of age)<br>Biallelic<br>MMRd* | NCT02992964 |
| Nivolumab | Single arm | mCRPC with mutations in DNA repair defects# | IV | 2 | ImmunoProst Trial<br>IO until progression or unacceptable toxicity | NCT03040791 |
| Avelumab<br>Ad-CEA vaccine<br>Standard of care | FOLFOX<br>Bevacizumab<br>Capecitabine | CRC | IV | 2 | CT and IO with maintenance | NCT03050814 |
| Pembrolizumab | Single arm | High-grade gliomas, diffuse intrinsic pontine gliomas, or hypermutated brain tumors | NA | 2 | IO for 34 courses | NCT02359565 |

FOLFOX: Fluorouracil, Leucovorin, and Oxaliplatin combination regimen. CRC: colorectal cancer. CT: chemotherapy. IO: immunotherapy. FOLFIRI: Fluorouracil, Leucovorin, and Irinotecan. MMRp: mismatch repair proficient profile. MMPd: mismatch repair deficient profile. NSCLC: non-small cell lung carcinoma. mCRPC: metastatic castration-resistant prostate cancer; °GVAX, cancer vaccine composed of irradiated tumor cells genetically modified to secrete granulocyte-macrophage colony-stimulating factor; §AZD9150, antisense oligonucleotide inhibitor of STAT3; +Poly-ICLC (carboxymethylcellulose, polyinosinic-polycytidylic acid, and poly-L-lysine double-stranded RNA), ligand of TLR3; ^DS-8273a, anti-human death receptor 5 (DR5) agonistic antibody; *patients must have evidence of biallelic mismatch repair deficiency either in their tumor tissue (by immunohistochemistry or sequencing) or in their germline (by sequencing) and/or evidence of hypermutant malignancy by whole exome sequencing with a mutation load > 100 per exome; #the germline and somatic DRD (BRCA1, BRCA2, ATM, PTEN, CHEK2, RAD51C, RAD51D, PALB2, MLH1, MSH2, MSH6, and PMS2) will be assessed by T-NGS of metastatic sites or by liquid biopsy.

is still poorly understood. A recent study examined 5.930 cancer exomes from 18 cancer types at more than 200.000 microsatellite loci, analyzing also cancer types for which MSI status has not been previously tested in clinical practice. The average number of unstable sites varied considerably by cancer type, ranging from a minimum of 765 unstable sites in thyroid carcinomas, to a maximum of 2.315 in colon cancers [56]. Endometrial, colon, and gastric cancer were confirmed to have the highest proportion of microsatellite instability; however most cancer types examined (14 of 18) included one or more representatives with microsatellite instability, suggesting that this could be a generalized, continuous rather than discrete, cancer phenotype. This heterogeneity adds further complexity to the scenario of potential predictive biomarkers of immunotherapy response. Interestingly, this analysis identified loci more likely to be unstable in specific cancer types, resulting in specific signatures in cancer-associated genes, suggesting that instability patterns may reflect selective pressures and can potentially identify novel cancer drivers [56].

## 5. Conclusion

Data from recent clinical studies suggest that immunotherapy with immune-checkpoint inhibitors may represent a promising therapeutic strategy for patients with MMR deficient tumors, independently of subtype. The proportion of candidate patients, however, is relatively small, because MMR deficiency has been observed only in about 4% of metastatic CRCs, 11% of ovarian carcinomas, 18% of endometrial cancers, and 1% of pancreatic cancers [25, 57]. A few reports showed promising results also in cancers not usually treated with immunotherapy, thus suggesting that screening for MMR deficiency should be potentially offered to all patients with advanced disease, independently from histology. Accordingly, some current ongoing studies are exploring the potential predictive role of MMR status, as summarized in Table 1. Most importantly, these results support an approach to treatment based on genetic status of tumor regardless of cancer subtype. Eventually, a better understanding of pathologic and genomic features of MMR deficient tumors may allow the identification of other biomarkers (such as TILs, immune-checkpoint proteins, and genomic mutations) potentially useful in clinical routine practice to predict response to immunotherapy or as surrogate markers of early response to therapy [17]. Indeed, microsatellite instability alone may not be sufficient to predict response to immune-checkpoint inhibitors, as, for example, not all tumor neoantigens may bind the major histocompatibility complex (MHC) class I. Additional immune-regulatory mechanisms may have a role as a contributor of anti-PD-1/PD-L1 response, as T cell absence and genetic/epigenetic alterations [58]. Accordingly, it has been demonstrated that PI3K/PTEN/AKT pathway hyperactivity may dampen antitumor immune activation when PTEN-null tumors are exposed to an immune-checkpoint inhibitor, thus suggesting a specific genetic regulatory mechanism [59].

A global concept has recently been summarized by Chen and Mellman [60] in the definition of an "immune set-point" as a global immune activation status potentially predictive of response to immune therapies as well as a tool to guide the choice of different strategies of treatment.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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*Review Article*

# Immunotherapy in Gastrointestinal Cancers

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Gastrointestinal cancers represent a major public health problem worldwide. Immunotherapeutic strategies are currently under investigation in this setting and preliminary results of ongoing trials adopting checkpoint inhibitors are striking. Indeed, although a poor immunogenicity for GI has been reported, a strong biological rationale supports the development of immunotherapy in this field. The clinical and translational research on immunotherapy for the treatment of GI cancers started firstly with the identification of immune-related mechanisms possibly relevant to GI tumours and secondly with the development of immunotherapy-based agents in clinical trials. In the present review a general overview is firstly provided followed by a focus on major findings on gastric, colorectal, and hepatocellular carcinomas. Finally, pathological and molecular perspectives are provided since many efforts are ongoing in order to identify possible predictive biomarkers and to improve patients' selection. Many issues are still unsolved in this field; however, we strongly believe that immunotherapy might positively affect the natural history of a subgroup of GI cancer patients improving outcome and the overall quality of life.

## 1. Gastrointestinal Cancers: Where Do We Stand?

Gastrointestinal (GI) cancers, including colorectal cancer (CRC), gastric cancer (GC), pancreatic cancer, and cancers of the liver (HCC) and of the biliary tract, are among the most frequent malignancies diagnosed annually in Europe and represent a major public health problem worldwide [1].

Although early-stage GI cancers are amenable to surgical resection with curative-intent, the overall 5-year relapse rate remains high. As a matter of fact, the addition of neoadjuvant or adjuvant chemotherapy and radiation therapy, when indicated, only modestly improves the overall long-term survival. Unfortunately, a large proportion of patients present with unresectable disease at the time of diagnosis: approximately 25% of GI cancers are diagnosed at advanced stage, whereas another 25 to 50% of patients will develop metastases during the course of the disease [2, 3]. In the last decade, meaningful improvement in the prognosis of patients with metastatic GI cancers derived from the development of new intensive and/or tailored therapies, which incorporated cytotoxic drugs and targeted therapies (cetuximab, panitumumab, bevacizumab, aflibercept, and regorafenib for mCRC; trastuzumab and ramucirumab for mGC; and sorafenib for HCC), and from the integration of medical treatments with more and more effective locoregional and surgical approaches [4]. Despite these advances, GI cancers are still a leading cause of cancer death [4]; thus,

it is imperative to develop novel therapeutic approaches for patients affected by those cancers.

In recent years, we assisted in a paradigmatic shift in the treatment of both solid tumours, such as melanoma, non-small cell lung cancer, and genitourinary cancers, as well as hematologic malignancies, thanks to the striking results with long lasting responses and increased overall survival (OS) obtained with immunotherapy-based agents [5–7]. In parallel, the clinical and translational research on immunotherapy for the treatment of GI cancers started firstly with the identification of immune-related mechanisms possibly relevant to GI tumours and secondly with the development of immunotherapy-based agents in clinical trials.

Undoubtedly, the progress made towards the development of effective antitumour immunotherapies for GI cancers has been relatively slow: the first practice changing clinical data came out only in 2015 and the most part of immunotherapies are still in early phase clinical testing. The main reason for having GI cancers as a kind of Cinderella in the landscape of tumoural immunotherapy resides in the lack of their effector T cell responses and in the their well-known poor immunogenicity [8]. Immunotherapy against cancer has been assumed to be beneficial mainly in tumours with high immunogenicity by nature [9]. However, some approaches to circumvent immunosuppression including programmed death-1 (PD-1)/programmed death ligand-1 (PD-L1) blockade were successful to achieve significant response, also in cancers that hardly retain immunogenic nature [10].

This article highlights the state of the art of immunotherapy in GI deepening recent scientific evidence regarding anti-PD-1/PDL-1 and anti-CTLA4 monoclonal antibodies, peptide based vaccine, DNA based vaccine, and pulsed dendritic cells (DC), also outlining current clinical trials and finally suggesting areas for future research.

## 2. The Rationale for Immunotherapy in GI Cancers

Accumulating evidences indicate that a dynamic cross-talk between tumours and the immune system can regulate tumour growth and metastasis [10]. The increased understanding of the biochemical nature of tumour antigens and of the molecular mechanisms responsible for innate and adaptive immune cell activation has revolutionized the fields of tumour immunology and immunotherapy.

The first notion of a role of immunity in cancer was postulated in 1909 by Ehrlich, speculating that the immune system could repress the growth of carcinomas recognising tumour cells as foreign. About 50 years later, the theory of tumour immune surveillance was proposed by Burnet [11]. However, this theory has been recently completed with the identification of the so-called immunoediting proposed by Schreiber et al. The immunoediting progresses through 3 main phases: (1) the elimination phase (or immunosurveillance), when the innate and adaptive immune cells remove the proliferating cells, thus protecting the host against cancer; (2) the equilibrium phase, when the tumour growth and the immunosurveillance enter into a dynamic balance; in this genetically instable phase, the increase of mutational load and the emergence of resistant clones among tumour cells lead to (3) the escape phase; at this point, tumour variants are able to avoid immune-mediated destruction and speed up tumour progression and clinical expression [12, 13].

A role for the immunoediting in gastroenteropancreatic tumour pathogenesis was suggested since the first observations that T cells infiltration was linked to a more favorable outcome in pancreatic cancer, CRC, and GC [13, 14]. The following studies regarding the molecular basis and regulation of immunoediting have identified the tumour cells, the tumour microenvironment, and the immune system as the key players of a complex network [15]. Defining the relationships between these key players has been critical in facilitating the development of successful immunotherapies.

(A) *Tumour cells* have developed several mechanisms that directly or indirectly block the activity of effector antitumour CD4+ and CD8+ T cells dampening local tumour-infiltrating immune responses [16, 17]. Examples include (1) the secretion of soluble immunosuppressive factors (TGF-beta, IL-10, VEGF, and indoleamine 2,3 dehydrogenase) [18, 19]; (2) the activation of negative costimulatory signals in the tumour microenvironment such as PD-L1 [20, 21]; (3) tumour-induced impairment of the antigen presentation machinery due to the accumulation of point mutations in the cell surface not recognised by cytotoxic T cells [22]; and finally (4) the downregulation of the major histocompatibility complex (MHC) class I expression which plays a crucial role in tumour antigens presentation to T cells [22].

(B) Mechanisms explaining the *tumour microenvironment* role in immunoediting are best illustrated in studies on human and mouse pancreatic cancer models, since desmoplasia is the pathologic hallmark of pancreatic cancer [23, 24]. This inflammatory environment consists of regulatory immune cells, extracellular matrix proteins, and all the above fibroblasts (cancer-associated fibroblasts, CAFs) [25]. These stroma players in turn secrete tumour-promoting factors that contribute to tumour invasion and neoangiogenesis [26, 27]. Interestingly, CAFs have a critical role in CRC immunosuppression [28]: their activity in *RAS* mutant tumours overcome effector T cells signalling leading to tumour progression thanks to the activation of epithelial mesenchymal transition and TGF-beta/SMAD signalling [28]. Actually, high levels of CAFs markers correlated with poor prognosis in CRC [29].

(C) *The immune system* plays a critical role in immunoediting thanks to the involvement of several innate and adaptive effectors such as myeloid-derived suppressor cells (MDSCs), mast cells, tumour associated macrophages (TAMs), mesenchymal stem cells (MSCs), CD4+/CD25+ regulatory T cells (Tregs), and DCs [30]. By modulating the tumour microenvironment through the secretion of selected chemokines, cancer cells can actively prevent the induction of antitumour immunity through the differentiation, expansion, and/or recruitment of Treg [30, 31]. It has been reported that a low percentage of Tregs in the circulation 1 year after resection of pancreatic cancer correlates with improved survival [32, 33]. In addition, DCs are critically important for the generation and the maintenance of a specific adaptive antitumour immune response [33, 34]. Data from many laboratories obtained during past few years indicate that

defects in DCs are among the main factors responsible for tumour escape [35].

Among immunosuppressive mechanisms the immune checkpoint modulation mediated by cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and Programmed Death-1 (PD-1) plays a crucial role. In the normal host setting, immune checkpoint molecules modulate the T-cells response to antigens by either upregulating costimulatory pathways or downregulating coinhibitory pathways of immune signalling. CTLA-4 is an inhibitory receptor expressed by T cells. It can bind to CD80 or CD86 on DCs and inhibit their capabilities to activate T cells. CTLA-4 plays a critical role in the initial phase of immune response. PD-1 is a cell surface coinhibitory receptor that downregulates T cell activity in peripheral tissues during inflammation, thus preventing increased collateral tissue damage during an immune response and the development of autoimmunity. PD-1 is widely expressed on T cells, B cells, monocytes, and natural killer cells and plays a critical role in subsequent phases of immune responses compared to CTLA-4. It has two known ligands, PD-L1 and PD-L2, which are both upregulated during an inflammatory response. Tumour cells of various malignancies have been shown to upregulate PD-L1 as a mechanism that dampens the local T cell response by decreasing cytokine production and T cell proliferation. In GI malignancies, PD-L1 upregulation has been demonstrated to occur in pancreatic, GC, and CRC [34, 35], thus correlating with poor prognosis [35].

Moving from such complex background, immunotherapeutic strategies in GI cancers have been developed and are described in the following paragraphs. In particular, a general overview is firstly provided followed by a focus on major findings on GC, CRC, and HCC. Finally, a pathological and a molecular perspective are provided.

## 3. Immunotherapeutic Strategies: A General Overview

Activation of immune system against cancer might derive from active immunotherapeutic strategies, such as the adoption of cytokines, cancer vaccines, and immune checkpoints inhibitors or from a passive immunization mediated by adoptive cellular therapy (ACT) or monoclonal antibodies [14, 36]

The first attempts of active host immunity stimulation were based on the adoption of cytokines, in particular interferon-$\gamma$ (IFN-$\gamma$), interleukin-2 (IL-2), IL-10, or GM-CSF. However, cytokines-based strategies are not adopted in clinical practice since results of trials are dated and inconclusive [37].

Cancer vaccines, as active immunotherapy, were firstly investigated 30 years ago. They are designed to activate and expand tumour-specific T cells with the potential to produce a persistent or even permanent anticancer effect. The ideal vaccine is easy to administer, offers prolonged protection, and induces relatively low toxicity. Although many trials investigated the possible role of peptide, protein, whole tumour cells, or DC-based vaccines in GI cancers [37], to date none entered the clinical practice. However, a renewed enthusiasm derived from a new class of recombinant immunogenic protein fused with a novel cell-penetrating peptide (Z12). This compound is able to promote efficient protein loading into the antigen processing machinery of DC and to lead to multiepitopic MHC class I and II restricted presentation. This novel vaccine elicited an integrated and multiepitopic immune response with persistent CD8+ and CD4+ stimulation in different tumour models [38] and will be soon investigated in human GI models.

Undoubtedly, immune checkpoints inhibitors are the real game changers. The anti-CTLA-4 ipilimumab and the anti-PD-1 mAbs, pembrolizumab and nivolumab, were firstly approved by the US FDA for the treatment of metastatic melanoma in 2011 and 2014, respectively [39, 40]. Several trials investigated or are currently investigating such mAbs in GI cancer with very promising preliminary results. As an example, pembrolizumab received the breakthrough therapy designation in mCRC cancers with microsatellite instability in November 2015 [41]. Despite some practice changing results have been obtained, many efforts are currently made in order to identify subgroup of patients benefitting from these agents and to design newer strategies involving the association of standard treatment with immunotherapy. Moreover, new molecules are under investigation such as the anti-PD-L1 avelumab and atezolizumab in GC and the anti-CTLA-4 mAb tremelimumab in HCC patients.

Among passive immunization strategies, adoptive cell therapy (ACT) is based on the passive transfer of tumour-specific T cells into a tumour-bearing host for the direct destruction of tumours. Briefly, T cells are collected from the tumour, draining lymph nodes or peripheral blood, and are activated and expanded in vitro. The first clinical trial of ACT in advanced cancers adopted lymphocytes-activated killer (LAK) cells. Since then, the innovative ACT with tumour-infiltrating immune cells (TILs) has been developed taking advantage of lymphocytes with demonstrated ability to recognise the tumour. ACT with TILs isolated from resected tumours, expanded ex vivo, and administered to patients in combination with IL-2 has demonstrated a 50% response rate in patients with metastatic melanoma [42, 43]. Since TILs have been isolated from a variety of GI cancers, this approach is currently under investigation in the metastatic setting [44]. The most recent ACT treatment adopts engineered T cells able to express chimeric antigen receptors (CARs) specific for CEA. CARs engage their target independently from antigen processing process and from MHC. Thus, CAR therapy is advantageous when MHC class I is downregulated [45]. Since T cells are ubiquitously expressed, targeting self-antigens might cause serious immune-related toxicities and safety concerns are still unsolved [45].

Finally, monoclonal antibodies (mAbs) commonly adopted in GI cancers represent the most relevant example of passive immunotherapy strategy. However, a wide body of literature is already available regarding this topic and it is not discussed in the present review.

## 4. Focus on Gastric, Esophageal, and Pancreatic Cancers

The first promising data about immunotherapy in GC or gastroesophageal junction cancer (GEJC) came from anti-PD-1 agents. The phase Ib study KEYNOTE-012 was designed

to assess the safety and activity of pembrolizumab in GC and GEJC and the predictive role of PD-L1 expression in those malignancies. Primary endpoints were safety and response rate (RR). Toxicity profile was manageable; among 36 evaluable patients, RR was 22% (95% CI 10–39). No association between PD-L1 expression and clinical responses to pembrolizumab was observed [46]. Moving from the promising KEYNOTE-012 results, two trials are currently ongoing: the KEYNOTE 061 is evaluating pembrolizumab versus paclitaxel after progression to a first-line platinum-based therapy [47] and the KEYNOTE 062 is randomizing patients to receive pembrolizumab as monotherapy or platinum and 5-FU in association with pembrolizumab or placebo in the first-line setting [48] (see Table 3).

Pembrolizumab as single agent was also tested in esophageal cancer (EC) in the multicohort, phase Ib KEYNOTE-028 trial. In this study, 23 pretreated patients with either squamous cell carcinoma (SCC) or adenocarcinoma of the esophagus or GEJC were treated. Encouraging results were reported: the ORR was 30.4% and 52.2% in SCC and adenocarcinoma patients, respectively. Six- and 12-month progression free (PF) rates were 30.4 and 21.7%, respectively [49].

In the randomized phase III trial ONO-4538/BMS-936558, the anti-PD-1 nivolumab was tested as monotherapy versus placebo in advanced GC and GEJC after second or later lines. This study met all its endpoints. In detail, mOS was 5.3 versus 4.1 months (HR = 0.63, 95% CI 0.50–0.78, $p < 0{,}0001$) and mPFS was 1.61 versus 1.45 months (HR = 0.60, 95% CI 0.49–0.75, $p < 0{,}0001$) in the nivolumab ($N = 330$) and in the placebo arm ($N = 163$), respectively [50]. The shape of the curve shows that only a subgroup of patients derives benefit from the treatment reaching a long lasting disease control and response to treatment.

Nivolumab has also been tested in 65 patients affected by advanced esophageal SCC in a Japanese single-arm phase II trial. Patients received one or more previous treatment and were not preselected by PD-L1 status. The preliminary results showed durable activity with a manageable safety profile, with median OS of 12.1 months in 64 evaluable patients [51].

Another promising immunotherapy agent is the anti-PD-L1 avelumab, which has been tested in patients with GC or GEJC in the phase Ib trial JAVELIN. Patients were eligible if treated with a first-line chemotherapy based regimen and grouped by progression status after first line: patients achieving disease control during first line received avelumab as switch maintenance ($N = 89$) and those with progressive disease after chemotherapy received it as second line ($N = 62$). Primary endpoint was safety. An acceptable safety profile was shown. ORR was 9.0% and 9.7% in the 2 subgroups, respectively [52]. Given the promising results of this trial, JAVELIN Gastric 100 and JAVELIN Gastric 300 phase III trials are now ongoing [53, 54].

Less encouraging results come from anti-CTLA-4 agents, which showed higher toxicity and lower efficacy than anti-PD-1 in gastric and esophageal malignancies. The reasons for these differences are still debated. No objective responses were observed with the anti-CTLA-4 tremelimumab, tested as second-line treatment in a phase II trial in advanced GC and EC [55]. Similarly, ipilimumab was compared to best supportive care (BSC) in a randomized phase II trial, in pretreated patients with metastatic or locally advanced GC or GEJC and survival parameters were similar between the two arms [56].

In order to enhance the activity of anti-CTLA-4 antibodies, combination treatments with anti-PD-1 have been tested. The checkMate-032 is a phase I/II multicohort trial that randomized 160 pretreated patients to receive (1) nivolumab alone 3 mg/kg, (2) nivolumab 3 mg/kg plus ipilimumab 1 mg/kg, or (3) nivolumab 1 mg/kg plus ipilimumab 3 mg/kg. A notable RR was seen in each arm, with an overall DCR of 38%. Of interest, the ORR in patients with PD-L1-positive (≥1%) and PD-L1-negative (<1%) tumours was 27% and 12%, respectively, suggesting that PD-L1 expression may increase response rates. The highest ORR (26%) and mOS (6.9 months) were observed in arm 3 (nivolumab 1 mg/kg and ipilimumab 3 mg/kg) [57]. Given these interesting findings, the phase III trial CheckMate-649 investigating nivolumab plus ipilimumab versus FOLFOX/XELOX in untreated patients is ongoing. Table 1 shows ongoing trials in this setting.

Pancreatic cancer models have been widely adopted in order to identify the immunotherapeutic rationale in GI cancer; however, data derived from early phase clinical trials yielded no benefit in pancreatic cancers. In particular, negative results derived from checkpoint inhibitor and vaccination trials [58]. Future clinical trials will test combination approaches in order to overcome immunosuppressive intratumour mechanisms and/or to increase the immunogenicity of microenvironment.

## 5. Focus on Colorectal Cancer

The first data on immunotherapy in CRC came from 1981, when the role of vaccines as immunotherapy was explored, based on the rationale of activating host defense against tumour-specific or tumour-associated antigens by means of the injection of autologous tumour cells with an immunomodulator (Bacillus Calmette-Guérin (BCG)). Preclinical models showed that the injection of BCG and tumour cells (OncoVAX®) was able to activate systemic immunity and stop the tumour burden [59].

The efficacy of OncoVAX was subsequently evaluated in the adjuvant setting in three phase III clinical trials, where patients were randomized to receive surgical resection only or surgical resection plus vaccination. The first study (8102) was initiated in 1981 and enrolled 98 patients with stages II and III CRC. The primary endpoints, OS and disease-free survival (DFS), were not reached (HR for OS = 1.75, $p = 0.68$; HR for DFS = 1.58, $p = 0.147$). However, in the subgroup analyses a significant benefit of OncoVAX was seen in patients with colon cancer (HR for OS = 2.83, $p = 0.02$; HR for DFS = 2.67, $p = 0.039$) and not in those with rectal cancer (HR for OS = 1.13, $p = 0.772$; HR for DFS = 1.05, $p = 0.905$) [60]. The phase III 5283 trial enrolled 412 colon cancer patients with stages II and III; no differences in OS and DFS were observed [61]. Lastly, in the phase 8701 III trial, 254 patients with stages II and III colon cancer patients were enrolled; the vaccine was centrally manufactured and was administered 4 times

Table 1: Ongoing studies on gastric, gastroesophageal junction, and esophageal cancers.

| NCT identifier | Setting | Phase | Study interventions | Number of patients | Primary endpoint |
|---|---|---|---|---|---|
| | | | *Checkpoint inhibitors* | | |
| NCT02689284 | Metastatic HER2+ GC/GEJC | Ib/II | Margetuximab+ pembrolizumab | 52 | MTD and MAD for margetuximab; duration of response; 12-month ORR |
| NCT02563548 | Metastatic GC after 1st line | Ib | PEGPH20 +pembrolizumab | 81 | DLT; 18-month ORR |
| NCT02443324 | Metastatic GC/GEJC and other tumours | I | Ramucirumab + pembrolizumab | 155 | DLT |
| NCT02589496 | Metastatic GC/GEJC after first line | II | Pembrolizumab | 40 | 2-year RR |
| NCT02901301 | First-line HER2 + GC | Ib/II | Pembrolizumab + trastuzumab + capecitabine + cisplatin | 49 | RP2D; 6-week ORR |
| NCT02954536 | First-line HER2+ GC/GEJC/EC | II | Pembrolizumab + trastuzumab + capecitabine + cisplatin | 37 | 6-month PFS |
| NCT02318901 | Unresectable HER2 + GC/GEJC | II | Pembrolizumab + ado-trastuzumab emtansine | 90 | RP2D |
| NCT02559687 | EC (adenocarcinoma or squamous cell)/GEJC after 2nd line | II | Pembrolizumab | 100 | 2-year ORR |
| NCT02494583 | First-line GC/GEJC | III (random) | Pembrolizumab versus pembrolizumab + cisplatin + 5-fluorouracil or capecitabine versus placebo + cisplatin + 5-FU or capecitabine | 750 | 44-month PFS and OS |
| NCT02370498 | Second-line GC/GEJC | III (random) | Pembrolizumab versus paclitaxel | 720 | PFS, OS |
| NCT02564263 | EC (adenocarcinoma or squamous cell) /GEJC after 1st line | III (random) | Pembrolizumab versus investigator's choice of standard therapy (paclitaxel, docetaxel, or irinotecan) | 600 | 3-year PFS and OS |
| NCT02872116 | Unresectable GC/GEJC | III (random) | Nivolumab + ipilimumab versus nivolumab + oxaliplatin + fluoropyrimidine versus oxaliplatin + fluoropyrimidine | 1266 | 40-month OS in patients PD-L1 + |
| NCT02864381 | Metastatic GC/GEJC | II (random) | GS-5745 + nivolumab versus nivolumab alone | 120 | 2-year ORR |
| NCT02340975 | Pretreated metastatic/GC/GEJC | Ib/II (random) | MEDI4736 + tremelimumab versus MEDI4736 versus tremelimumab | 135 | Phase Ib: DTL, Phase II: ORR and 6-month PFS |
| NCT02625623 | 3rd-line GC/GEJC | III (random) | Avelumab+ BSC versus chemotherapy (paclitaxel or irinotecan)+BSC or BSC alone | 330 | 2-year OS |
| NCT02625610 | 1st-line GC/GEJC | III (random) | Maintenance with avelumab versus continuation of 1st-line chemotherapy | 666 | 3-year OS and PFS |
| | | | *Immunotherapy + radiotherapy* | | |
| NCT02642809 | 1st-line EC | I | Pembrolizumab + brachytherapy | 15 | Tolerability and toxicity |
| NCT02830594 | Pretreated EC/GC(GEJC | II | Pembrolizumab + external beam palliative radiation therapy | 14 | Biomarkers |
| NCT02735239 | Metastatic EC | I/II | Durvalumab + oxaliplatin/capecitabine | 75 | AE, DLT, laboratory evaluations |

Table 1: Continued.

| NCT identifier | Setting | Phase | Study interventions | Number of patients | Primary endpoint |
|---|---|---|---|---|---|
| *Vaccines* | | | | | |
| NCT02276300 | Metastatic HER 2 + GC | I | HER2-derived peptide vaccination | 12 | Safety and tolerability |
| NCT02317471 | Stage III gastric cancer | I/II | Vaccination with autologous tumour derived heat shock protein gp96 | 45 | DFS |
| NCT02795988 | Metastatic HER 2 + GC/GEJC | Ib/II | IMU-131 HER2/Neu peptide vaccine+ cisplatin and either 5-FU or capecitabine chemotherapy | 18 | RP2D, AE |
| *Cytokines* | | | | | |
| NCT01691664 | Locally advanced EC | NS (random) | Radiation therapy alone or with DC-CIK cellular therapy | 40 | DFS |
| NCT01691625 | Locally advanced EC | NS (random) | Concurrent chemoradiation with or without DC-CIK | 50 | Quality of life |
| NCT02504229 | Metastatic refractory GC | II (random) | Chemotherapy with or without DC-CIK | 80 | PFS |
| NCT01783951 | Metastatic refractory GC | I/II | S-1 with or without DC-CIK | 30 | PFS |
| | | | *CAR-T cells* | | |
| NCT02713984 | Metastatic refractory HER 2 + GC | I/II | Anti-HER2 CAR-T cells | 60 | Toxicity |
| NCT02725125 | Metastatic refractory GC | I/II | EPCAM-targeted CAR-T cells | 19 | DCR |
| NCT02617134 | Metastatic refractory MUC1+ GC | I/II | Anti-MUC1 CAR-T cells | 20 | Toxicity |
| NCT02349724 | Metastatic refractory CEA+ GC | I | Anti-CEACAR-T cells | 75 | Toxicity |
| NCT02862028 | Metastatic refractory EGFR+ GC | I/II | Anti-PD-1CAR-T cells | 20 | ORR, DCR, OS, PFS |
| NCT03013712 | Metastatic refractory EpCAM+ GC/EC | I/II | Anti-EpCAMCAR-T cells | 60 | Toxicity |

GC, gastric cancer; GEJC, gastroesophageal junction cancer; EC: esophageal cancer; NA, not assessed; MTD, maximum tolerated dose; MAD, maximum administered dose; DTL, dose limiting toxicity; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; DFS, disease-free survival; NS, not specified; RP2D, recommended dose of phase II; AE, adverse events; DCR, disease control rate; BSC, best supportive care; DCR disease control rate.

instead of 3. A 44% risk reduction for disease recurrence was observed in patients treated with OncoVAX ($p = 0.023$). In the subgroup analyses, the efficacy was only observed in stage II patients (61% risk reduction for disease recurrence) [62].

A meta-analysis including the 3 above reported trials showed an improvement in recurrence-free interval by OncoVAX with an annual odds reduction of 25 ± 13% ($p = 0.05$). The subgroup analysis by stage showed a predominant improvement in stage II patients ($p = 0.05$) [61]. According to these promising data, a multicenter phase III trial with OncoVAX in stage II patient is currently ongoing (Table 1).

Virus modified vaccines were also investigated in CRC. In particular, the Newcastle disease virus-infected (NDV) autologous modified vaccine, obtained admixing a nononcolytic strain Ulster of NDV with irradiated autologous tumour cells was tested in patients undergoing radical liver resection [63]. The results of a randomized phase III trial with NDV autologous modified vaccine in patients who undergone radical resection of CRC liver metastases were published at the end of 2000s. In this study, 51 patients were enrolled. No differences in OS (primary endpoint) and in DFS (secondary endpoint) were detected. However, in the subgroup analyses, a significant advantage was observed in patients with colon cancer with respect to OS (HR 3.3, $p = 0.042$) and DFS (HR 2.7, $p = 0.047$) but not in those with rectal cancer [64].

Data emerging from cancer vaccines have not yet altered the clinical practice. Several trials are currently ongoing in adjuvant and in metastatic settings with the aim of improving vaccines immunogenicity and of identifying a subset of patients amenable of this kind of treatments.

Conversely, the striking results obtained from immune checkpoint inhibitors trials lead to the introduction of new therapeutic options in mCRC. The first data regarding initial success of immune checkpoint inhibitors in mCRC were presented in mid-2015, when the results of the phase II KEYNOTE 016 trial with pembrolizumab in patients with refractory metastatic tumours were published. In this study, three cohorts of patients were recruited: (1) cohort A: patient with high microsatellite instability (MSI-H) or deficient mismatch repair (dMMR) mCRC ($n = 11$); (2) cohort B: patients with microsatellite stability (MSS) or proficient (p)MMR mCRC ($n = 21$); and (3) cohort C: patients with MSI-H non-mCRC cancers ($n = 9$). Immune-related objective response (iORR) rates were 40%, 0%, and 71% in the 3 groups, respectively; the median PFS and OS were not reached in cohort A; 2.2 and 5.0 months, respectively, in cohort B (HR for PFS = 0.10, $p < 0.001$, HR for OS = 0.22, $p = 0.05$) [41]. For the first time the activity of an anti-PD1 was demonstrated in patients with MSI-H while no effect was observed in MSS mCRC patients. As possible explanation of such results, it was demonstrated that tumours with MSI-H are characterized by a high burden of somatic mutations that can be recognised by the patient's immune system. As a supplementary proof, MSI-H tumours were found to be characterized by a dense immune infiltration and a cytokine-rich environment [65].

Based on these results, on November 2, 2015, the FDA granted "breakthrough therapy designation" for pembrolizumab in advanced CRCs with high microsatellite instability (MSI-H). To further explore this strategy, the KEYNOTE 164 trial was planned [66]; in this trial, pretreated MSI-H mCRC patients are candidate to receive pembrolizumab 200 mg every 3 weeks. Moreover, a phase 3 study of pembrolizumab versus investigator choice chemotherapy for MSI-H mCRC in first line is ongoing (KEYNOTE 177) [67].

One year later, at the 2016 ASCO Annual Meeting, several encouraging preliminary data on immune-checkpoint inhibitors in the treatment of mCRC were presented, including the update of the KEYNOTE 016 trial [68], a new treatment strategy adopting a combination of anti-CTLA4 and anti PD1 (the CHECKMATE 142 trial) [69], and a phase Ib study combining a MEK inhibitor and an anti-PD-L1 in patients with microsatellite stable (MSS) tumours [70].

The phase II CHECKMATE 142 trial investigates nivolumab plus or minus ipilimumab in patients with MSS and MSI-H mCRC patients in advanced lines of treatment. In the MSI-H cohort, ORR was 25,5% in patients receiving nivolumab ($N = 47$) and 33,3% in those receiving ipilimumab plus nivolumab ($N = 27$). Data presented at ASCO GI 2017 on 72 patients treated with nivolumab showed encouraging results for ORR, 12-month PF rate, and 12-month survival rate (31%; 48,4%; and 73.8%, resp.). Responses were observed regardless of tumour or immune cell PD-L1 expression, *BRAF*, *KRAS* mutation status, or clinical history of Lynch syndrome. Centrally revised data identified 2 patients experiencing complete response. This data represents a big step forward in the treatment of advanced mCRC and we perfectly agree with the conclusion of the authors stating that nivolumab should be considered a new standard of care for patients with previously treated MSI-H advanced CRC [70]. A new cohort of the trial is evaluating the activity of nivolumab and ipilimumab as first-line treatment (Table 1).

Data presented so far are highly significant in the subgroup of MSI-H patients while results in MSS cases are disappointing. RRs in patients with MSS treated with nivolumab or ipilimumab plus nivolumab were 10% and 0%, respectively, with overall poor PFS and OS [70]. Thus, many efforts are ongoing in order to identify possible immunotherapeutic strategies in MSS. In preclinical models, MEK inhibition alone increased the tumour-infiltrating $CD8^+$ T cells and induced MHC-I upregulation, the combination of MEK inhibition with an anti-PD-L1 resulted in synergistic and durable tumour regression [71]. In a cohort of 23 mCRC patients receiving the MEK inhibitor cobimetinib and the anti-PD-L1 antibody atezolizumab, the ORR was 17% with 4 partial responses and 5 disease stabilizations. Among responders 3 out of 4 were MSS, thus leading to hypothesizing a possible effect for such strategy in this group of patients. A phase III trial is currently investigating atezolizumab and cobimetinib versus regorafenib in refractory mCRC [71, 72]. Other association strategies of checkpoints inhibitors with chemotherapy or anti-VEGF are also under investigation in mCRC patients irrespective of MSI status. All immune strategies under investigation in CRC are summarized in Table 2.

Table 2: Ongoing studies on colorectal cancers.

| NCT identifier | Setting | Phase | Study interventions | Number of patients | Primary endpoint |
|---|---|---|---|---|---|
| | | | *Checkpoint inhibitors* | | |
| NCT03026140 | Early stage colon cancer | II | Nivolumab + ipilimumab versus nivolumab + ipilimumab + celecoxib | 60 | Safety |
| NCT02260440 | Chemorefractory mCRC | II | Pembrolizumab + azacitidine | 40 | ORR |
| NCT02997228 | MSI-H mCRC | III | mFOLFOX6 bevacizumab versus atezolizumab versus atezolizumab + mFOLFOX6 bevacizumab | 439 | PFS |
| NCT02870920 | Chemorefractory mCRC | II | BSC + durvalumab + tremelimumab versus BSC | 180 | OS |
| NCT02788279 | Chemorefractory mCRC | III | Atezolizumab versus cobimetinib + atezolizumab versus regorafenib | 360 | OS |
| NCT02991196 | mCRC | I | DS-8273a + nivolumab | 20 | DLTs; MTD; ORR; DCR; TTP; PFS; |
| NCT02713373 | Unresectable mCRC | I-II | Cetuximab + pembrolizumab | 42 | PFS; ORR; safety and tolerability |
| NCT02981524 | mCRC | II | CY/GVAX + pembrolizumab | 30 | ORR |
| NCT02754856 | mCRC with resectable CLM | I | Tremelimumab + MEDI4736 + FOLFOX bevacizumab | 35 | Feasibility |
| NCT02933944 | RAS mutmCRC | I | TG02 versus TG02+ pembrolizumab | 20 | Safety; irORR |
| NCT02860546 | MSS mCRC | II | TAS-102 + nivolumab | 35 | irORR |
| NCT02948348 | Locally advanced RC | I-II | Chemoradiotherapy with capecitabine → nivolumab → surgical therapy | 50 | PCR |
| NCT02437071 | mCRC | II | Pembrolizumab+ radiotherapy versus pembrolizumab+ ablation | 48 | ORR |
| NCT02060188 (CheckMate 142) | mCRC | II | Nivolumab/nivolumab + ipilimumab/nivolumab + ipilimumab 1st line/nivolumab + ipilimumab + cobimetinib/nivolumab + BMS-986016/nivolumab + daratumumab | 260 | ORR |
| NCT02512172 | mCRC | I | Oral CC, 486 & MK-3475 versus romidepsin & MK-3475, versus oral CC, 486 & romidepsin & MK-3475 | 30 | Degree of change in TIL |
| NCT02227667 | mCRC | II | MEDI4736 | 48 | ORR |
| NCT02563002 (KEYNOTE 177) | MSI-H mCRC | III | Pembrolizumab versus investigator's choice of standard of care | 270 | PFS |
| | | | *Vaccines* | | |
| NCT02448173 | Stage II | III | OncoVAX + surgery versus surgery | 550 | DFS |
| NCT01890213 | Stage III | I | AVX701 | 12 | AE |
| NCT02718430 | mCRC with CLM | I | VXM01 | 24 | Safety and tolerability |
| NCT01741038 | mCRC | II-III | AlloStim® + cryoablation versus AlloStim + physician's choice (PC) | 450 | OS |
| NCT02615574 | Refractory mCRC | II | áDC1 vaccine+ CKM | 44 | OS |
| | | | *Cytokines* | | |
| NCT02415699 | Stage III | II-III | DC-CIK + chemotherapy versus chemotherapy | 100 | DFS |
| NCT02280278 | Stage III | III | Adjuvant CT → CIKCC versus adjuvant CT | 550 | DFS |
| NCT01929499 | Stages II-III | II | Adjuvant CT + synchronous CIKCC versus adjuvant CT → CIKCC versus adjuvant CT | 210 | DFS |
| NCT02466906 | Stage III | II | RhGM-CSF versus placebo | 60 | DFS |
| *Oncolytic virus* | | | | | |
| NCT01274624 | KRAS | II | REOLYSIN® + FOLFIRI, bevacizumab | 32 | DLTs |
| NCT01622543 | mCRC | II | FOLFOX + bevacizumab + reolysin versus FOLFOX + bevacizumab | 109 | PFS |

Table 2: Continued.

| NCT identifier | Setting | Phase | Study interventions | Number of patients | Primary endpoint |
|---|---|---|---|---|---|
| | | | *Adoptive cell therapy study* | | |
| NCT03008499 | mCRC | I-II | High-activity natural killer versus no special treatment | 18 | Relief degree of tumours evaluated by RECIST |
| NCT02577588 | mCRC | I | Reactivated T cells | 10 | DLTs |
| | | | *Adjuvant therapy* | | |
| NCT01545141 | Resectable CRC | I-II | Surgery versus chemokine modulatory regimen (a combination of IFN, celecoxib, and rintatolimod prior to surgery | 50 | Change in the number of tumour-infiltrating CD8+ cells |
| | | | *Immune modulators therapy* | | |
| NCT02077868 (IMPALA) | mCRC | III | Maintenance versus MGN1703 | 540 | OS |
| NCT02413853 (PRIMIER) | mCRC | II | PRI-724 + mFOLFOX6/bevacizumab versus mFOLFOX6/bevacizumab | 100 | PFS |

GC, gastric cancer; GEJC, gastroesophageal junction cancer; EC: esophageal cancer; NA, not assessed; MTD, maximum tolerated dose; MAD, maximum administered dose; DTL, dose limiting toxicity; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; DFS, disease-free survival; NS, not specified; RP2D, recommended dose of phase II; AE, adverse events; DCR, disease control rate; BSC, best supportive care; DCR disease control rate.

## 6. Focus on Hepatocellular Carcinoma

Over the last decades, recombinant human interferon-alfa (IFN-$\alpha$) has been extensively studied in patients with HCC, due to its previous use as immune-stimulatory antiviral agent. Both adjuvant and advanced settings have been investigated [73, 74].

Given the association of a specific HCC-directed immune response with a prognosis improvement, various targets among tumour associated antigens (TAA) or neoantigens have been investigated. Peptide based, DNA/RNA based, and DCs based vaccines have been tested in clinical setting, but efficacy data have been to date disappointing [75, 76]. In a phase 1 study published in 2012, two glypican-3 (GPC3) derived peptides restricted for HLA-A phenotypes induced specific CD8+ cells tumour infiltration; a peptide-specific cytotoxic T response was associated with longer OS, but only one out of 33 treated patients reached an objective response [77]. The same treatment was investigated in a more recent phase II single-arm study in the adjuvant setting after resection or RFA: among 41 patients evaluable, 31 (75,6%) experienced a recurrence, with a mOS of 20.1 months [78]. Telomerase-derived peptide [79], DNA, RNA, and DCs based vaccines have also been studied with overall negative results [80–86]. A new phase I clinical trial to evaluate the safety of an allogenic dendritic cell vaccine-COMBIG-DC in HCC patients is now recruiting participants (NCT01974661).

As for other GI cancers, most exciting results derived from data on immune checkpoint inhibitors. The first report came in 2013 from a pilot phase II study of tremelimumab, an anti-CTLA-4 mAb, in 20 patients with HCC and chronic HCV. Efficacy data showed a good antitumour activity, with a 17.6% RR, 76.4% DCR, and a median PFS of 6.5 months [87]; these results are remarkable when considering that the majority of patients were pretreated with sorafenib and had a Barcelona Clinic Liver Cancer (BCLC) stage C and 43% had a Child-Pugh stage B.

Several phase II/III trials with anti-PD-1/PDL-1 agents, alone or in combination with other compounds, are ongoing, with some preliminary data already reported. The most robust data presented so far are about the anti-PD-1 nivolumab, investigated alone or in combination with ipilimumab in a multicohort phase I/II study opened in 2012 for advanced HCC patients (CHECKMATE040). Four out of 5 scheduled cohorts have completed enrollment for a total of 576 HCC patients treated. Among 262 patients treated with nivolumab monotherapy, across dose escalation ($n = 48$) and dose expansion cohorts ($n = 214$), [88] a 20% RR was observed irrespective of dose, HCV, or HBV infection status and PDL-1 expression on tumour cells. Median duration of response was 9.9 months; median OS was 15.0 months and 13.2 months in dose escalation and expansion cohort, respectively. These data are very promising, especially considering the overall poor prognosis of HCC patients. In the Sorafenib Hepatocellular Carcinoma Assessment Randomised Protocol (SHARP) RR, survival rate at 12 months was 2% and 44%, respectively, and mOS was 10.7 months [89, 90]. Thus, a phase III study of nivolumab versus sorafenib in treatment-naïve patients has been planned and is already ongoing (CHECKMATE 459) [91].

The anti-PDL-1 durvalumab demonstrated clinical activity in several solid tumours, including 19 HCC patients, in a phase 1 study published in 2014 [92]; a randomized open-label phase II study is currently ongoing with durvalumab, tremelimumab, or the combination of the two compounds in patients with unresectable HCC. Pembrolizumab is also under investigation in HCC: a phase II open-label study just completed the enrollment with sorafenib intolerant or progressed patients (Keynote 224), and a phase III study planning to enroll 408 second-line patients is recruiting patients (Keynote 240). Ongoing studies are summarized in Table 3.

## 7. Possible Biomarkers for Immunotherapy: The Pathologist Perspective

The introduction of immunotherapies as possible treatment options in GI cancers made the assessment of MSI status (especially for CRC) and PD-L1 expression crucial in the pathologic assessment of GI cancers. Overall, an adequate characterization of the immune microenvironment in cancer samples emerged as the driver diagnostic element for the identification of patients likely to benefit from specific immunotherapies [93, 94].

Among the others, the greatest focus has been on PD-L1 expression [95]. Increasing evidences pointed out to the association between PD-L1 and a higher burden of disease, more extensive metastatic involvement of lymph nodes, and poorer survival, in both esophageal and gastric cancers. Although PD-L1 testing by immunohistochemistry has been associated with a significant enrichment for populations with clinical benefit to anti-PD1 or anti-PDL1 therapies, no conclusive date have been reported so far [46, 57] and several factors are limiting its use in the clinical practice. Above all, different threshold levels have been adopted for the identification of positive samples in different tumour types [96]. Several reports pinpointed the predictive value of PD-L1 expression on infiltrating immune cells instead of tumour cells [97]. Most companies have developed their own companion PD-L1 immunohistochemistry diagnostic assay characterized by different antibody-specific features. Of course, this diversified request for immunohistochemical testing and the related need of antibody/company-specific immunostainers is inconsistent with the current practice of most surgical pathology laboratories.

From a general perspective, the identification of consistent biomarkers to be introduced into clinical practice is affected by (i) the inherent biological heterogeneity of tumour microenvironment; (ii) the complexity of novel immunotherapeutic regimens and the combination of immunotherapy with other target therapeutics; (iii) the variability on molecular biology testing; (iv) the inconsistent aptitude of formalin-fixed paraffin-embedded (FFPE) preparations with many downstream molecular biology techniques [98]; (v) the significant discrepancies in the proposed biomarker evaluation systems [99]; (vi) the need of integrated diagnostics (i.e., histology, immunophenotyping, and molecular profiling), not always available in "spoke" surgical pathology units.

Table 3: Ongoing studies on hepatocellular carcinoma.

| Trial (*ClinicalTrials.gov* identifier) | Setting | Phase | Study interventions | Number of patients | Primary endpoint |
|---|---|---|---|---|---|
| *Checkpoint inhibitors* | | | | | |
| NCT02576509 CheckMate459 | 1st line | III | Nivolumab versus sorafenib in first line | 726 | OS, ORR |
| NCT02702414 Keynote 224 | 2nd line | II | Pembrolizumab in second line | 100 | ORR |
| NCT02702401 Keynote 240 | 2nd line | III | Pembrolizumab versus BSC in second line | 408 | PFS, OS |
| NCT01658878 CheckMate 040 | | I/II | Nivolumab versus nivolumab + Ipilimumab nivolumab | 620 | AEs, SAEs, ORR |
| NCT02519348 | Advanced | II | Tremelimumab + MEDI4736 versus tremelimumab versus MEDI4736 | 144 | AEs, SAEs, DLTs |
| *Vaccines* | | | | | |
| NCT01974661 | Advanced | I | COMBIG-DC (allogeneic dendritic cells) cancer vaccine | 18 | Safety and tolerability |
| *Cytokines* | | | | | |
| NCT02632188 | Resected | I/II | SURGERY → DC-PMAT treatment | 60 | PFS |
| NCT02873442 | Advanced | I/II | TACE versus TACE + precision cell immunotherapy | 40 | PFS, OS |
| NCT02487017 | Advanced | II | TACE versus TACE + DK-CIK | | OS |
| *CAR-T cells* | | | | | |
| NCT02729493 | Advanced | II | EPCAM-targeted CAR-T cells | 25 | DCR |

Similar considerations might be drawn for MSI status assessment that represents the only well-established predictive biomarker for immunotherapy response in mCRC. MSI status is assessed by means of immunohistochemistry evaluating altered expression of mismatch repair proteins (i.e., MLH1, PMS2, MSH2, and MSH6) or by means of PCR techniques detecting mutations on BAT25, BAT26, D2S123, D5S346, and D17D250, according to the Bethesda panel guidelines [100].

Due to the association of MSI status with a higher mutational and neoantigen burden, also more sophisticated next-generation sequencing approaches have been successfully applied in the evaluation of mutational load in immunotherapy clinical trials, and these methods allow the identification of other hypermutated tumour classes such as those characterized by dysfunctions in DNA polymerases (POLE) [101]. However, even these promising data, neither mutational load analysis nor the evaluation of the mismatch repair machinery status, have been included in the clinical selection of the patients undergoing immunotherapy, so far.

Both PD-L1 expression and MSI might play a role as positive predictive factors for GC and immunotherapy according to the data from the Cancer Genome Atlas (TCGA) Research Network project [22].

The presence of tumour-infiltrating lymphocytes (TILs) is an indirect sign of disease control through immune mechanisms and has been evaluated as a predictive biomarker for checkpoint inhibitor immunotherapy [102, 103]. Beside these therapeutic implications, the landmark studies of Jérôme Galon identified the prognostic value of the global assessment of the immune infiltrate (also known as immunoscore) in colorectal cancer and in other solid tumours [104, 105]. However, infiltrating lymphocytes evaluation still lacks intralaboratory and intrapathologist standardization and is not yet a widespread practice among the pathologists' community.

Because of all these challenging problems in the definition of ultimate optimized model for predicting tumour response to anti-PD1 or anti-PD-L1-based therapies, more technically complex combined biomarker strategies and/or comprehensive immune gene signatures have been also successfully tested. The limited amount of analysable material in preneoadjuvant biopsy specimens and the use of FFPE samples are, however, currently affecting the improvement of these approaches in the clinical setting.

Overall these data underline that an adequate personalized immunotherapy will be obtained only with the integration of traditional microscope-based biomarkers (such as the immunoscore) to more advanced FFPE-compatible genetic, genomic, and expression profiling strategies. A new revolutionizing era of diagnostic surgical pathology has started.

## 8. Back to the Bench: Biomarkers and Genetic Signatures as Predictive Factors

As stated above, traditional microscope-based techniques are not adequate to comprehensively assess the intriguing landscape of tumour benefitting from immunotherapy. Genetic signatures might be useful tools to identify predictive biomarkers able to help patients' selection.

Among proposed biomarkers, MSI-H status represents the only validated positive predictive factor for immunotherapy response in mCRC; however, it occurs in only 6% of patients. Given the high benefit deriving from immune checkpoint inhibitors in this setting in terms of OS, responses, and symptoms relief, recently, several papers have pointed out the importance of using gene expression profile to better identify those tumours that behave as MSI-H.

Tian et al. [106] adopted full genome expression data of stage II and stage III CRC to identify genes that correlate with MSI status. An MSI gene signature was developed and further validated in other external data set with an overall accuracy of about 90.6%. The strength of the MSI-signature is that it can identify the true MSI-H patients as well as a group of patients that are not MSI by conventional clinical tests but they are by signature. Those patients are defined as MSI-like and share the hypermutated status as pure MSI-H patients. Furthermore, they seem to not respond to 5-FU regimen as stage II MSI-H patients. If MSI-H mCRC patients benefit from immunotherapy, we can assume that also MSI-like patients will. Indeed, this is the rationale of one of the trials that will soon be run in the frame of the MoTriColor consortium (http://www.motricolor.eu/).

In line with these findings, more recently, Mlecnic et al. [107] performed a comprehensive analysis of the tumour microenvironment, immune gene expression, and mutational status in CRC, so-called immunoscore. They identified a high number of genes upregulated in MSI tumours (high immunoscore) versus MSS (low immunoscore). These genes were mainly associated with INF$\gamma$ signalling, Th1 related cytokines, antigen presentation pathways, chemokine receptors, and chemokine and leucocyte migration. However, a high immunoscore was identified also in a subset of MSS tumours and was not observed in a certain number of MSI tumours. Both MSI-H and high immunoscore predicted favorable prognosis among CRC patients; however, data derived from multivariate analyses identified the immunoscore as a stronger predictor of good CRC patients' survival than MSI and proposed it as a stronger predictor of immunotherapy response than MSI.

Although those two studies do not question the role of MSI status in CRC in terms of increased immune infiltrates, higher frequencies of frame shift mutations, and favorable outcome, still they demonstrate that the canonical MSI tests may be not sufficient to fish out all the patients that have a common "MSI phenotype" and could benefit from immunotherapy. Interestingly enough, Zhao et al. [108] also identified a MSI-H mutation signature by using whole genome and whole exome sequencing. They confirmed this signature to be similar to germline DNA, thus meaning that a fraction of genetic variations arises through mutations escaping MSI. Most importantly, they identified a large number of recurrent indels that can be used to detect MSI and that are currently under implementation for clinical application. Moreover, they found that recurrent indels are enriched for the double-strand break repair (DBS) by homologous recombination (HR) pathway. All in all, these data indicate

that the MMR pathway is not yet completely known and that in the future new biomarkers belonging to this pathway will need to be validated and used as predictive of response to immunotherapy. Moreover, the importance of those gene signatures has been shown only in CRC. Thus, further studies will be required to know if this applies to other tissues types that show microsatellite instability, such as gastric cancer, genitourinary tract malignancies, and esophageal cancer. Since MSI-H tumours are not that frequent especially in the metastatic setting, the use of gene expression profile could help in enlarging the group of patients who will benefit from such treatment. At the same time, this will also avoid that useless toxicity will be given to patients who seem to carry an MSI-H tumours but that by gene expression it is not defined as MSI-like or immunoscore positive or positive for the MMR-deficient mutation signature. A proof that a response to immunotherapy can also be observed in MSS tumours is provided by the case report published by Chen et al. [109]. Authors report indeed the case of a 64-year-old man who received pembrolizumab as second-line treatment for a HER2 positive metastatic gastric cancer. Clinical tests reported the tumour to be MSS and Epstein Barr negative and to not carry any mutations in the POLE gene, thus meaning carrying all the biomarkers that so far have been identified as negative predictors of response to immunotherapy. Although the authors did not investigate other biomarkers to understand why the patient responded to immunotherapy, based on the data here summarized, we can hypothesize that MSI clinical tests are not sensitive enough and that the integration of multiple tumour and immune response parameters such as protein expression, genomics, and transcriptomics may be necessary for accurate prediction of clinical benefit.

Finally, response to immunotherapy might not only be driven by the "genetic makeup" of the tumour and the way how the immune system reacts to it, but also by its regulation via other mechanisms such as the gut microbiota. Indeed two recent papers show its role in modulating the anticancer activity of CTLA4 and PD1 blockade. Vétizou et al. [110] elegantly showed that the gut microbiota can itself reduce the tumour volume and when combined with immunotherapy it further reduces the tumour size. This effect is driven only by certain microbiota composition, like the Bacteroides spp., which seem to be also regulated by ipilimumab itself. Life style and immunotherapy could change the gut microbiota. This in turn affects interleukin 12 dependent Th1 immune response which facilitates tumour control both in mice and in patients while sparing intestinal integrity. Moreover, the oral administration of *Bifidobacterium* associates with tumour effect and when combined with anti-PDL1 therapy nearly abolishes tumour outgrowth. Whether the role of the microbiota in modulating the response to immunotherapy is tissue specific is not yet clear. Thus, further investigation is required. If those data will be confirmed, we might consider in the future the use of stool to identify biomarkers and fecal transplantation to modulate the immune response.

## 9. Future Perspectives

Recently, ASCO proclaimed immunotherapy against cancer as "the advance of the year." In particular, immune checkpoint blockade was heralded as a major breakthrough in cancer therapy in the last years [111]. However, there are still many challenges that must be overcome; in particular, in GI cancer many drugs are still in the early phase of development.

First of all, biomarkers identification and validation represent a major issue as discussed in the last 2 paragraphs of the review.

Secondly, clinicians still need to learn how to deal with response assessment in patients receiving immunotherapy. Conventional and nonconventional responses have been reported. As an example, patients experiencing a rapid disease progression need to be carefully evaluated with an expert radiologist, to exclude the occurrence of a pseudoprogression, identified as the burning of an inflammatory response that can simulate the onset of new lesions. Moreover, we have to be aware that a RECIST response might be observed after more than three months of treatment but can be persistent after occurrence [112]. It has also been proposed that RECIST criteria might not be adequate to assess immune response; although immune-related response criteria have been developed [112], they are still not universally adopted especially in clinical trials on GI immunotherapy [112].

Toxicity profile of immunotherapeutic agents represents another thorny issue. GI oncologists involved in clinical trials are facing a different adverse event scenario compared to the traditional chemotherapy one and need to improve their knowledge and skills to treat immune-related toxicities in a subset of patients who may already have baseline GI, liver function, and endocrine abnormalities from their underlying cancer or as complications from prior treatments. [113].

Finally the most efforts are focusing on the development of novel approaches to enhance this innovative strategy. All ongoing trials are shown in Tables 1–3. Promising trials have been evaluating innovative combination treatments (so-called "combo-immunotherapy"), that is, PD-1 or PD-L1 blockade in combination with (1) anti-CTLA4, (2) adaptive immunotherapy such as anti-LAG3, (3) innate immunotherapy such as TLRs agonists, (4) chemo- or radiotherapy, (5) drugs able to increase antigen presentation such as the COX-2, JAK1/2 inhibitor or the MEK inhibitor cobimetinib, and (6) targeted therapy (anti-HER2, anti-VEGFR2) [69, 114, 115].

From a clinician perspective, the use of immunotherapies in recent clinical trials gave us the opportunity to contribute to a paradigmatic shift in the treatment of GI cancers. We are glad to observe highly pretreated patients experiencing a dramatic clinical benefit after treatment start, with symptoms relief, long lasting disease stabilization, and an overall manageable safety profile. We are really feeling a revolution in the daily life of our patients. Every day we ask questions about future availability of clinical trials involving immunotherapeutic agents for GI cancers from our new and historical patients. We strongly believe that further steps of drugs development such as larger phases II and III clinical trials are warranted in order to answer unsolved question and to establish the efficacy of immunotherapeutic agents. A wide international involvement of experienced centers in the next clinical trials will break a potential unequal distribution of immunotherapeutic resources.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

## Authors' Contributions

Letizia Procaccio and Marta Schirripa equally contributed as first authors. Vittorina Zagonel and Sara Lonardi equally contributed as senior authors.

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*Review Article*

# Immunotherapy for Patients with Advanced Urothelial Cancer: Current Evidence and Future Perspectives

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In recent years, immunotherapy has produced encouraging results in a rapidly increasing number of solid tumors. The responsiveness of bladder cancer to immunotherapy was first established in nonmuscle invasive disease in 1976 with intravesical instillations of bacillus Calmette-Guérin (BCG). Very recently immune checkpoint inhibitors demonstrated good activity and significant efficacy in metastatic disease. In particular the best results were obtained with programmed death-ligand-1 (PD-L1) and programmed death-1 (PD-1) inhibitors, but many other immune checkpoint inhibitors, including anti-cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) antibodies, are currently under investigation in several trials. Simultaneously other therapeutic strategies which recruit an adaptive immune response against tumoral antigens or employ externally manipulated tumor infiltrating lymphocytes might change the natural history of bladder cancer in the near future. This review describes the rationale for the use of immunotherapy in bladder cancer and discusses recent and ongoing clinical trials with checkpoint inhibitors and other novel immunotherapy agents.

## 1. Introduction

As well documented by a large body of research, tumor cells are able to avoid control and destruction by the immune system using a range of complex and often overlapping mechanisms that lead to disruption of key components involved in the effective antitumor response [1–4]. Immune system should recognize and eliminate tumor cells that can avoid this immune response by disrupting antigen presentation, either through downregulation of major histocompatibility complex (MHC) class I molecules or by disabling antigen-processing machinery. Alternatively, or in addition, tumors can be able to suppress the immune system by a disruption of molecular pathways involved in controlling T-cell inhibition and activation or by recruiting immunosuppressive cell types, such as regulatory T-cells (Tregs) and myeloid-derived suppressor cells. Another mechanism that tumor cells may use in order to suppress immune activity is the release of factors, including adenosine and prostaglandin E2 and the enzyme indoleamine 2,3-dioxygenase (IDO) [3].

The robust progress in the understanding of these tumor immune-evasion strategies has resulted in the evaluation of various approaches to target and harness the patient's immune system directly to kill tumor cells. Consequently, in recent years, new generation of immunotherapy has produced relevant results in a rapidly increasing number of solid tumors. With the exception of the therapeutic vaccine sipuleucel-T that was approved for the treatment of prostate cancer in 2010, all these practice-changing results have been obtained with immune checkpoint inhibitors. Two major classes of drugs have been tested: anti-cytotoxic T-lymphocyte-associated protein (CTLA)-4 antibodies and anti-programmed death-1 (PD-1) or anti-programmed death-ligand-1 (PD-L1) antibodies. Starting from melanoma, these

drugs have produced positive results in many solid tumors. Differently from classical chemotherapy and from the majority of molecularly targeted agents that act by directly targeting tumor cells, all the immune checkpoint inhibitors act by targeting the patient's immune system against tumor cells.

First important results have been obtained with ipilimumab in patients affected by malignant melanoma [5, 6]. Subsequently, also nivolumab and pembrolizumab demonstrated efficacy in these patients [7–9].

Following the results obtained in patients with malignant melanoma, immune checkpoint inhibitors have produced clear evidence of efficacy, within randomized controlled trials, in the treatment of patients with advanced non-small-cell lung cancer (NSCLC). Namely, in patients who have failed first-line platinum-based chemotherapy, nivolumab, pembrolizumab, and atezolizumab, all given as single agents, demonstrated an improvement in overall survival compared to docetaxel [10–13]. In addition, pembrolizumab has also shown superiority compared to platinum-based chemotherapy, when given as first-line in a population of advanced NSCLC patients, selected for the high expression of PD-L1 in tumor cells [14].

Nivolumab has also been approved for the second-line treatment of advanced renal cell cancer, following the results of a randomized phase III trial showing an improvement in overall survival compared to everolimus [15].

Furthermore, the list of other solid tumors where immune checkpoint inhibitors have already produced evidence of activity and efficacy and where these drugs are currently under investigation is long.

## 2. Rationale for Immunotherapy in Urothelial Cancer

The efficacy of immunotherapy in bladder cancer was first established in 1976 when Morales et al. proved for the first time that intravesical instillations of bacillus Calmette-Guérin (BCG) were efficient in preventing recurrences of high-risk nonmuscle invasive urothelial bladder cancer and in treating carcinoma in situ [16]. Although the mechanism of action of BCG is not yet clear even after forty years from the first evidence, it seems to stimulate a cytotoxic response trough the combination of antigenic fragments, processed by bladder cancer cells, with the histocompatibility complex on the tumor cells surface [17].

After this initial success, many other attempts have been made to take advantage of directing T-cells against bladder cancer cells both in the localized and advanced disease, using activating cytokines such as interleukin- (IL-) 2 and interferon- (IFN-) alfa-2B [18, 19]. These drugs have shown limited benefits in achieving disease control.

A turning point took place on the second decade of this century when immune checkpoint inhibitors arrived on the scene. Contrary to the previous strategy this new class of monoclonal antibodies aims to reduce inhibitory signaling instead of directly stimulating T-cells.

The first receptor to be targeted was CTLA-4, a molecule expressed on activated CD4 and CD8 T-cells. CTLA-4 competes with CD28 for the interaction with the costimulatory CD80-CD86 molecules on antigen presenting cells (APCs). While the latter interaction promotes T-cells activation and effector functions, CTLA-4-CD80/86 inhibits T-cell activation in lymphoid tissues [20]. Two monoclonal antibodies targeting CTLA-4 have been developed: ipilimumab and tremelimumab, whose effect is to shift T-cell equilibrium toward activation.

It has been further observed that tumor cells might evade immune system surveillance through the interaction between PD-L1 and PD-L2 with their receptor PD-1, which is expressed on CD4 and CD8 T-cells, Tregs, B-cells, and natural killer (NK) cells. Acting directly among tumor microenvironment, drugs directed against either PD-1 or PD-L1 are usually characterized by lower adverse effects than CTLA-4 inhibitors [21].

Furthermore, many other immune checkpoint receptors are currently under investigation in several trials, as potential therapeutic targets. Simultaneously other therapeutic strategies which recruit an adaptive immune response against tumoral antigens might change the natural history of bladder cancer in the near future [20].

Bladder cancer usually shows some biological features that have been associated with better response to immunotherapies. First of all, an adaptive immune response against cancer cells requires the presence tumor antigens endowed with a good immunogenicity. More the mutation board, more likely this kind of antigens is expressed in tumor microenvironment. Bladder cancer is often characterized by a high mutation load. Moreover PD-L1 expression on the surface of tumor cells has been correlated with a higher-stage, suggesting good response to PD1/PD-L1 antagonist, although the results of different trials did not observe the association between PD-L1 expression and tumor response rate [22].

Indeed, the major challenge that is going to be faced in the next years is to find predictive factors granted by greater sensibility and specificity.

## 3. PD-L1 Inhibitors

*3.1. Atezolizumab.* Atezolizumab is an engineered human monoclonal antibody against PD-L1, able to inhibit the interaction between PD-L1 and its receptor PD-1. A multicentre, nonrandomized, phase II trial (IMVigor 210) evaluated the efficacy and safety profile of intravenous atezolizumab (given every three weeks at the dose of 1200 mg) in two different cohorts of locally advanced or metastatic urothelial carcinoma: cohort A included treatment naïve patients, ineligible for cisplatin; cohort B included patients progressing during or after platinum-based chemotherapy. PD-L1 expression on tumor infiltrating immune cells was assessed prospectively by immunohistochemistry. On the basis of PD-L1 expression, patients were categorized in three subgroups: IC0 (<1%), IC1 (≥1% but <5%), and IC2/3 (≥5%).

In cohort B, among the 310 evaluable patients overall response rate (ORR) was 15% (95% CI, 11–20) with 5% of complete responses. High levels of PD-L1 expression were associated with better ORR (27%; 95% CI, 19–37). After a median follow-up of 11.7 months, the median duration of response has not yet been reached, and durable responses

have been recorded also in patients with poor prognostic features [23] (Table 1). Median overall survival was 11.4 months in patients in the IC2/3 group, 8.8 months in the IC1/2/3 group, and 7.9 months in the whole cohort of patients.

Due to these positive results, Food and Drug Administration (FDA) approved in May 2016 atezolizumab for the treatment of patients with locally advanced or metastatic urothelial carcinoma progressing during or following platinum-containing chemotherapy or within 12 months of either adjuvant or neoadjuvant platinum chemotherapy. The recommended dose is 1200 mg, given as an intravenous infusion every three weeks.

As for the cohort A of patients who were not eligible for cisplatin ($n = 123$), the ORR was 23% (95% CI, 16–31) in all patients, with slight but not statistically significant differences among PD-L1 subgroups. ORR was 28% (95% CI; 14–47) in patients with high PD-L1 expression and 21% (95% CI; 9–36) in patients PD-L1 negative. After a 17.2 months median follow-up duration, median overall survival (OS) was 15.9 months (95% CI; 10.4 to not estimable) in all patients [24] (Table 1).

In the attempt of identifying predictive factors of activity and efficacy of atezolizumab, in addition to PD-L1 determination, authors evaluated also Cancer Genome Atlas gene expression and mutation load. In both cohorts, responses were more frequent in the Luminal II subtype and in patients with higher mutation load, irrespective of PD-L1 expression. Moreover, in cohort B, PD-L1 expression and responses to atezolizumab were most closely associated with immune activation gene expression (e.g., interferon-$\gamma$-inducible T-helper-1-type chemokines: CXCL9 and CXCL10) and CD8 T-cell infiltration [23, 24].

Treatment with atezolizumab was well tolerated in both cohorts, with serious adverse events (AEs) occurring in 15-16% of patients, and only a treatment-related death for sepsis occurred in cohort A [23, 24].

A phase 3 trial is evaluating the efficacy of atezolizumab compared to second-line chemotherapy in patients with locally advanced or metastatic urothelial carcinoma progressing to platinum-based treatment; furthermore, several studies are ongoing investigating atezolizumab monotherapy or in combination with chemotherapy or other immunological agents in different stages of disease (Table 2).

*3.2. Durvalumab.* Durvalumab (MEDI4736) is a selective, high-affinity, human monoclonal antibody directed against PD-L1. A phase I/II multicentre dose escalation and dose-expansion study is ongoing in patients with advanced solid tumors, to evaluate safety, tolerability, and antitumor activity of durvalumab monotherapy. In June 2016, Massard et al. published first results about patients with urothelial carcinoma progressing on or ineligible for cisplatin-based therapy ($n = 61$). Durvalumab, at the dose of 10 mg/kg, was administered intravenously every two weeks, for up to twelve months. Patients were categorized on the basis of PD-L1 expression, assessed either on tumor cells or on immune cells (adopting a cutoff of 25%). Among the 42 patients evaluable for response, the ORR was 31% (95% CI, 17.6–47.1). A greater antitumor activity was observed in the PD-L1 positive subgroup (46.4%; 95% CI, 27.5–66.1); in the PD-L1 negative patients the ORR was 0% (95% CI, 0.0–23.2). At the time of analysis, responses were ongoing in 12 of 13 patients with a median duration of response not yet reached (range: 4.1 to 49.3 weeks). Treatment tolerance was optimal; serious AEs occurred in 4.9% of patients, with no treatment-related deaths [25] (Table 1).

An update of this study has been presented at 2017 ASCO Genitourinary Cancer Symposium. Efficacy analysis included 103 patients with a median follow-up of 7.3 months. The ORR was 20.4% (13.1–29.5) in the overall population and 29.5% (18.5–42.6) in the PD-L1 positive subgroup versus 7.7% (1.6–20.9) in the PD-L1 negative patients [26].

In February 2016 the FDA granted a breakthrough therapy designation to durvalumab as a treatment for PD-L1-positive inoperable or metastatic urothelial bladder cancer patients progressing on platinum-based treatment.

Several trials are ongoing in urothelial carcinoma patients investigating activity of durvalumab, alone or in combination with the anti-CTLA4 tremelimumab (Table 2).

*3.3. Avelumab.* Avelumab is a fully human anti-PD-L1 monoclonal antibody. A large phase Ib trial is ongoing, investigating safety, tolerability, and clinical activity of avelumab in patients with locally advanced or metastatic solid tumors, including patients with urothelial carcinoma whose disease progressed after platinum-based chemotherapy or who were platinum ineligible. Avelumab showed preliminary safety and efficacy in a cohort of 44 patients [27] (Table 1), so an additional cohort of 129 eligible urothelial carcinoma patients was enrolled and received avelumab, 10 mg/kg, every two weeks until confirmed progression, unacceptable toxicity, or withdrawal. Preliminary data about 109 patients with at least four months of follow-up were presented at 2016 ESMO Congress: confirmed ORR was 16.5% (95% CI, 10.1–24.8), with 3 complete and 15 partial responses. PFS rate at 12 weeks was 35.6 (95% CI; 26.5–44.7). Treatment was well tolerated; grade 3-4 treatment-related adverse events occurred in 9% of patients; and pneumonitis resulted in one treatment-related death [28]. An update of this study was reported at 2017 ASCO Genitourinary Cancer Symposium. Data were available in 153/241 patients with at least six months of follow-up: ORR was 17.6% (95% CI, 12.0–24.6), 88.9% of responses were ongoing at the time of analysis, and median OS was 7.0 months (95% CI, 5.6–11.1). Based on a ≥5% PD-L1 expression cutoff assessed prospectively on tumor cells, ORR was significantly higher in PD-L1 positive patients (25.0%; 95% CI, 14.4–38.4) compared with PD-L1 negative subgroup (14.7%; 95% CI, 7.6–24.7; $p = 0.178$). Treatment was well tolerated, with only 7.5% grade ≥ 3 treatment-related AEs [29].

A randomized, open-label phase 3 trial of avelumab + best supportive care (BSC) versus BSC alone as maintenance therapy after first-line platinum-based chemotherapy is ongoing in patients with advanced urothelial cancer (Table 2).

## 4. PD-1 Inhibitors

*4.1. Nivolumab.* Nivolumab is a fully human anti-PD-1 monoclonal antibody, currently approved for the treatment for

Table 1: Clinical trials with anti-PD-L1 and anti-PD-1 immune checkpoint inhibitors in metastatic urothelial cancer.

| Drug | Trial | Phase | Indication | Sample size ($n$) | Control arm | Results in all pts | Results according to PDL1 |
|---|---|---|---|---|---|---|---|
| Atezolizumab 1200 mg IV q3w* | ImVigor 210 | II | Cohort A: 1° line, cisplatin ineligible [24] | 123 | | **IR-ORR** 23% (95% CI 16–31)<br>*IA-ORR:* 25% (18–34)<br>*DOR* not reached (range 3.7–21.0+)<br>*PFS* 2.7 months (95% CI 2.1–4.2)<br>*OS* 15.9 m (95% CI 10.4 to N.E.) | **IR-ORR** 28% (14–47) in IC2/3, 24% (15–35) in IC1/2/3, 21% (95% CI 10–35) in IC1, and 21% (95% CI 9–36) in IC0<br>*IA-ORR:* 31% (16–36) in IC2/3, 25% (16–36) in IC1/2/3, 21% (95% CI 11–35) in IC1, and 26% (95% CI 13–42) in IC0<br>*PFS* 4.1 m (2.3–11.8) in IC2/3, 2.1 m (2.1–5.4) in IC1, and 2.6 m (2.1–5.7) in IC0<br>*OS* 12.3 m (6.0 to N.E.) in IC2/3, and 19.1 m (9.8 to N.E.) in IC0/1 |
| | | | Cohort B: postplatinum [23] | 310 | | **IR-ORR**: 15% (95% CI 11–20)<br>**IA-ORR**: 19% (95% CI 15–24)<br>*DOR:* not reached (range 2.0–13.7 m)<br>*IR-PFS:* 2.1 m (95% CI 2.1-2.1)<br>*IA-PFS:* 2.7 m (95% CI 2.1–3.9)<br>*OS:* 7.9 m (95% CI 6.6–9.3)<br>*OS at 12 m:* 36% (95% CI 30–41)<br>*Safety:* Any grade TRAE 215 (69%), serious TRAE 50 (16%) | **IR-ORR**: 26% (95% CI 18–36) in IC2/3, 18% (95% CI 13–24) in IC1/2/3<br>**IR-ORR**: 27% (95% CI 19–37) in IC2/3, 22% (95% CI 16–28) in IC1/2/3<br>*DOR:*<br>*IC-PFS:* 2.1 m (95% CI 2.1–4.1) in IC2/3, 2.1 m (95% CI 2.1-2.1) in IC1/2/3<br>*IA-PFS:* 4.0 m (95% CI 2.6–5.9) in IC2/3, 2.9 m (95% CI 2.1–4.1) in IC1/2/3<br>*OS:* 11.4 m (95% CI 9.0–N.E.) in IC2/3, 8.8 m (95% CI 7.1–10.6) in IC1/2/3<br>*OS at 12 m:* 48% (95% CI 38–58) in IC2/3, 39% (95% CI 32–46) in IC1/2/3 |
| Durvalumab 10 mg/kg q2w [25] | NCT01693562. | I/II | Unresectable or metastatic | 61 | | **Safety**: Any grade AE 39 (63.9%), serious AE 3 (4.9%)<br>*IA-ORR* 31.0% (95% CI, 17.6 to 47.1) in all pts | *IA-ORR:* 46.4% in PD-L1+ and 0% in PD-L1–<br>*DCR at 12 w:* 57.1% in PD-L1+ and 28.6% in PD-L1– |
| Avelumab 10 mg/kg q2w [27] | NCT01772004 | I | Postplatinum or cisplatin ineligible | 44 | | **Safety**: Any grade AE 29 (65.9%), serious AE 3 (6.8%)<br>*IR-ORR:* 18.2% (95% CI, 8.2%–32.7%)<br>*OS:* 13.7 m (95% CI, 8.5–N.E.)<br>*PFS:* 11.6 w (95% CI, 6.1–17.4) | *ORR:* 53.8% in PD-L1 ≥ 5% versus 4.2% in PD-l1 < 5% |

Table 1: Continued.

| Drug | Trial | Phase | Indication | Sample size ($n$) | Control arm | Results in all pts | Results according to PDL1 |
|---|---|---|---|---|---|---|---|
| Nivolumab 3 mg/kg IV q2w | Checkmate 032 NCT01928394. [30] | I/II | Postplatinum or refusing it | 78 | | **IA-ORR**: 19 (24.4%, 95% CI 15.3–35.4)<br>*DOR:* 1.5 m (1.2–4.1)<br>*PFS:* 2.8 m (95% CI 1.5–5.9)<br>*OS:* 9.7 m (95% CI 7.3–16.2)<br>*Safety:* Any grade TRAE 63 (81%), serious TRAE 17 (22%) | **IA-ORR**: 24% (95% CI 9–45) in PD-L1 ≥ 1%, 26% (14–42) in PD-L1 < 1%, 24.5 (13.8–38.3) in PD-L1 < 5%, 28.6 (8.4–58.1) in PD-L1 ≥ 5%<br>*PFS:* 5.5 m (95% CI 1.4–11.2) in PD-L1 ≥ 1%, 2.8 m (1.4–6.5) in PD-L1 < 1%, 2.8 (1.5–7.0) in PD-L1 < 5%, 5.5 (1.2–11.2) in PD-L1 ≥ 5%<br>*OS:* 16.2 m (95% CI 7.6–N.E.) in PD-L1 ≥ 1%, and 9.9 m (7.0–N.E.) in PD-L1 < 1%, 10.4 (7.0–N.E.) in PD-L1 < 5%, 12.9 (2.8–N.E.) in PD-L1 ≥ 5% |
| | Checkmate 275 NCT02387996 [31] | II | Postplatinum (no liver metastasis if ≥2 CT lines) | 270 | | **IR-ORR**: 19.6% (95% CI 15.0–24.9)<br>*IA-ORR:* N.A.<br>*PFS:* 2.00 m (95% CI 1.87–2.63)<br>*OS:* 8.74 m (95% CI 6.05–N.E.) | **IR-ORR:** 28.4% (95% CI 18.9–39.5) in PD-L1 ≥ 5%, 23.8% (95% CI 16.5–32.3) in PD-L1 ≥ 1%, 16.1% (95% CI 10.5–23.1) in PD-L1 < 1%<br>*IA-ORR:* N.A.<br>*PFS:* N.A.<br>*OS:* 11.30 m (8.74–N.E) in PD-L1 ≥ 1%, 5.95 m (4.30–8.08) in PD-L1 < 1% |
| Nivo 240 mg q2w → Nivo 3 mg/kg + Ipi 1 mg/kg q3w, then Nivo 240 mg q2w | CA209-260 NCT02553642 [32] | 2 | Metastatic, option of treatment with the combination if confirmed PD with Nivo | 40 (10 treated with Nivo + Ipi) | | **DCR**: 4/10 (1 partial response and 3 stable diseases) | |
| Pembrolizumab 200 mg q3w | Keynote 012 NCT01848834 [33] | Ib | Unresectable or metastatic | 33 | | **IR-ORR**: 26% (95% CI 11–46)<br>**Safety**: Any grade TRAE 20 (61%), serious TRAE 5 (15%)<br>*IA-ORR:*<br>*PFS:* 2 m (95% CI 2–4)<br>*DOR:* 10 m (range 4–22+)<br>*OS:* 13 m (95% CI 5–20) | Patients were required to have ≥1% PD-L1 expression |
| | Keynote 052 NCT02335424 [34] | II | Cisplatin ineligible | 370 | | **IR-ORR**: 27% (22–32)<br>*DOR:* (1+ to 14+ m)<br>*PFS 6 m:* 31%<br>*OS 6 m:* 67% | |
| | Keynote 045 NCT02256436 [36] | III | Postplatinum | 542 | Investigator's choice CT with paclitaxel, docetaxel, or vinflunine | **OS**: 10.3 versus 7.4 m (HR: 0.73; $p = 0.002$)<br>**PFS**: 2.1 versus 3.m (HR: 0.98; $p = 0.42$)<br>*ORR:* 21.1 versus 11.4% ($p = 0.001$)<br>*DOR:* not reached versus 4.3 m | **OS**: 8 versus 5.2 (HR: 0.57; $p = 0.005$) in PD-L1 ≥ 10%<br>**PFS**: HR: 0.89 $p = 0.24$<br>*ORR:* N.A.<br>*DOR:* N.A. |

AE: adverse events, CT: chemotherapy, DOR: duration of response, IA: investigator-assessed, IC: immune cells, IR: independently reviewed, m: months, N.A.: not available, N.E.: not estimable, ORR: overall response rate, OS: overall survival, PFS: progression free survival, TRAE: treatment-related adverse events, w: weeks; bold refers to primary endpoint; italics refers to secondary endpoint; * PD-L1 expression status was defined by the percentage of PD-L1+ IC: IC0 (<1%), IC1 (≥1% but <5%), and IC2/3 (≥5%).

Table 2: Ongoing clinical trials of anti PD-L1 and anti PD-1 immune checkpoint inhibitors in metastatic urothelial cancer.

| | Study | Phase | Regimen | Primary endpoints | Planned number of pts or pts enrolled | Status |
|---|---|---|---|---|---|---|
| Atezolizumab | NCT02302807 (IMVigor 211) | III | Atz 1200 mg IV d1 q3w versus CT (Vnf 320 mg/m$^2$ or Txl 175 mg/m$^2$ , or Txt 75 mg/m$^2$ ) IV d1 q3w | OS | 932 | Active, not recruiting |
| | NCT02807636 (IMVigor 130) | III | Atz 1200 mg IV d1 + CT (Crb AUC 4.5 IV d1 + Gem 1000 mg/m$^2$ IV d1,8 q3w) versus Placebo + CT | OS, PFS and Safety | 435 | Currently recruiting |
| | NCT02989584 | II | Atz 1200 mg IV d8 q3w + Gem 1000 mg/m$^2$ IV d1,8 + Cis 70 mg/m$^2$ d1 q3w (maintenance in phase II) | Safety | 30 | Currently recruiting |
| | NCT02298153 (ECHO-110) | I | Atz 1200 mg IV q3w + Epacadostat 25 mg OS BID as starting dose, followed by dose escalations. | Safety | 118 | Currently recruiting |
| | NCT02928406 | III | Atz 1200 mg IV q3w | Safety | 1000 | Active, not recruiting |
| | NCT02655822 | I | CPI-444 in 3 different schedules versus CPI-444 + Atz IV | Safety, ORR, median AUC of CPI-444 | 534 | Currently recruiting |
| | NCT02543645 | I/II | Varlilumab 0.3 or 1 or 3 mg/kg + Atz 1200 mg IV q2w | Safety, ORR | 55 | Currently recruiting |
| Durvalumab | NCT02516241 | III | IV Drv +/− IV Trm versus CT (platinum + Gem) | PFS, OS | 1005 | Active, not recruiting |
| | NCT02546661 (Biscay) | I | (A) Drv + AZD4547<br>(B) Drv + olaparib<br>(C) Drv + AZD1775<br>(D) Drv<br>(E) Drv + Vistusertib | Safety | 110 | Currently recruiting |
| | NCT02527434 | II | IV Trm versus IV Trm + IV Drv versus IV Drv | ORR | 66 | Currently recruiting |
| | NCT02643303 | I/II | IV Drv + IV Trm +/− IT/IM PolyICLC | Recommended combination dose, safety, ORR, PFS and OS | 102 | Active, not recruiting |
| | NCT02318277 | I/II | Drv IV q2w + OS INCB024360 25 mg BID followed by dose escalations. | DLT, ORR | 185 | Currently recruiting |
| Avelumab | NCT02603432 (JAVELIN Bladder 100) | III | Avl IV q2w + BSC versus BSC | OS | 668 | Currently recruiting |
| Nivolumab | NCT02387996 | II | IV Niv | ORR | 242 | Active, not recruiting |
| | NCT02897765 | I | Niv IV 240 mg q2w +/− NEO-PV-01 SC + Adj | Safety | 90 | Currently recruiting |
| | NCT02496208 | I | OS cabozantinib-s-malate + IV Niv +/− IV Ipi | Safety and DLT | 66 | Currently recruiting |
| | NCT01928394 (Checkmate 032) | I/II | IV Niv +/− IV Ipi (different schedules) +/− OS Cobimetinib | ORR | 1150 | Currently recruiting |
| | NCT02636036 (SPICE) | I | IV Niv + IV Enadenotucirev | MTD | 30 | Currently recruiting |

Table 2: Continued.

| | Study | Phase | Regimen | Primary endpoints | Planned number of pts or pts enrolled | Status |
|---|---|---|---|---|---|---|
| | NCT02834013 (DART) | II | Niv IV d1,15,29 + Ipi IV d1 q6w | ORR | 334 | Active, not recruiting |
| | NCT02614456 | I | Phase 1: IFN-$\gamma$ SC 50 $\mu$g/m$^2$ d1–7<br>Phase 2: IFN-$\gamma$ SC QD + Niv IV d1 q2w<br>Phase 3: Niv IV d1 q3w | Safety, DLT | 15 | Currently recruiting |
| Pembrolizumab | NCT02717156 | II | Pmb IV d1 + EphB4-HSA IV d1,8,15 q3w | Safety | 60 | Active, not recruiting |
| | NCT02925533 | I | IV B-701 + IV Pmb q3w | Safety | 12 | Currently recruiting |
| | NCT02560636 (PLUMMB) | I | IV Pmb + RT | MTD, Safety | 34 | Currently recruiting |
| | NCT02351739 (Keynote 143) | II | IV Pmb +/− ACP-196 | ORR | 75 | Active, not recruiting |
| | NCT02500121 | II | Pmb 200 mg IV d1 q3w versus placebo | 6 months PFS | 200 | Currently recruiting |
| | NCT02853305 (Keynote 361) | III | Pmb 200 mg IV d1 q3w +/− CT versus CT (platinum + Gem) | PFS, OS | 990 | Currently recruiting |
| | NCT02619253 | I/II | Pmb 200 mg IV d1 q3w + Vorinostat OS d1–14 q3w | Safety | 42 | Currently recruiting |
| | NCT02826564 | I | Stereotactic body radiotherapy prior to or concurrent with IV Pmb | Safety, selection of the sequence arm with a DLT < 20% | 20 | Currently recruiting |
| | NCT02880345 (Radvax) | Pilot | IV Pmb + hypofractionated RT (2 different regimens) | Safety | 14 | Active, not recruiting |
| | NCT02437370 | I | IV Pmb + IV Txt versus IV Pmb versus IV Gem | MTD | 38 | Currently recruiting |
| | NCT02043665 (Keynote 200) | I | (A) CVA21<br>(B) CVA21 + Pmb | ORR | 60 | Currently recruiting |
| | NCT02581982 | II | Pmb 200 mg IV d1 + Txl IV d1,8 q3w | ORR | 27 | Currently recruiting |
| | NCT01174121 | II | Cyclophosphamide and fludarabine + Pmb + young TIL | Rate of tumor regression | 290 | Currently recruiting |
| | NCT03006887 | I | Pmb 200 mg IV d1 + Lenvatinib OS 20 mg QD q3w | Safety, DLT | 10 | Active, not recruiting |
| | NCT02501096 | I/II | Pmb 200 mg IV d1 + Lenvatinib OS QD q3w | MTD, ORR, DLT | 250 | Currently recruiting |
| | NCT02346955 (MK-6018-001) | I | Multidose escalation of CM-24 +/− Pmb 200 mg IV | Safety, DLT | 196 | Currently recruiting |
| | NCT02452424 | I/II | Dose escalation of OS PLX3397 + Pmb 200 mg IV | Safety | 400 | Currently recruiting |
| | NCT02432963 | I | IV Pmb + SC MVA-p53 Vaccine | Tolerability | 19 | Currently recruiting |
| | NCT02393248 | I/II | Phase 1: dose escalation/expansion of INCB054828<br>Phase 2: INCB054828 + Pmb/CT (Txt or Cis + Gem) | MTD, pharmacodynamics | 150 | Currently recruiting |
| | NCT02443324 | I | IV Pmb + Ramucirumab IV d1 q3w | DLT | 155 | Currently recruiting |
| | NCT02856425 | I | IV Pmb + OS Nintedanib | MTD | 18 | Currently recruiting |

Atz: atezolizumab; Avl: avelumab; Cis: cisplatin; Drv: durvalumab; Gem: gemcitabine Ipi: ipilimumab; Trm: tremelimumab; Txl: taxol; Txt: taxotere; Niv: nivolumab; Pmb: pembrolizumab.

Table 3: Ongoing clinical trials of anti-CTLA-4 immune checkpoint inhibitors in metastatic urothelial cancer.

| | Study | Phase | Regimen | Primary endpoints | Planned number of pts or pts enrolled | Status |
|---|---|---|---|---|---|---|
| Ipilimumab | NCT01524991 | II | IV gemcitabine 1000 mg/m$^2$ d 1,8 + cisplatin 70 mg/m$^2$ d1 q3w. IV Ipi 10 mg/kg d1 (start c3) | 1 year OS | 36 | Active, not recruiting |
| | NCT02496208 | I | OS cabozantinib-s-malate + IV Niv +/− Ipi | Safety and DLT | 66 | Currently recruiting |
| | NCT01928394 | I/II | IV Niv +/− Ipi (different schedules) +/− cobimetinib | ORR | 1150 | Currently recruiting |
| | NCT02381314 | I | IV Ipi d1 q3w + IV enoblituzumab weekly | Safety | 84 | Currently recruiting |
| | NCT02834013 (DART) | II | IV Niv d 1,15,29 + IV ipilimumab d1 q6w | ORR | 334 | Active, not recruiting |
| Tremelimumab | NCT02516241 | III | IV Drv +/− IV Trm versus CT (platinum + gemcitabine) | PFS, OS | 1005 | Active, not recruiting |
| | NCT02527434 | II | IV Trm versus IV Trm + IV Drv versus IV Drv | ORR | 66 | Currently recruiting |
| | NCT02643303 | I/II | IV Drv + IV tremelimumab +/− IT/IM PolyICLC | Recommended combination dose, safety, ORR, PFS, and OS | 102 | Active, not recruiting |

different malignancies, as front-line (melanoma) or second-line monotherapy (NSCLC, renal cell cancer) or in combination with ipilimumab (melanoma). An ongoing open-label, two-stage, multiarm, phase I/II trial, Checkmate 032, is evaluating safety and activity of nivolumab alone or in combination with ipilimumab in subjects with advanced or metastatic solid tumors. First results about a cohort of patients with advanced urothelial carcinoma, who progressed during or after platinum-based chemotherapy, treated with nivolumab alone (3 mg/kg intravenously every two weeks), were published in October 2016. Eligible patients were enrolled regardless of tumor cells PD-L1 expression that was assessed retrospectively in pretreatment tumor biopsy specimens collected within three months before treatment start. A confirmed ORR was achieved in 24.4% (95% CI, 15.3–35.4) of 78 patients treated with nivolumab monotherapy, regardless of PD-L1 tumor expression. There was no difference in the ORR between patients with PD-L1 expression lower than 1% (26.2%) and patients with PD-L1 expression ≥ 1% (24.0%). However median OS was over 16.2 months in PD-L1 positive tumors and 9.9 months in PD-L1 negative ones [30] (Table 1).

These data were recently confirmed by positive results of phase II study, Checkmate 275, evaluating activity and safety of nivolumab in 270 patients with metastatic bladder cancer progressing during or after first-line platinum-based chemotherapy. Confirmed ORR was 19.6% (95% CI, 15.0–24.9) for all patients, 28.4% for patients with PD-L1 expression of 5% or greater, 23.8% for patients with PD-L1 expression of 1% or greater, and 16.1% for patients with PD-L1 expression of less than 1%. After a median follow-up equal to 7 months, 24.4% of patients were still on treatment. Median OS was 8.74 months in the whole study population; in the subgroup of patients expressing PD-L1 ≥ 1% on tumor cells median OS was 11.3 months, while in PD-L1 negative patients it was 5.95 months. Cancer Genome Atlas gene expression was also analysed on pretreatment tumor tissue: responses were more frequent in the Basal I subtype according to Atlas classification, which showed the strongest association with interferon-y signature and the highest CD8 expression [31] (Table 1).

At 2017 ASCO Genitourinary Cancer Symposium, preliminary data about combination of nivolumab and ipilimumab have been presented. Ten patients with advanced or metastatic urothelial cancer, refractory to nivolumab monotherapy, were treated. Despite a slight increase of immune-related toxicities, treatment was well tolerated and showed a promising activity, with a disease control rate of 40% (one partial response and three stable disease were reported) [32]. Of course, the number of patients described in this preliminary experience is still too small to comment the activity of the combination. Trials ongoing evaluating nivolumab in combination with ipilimumab are shown in Table 3 and will clarify the real potential of the immunetherapy combination.

*4.2. Pembrolizumab.* Pembrolizumab is a humanized monoclonal antibody directed against PD-1, which has shown promising results for treatment of metastatic bladder cancer. Results about urothelial cancer patients' cohort of the nonrandomized, multicohort, open-label, phase 1b Keynote 012 basket trial were published in January 2017. Thirty-three patients with advanced or metastatic urothelial cancer with at least 1% PD-L1 expression in tumor cells or stroma

were enrolled and treated with 10 mg/kg intravenous pembrolizumab every two weeks, until progressive disease or unacceptable toxicity. Treatment was generally well tolerated, and only 9% of patients experienced serious adverse events. Seven of 27 evaluable patients (26.0%; 95% CI, 11.0–46.0) achieved partial or completed responses [33] (Table 1).

At 2017 ASCO Genitourinary Cancer Symposium, preliminary data of phase II Keynote 052 trial have been presented. In detail, this trial evaluated activity and safety of pembrolizumab in cisplatin-ineligible patients with metastatic or locally advanced bladder cancer, enrolled regardless of PD-L1 expression. However, PD-L1 expression was prospectively assessed in tumor and immune cells, to better characterize responding and nonresponding patients. Patients received pembrolizumab 200 mg intravenously every three weeks, for up to 24 months of treatment. Among patients with at least four months of follow-up, ORR was 27% (95% CI, 22–32); no data about activity according to PD-L1 expression were reported. The only data available have been presented at ESMO 2016 Congress: ORR was 36.7% (95% CI, 19.9–56.1) in patients with 10% or greater PD-L1 expression [34, 35] (Table 1).

Of note, a randomized phase III trial, Keynote 045 study, compared pembrolizumab to chemotherapy (consisting of either paclitaxel, docetaxel, or vinflunine according to Investigator's choice) in 542 patients with locally advanced unresectable or metastatic urothelial carcinoma recurring or progressing following platinum-based chemotherapy. A survival benefit was shown in the pembrolizumab group (median OS was 10.3 versus 7.4 months, hazard ratio for death, 0.73; 95% CI, 0.59 to 0.91; $p = 0.002$), regardless of PD-L1 expression; also ORR was significantly improved in the pembrolizumab group (21.1% versus 11.4%; $p = 0.001$). These benefits were similar across almost all subgroups examined, regardless of the type of chemotherapy or the presence of poor prognostic factors, such as hepatic metastases. Fewer adverse events for any grade occurred in the pembrolizumab group compared to patients treated with chemotherapy (Table 1) [36].

Various studies are ongoing investigating pembrolizumab activity in combination with other systemic therapies and radiotherapy (Table 2).

## 5. Drugs That Target CTLA-4

*5.1. Ipilimumab and Tremelimumab.* Safety and immunologic pharmacodynamic effects of ipilimumab, an anti-CTLA-4 monoclonal antibody, have already been evaluated in the neoadjuvant setting in a small phase II clinical trial. Twelve patients with localized, high grade, urothelial carcinoma of the bladder were treated with ipilimumab, at the dose of 3 or 10 mg/kg. Safety profile of treatment was good. In all patients, an increase in CD4 + T-cell population in both tumor tissue and peripheral blood was found, probably positively related to clinical benefit. Of note, eight patients showed tumor regression: on radical cystectomy specimens, obtained after neoadjuvant treatment, lower stages of disease were found [37].

Several trials are now ongoing, to evaluate anti CTLA-4 antibody ipilimumab or tremelimumab, alone or in combination with nivolumab or durvalumab or chemotherapy or other target therapies (Table 3). Results are not yet available.

## 6. Other Immunotherapies

Immunotherapy includes treatments that work in different ways, not only limited to anti-PD-1, anti-PD-L1, or anti-CTL A4 antibodies. There are many potential targets under study: antigens on tumor cells surface, new immune-checkpoints, and tumor microenvironment. Against some of these targets, vaccines and monoclonal antibody are on development, even if few results from clinical trials are available at the time.

*6.1. Immune System Targets*

*6.1.1. Recombinant Interleukin-2.* One of the first attempts of immunotherapy foresaw the use of recombinant interleukin-2 (rIL-2), a cytokine whose main function is to promote T-cell differentiation and activation. In 1991, nine patients with metastatic transitional bladder cancer were treated with a continuous infusion of rIL-2 associated with lymphocytes previously stimulated in vitro with the same cytokine. Unfortunately none of the patients benefited from that treatment: at the first radiological evaluation eight patients showed progression disease and one patient had stable disease [18].

*6.1.2. ALT-801.* More recently at ASCO 2015 annual meeting, preliminary results of a phase Ib/II study of cisplatin and gemcitabine in combination with ALT-801, an IL-2/T-cell receptor fusion protein, in advanced or metastatic urothelial carcinoma were presented. Dose escalation expansion cohort phase Ib study included both chemonaïve and chemorefractory patients (group 1), whereas phase II expansion study included only chemorefractory patients (group 2). 34 of the 62 enrolled patients were chemorefractory. Among these patients, ORR was 35% (95% CI: 20–54%), and median OS was 12.3 months for group 1 (data not available for group 2 and for chemonaïve patients). Almost all patients experienced severe hematological toxicities [38].

*6.1.3. B7-H3.* B7H3, also known as CD276, is a ligand of the B7 family, which also includes the better known PD-1 and PD-L1. Even if its receptor remains unidentified, B7H3 acts as coinhibitor of peripheral immune response, and its expression seems to be particularly intense in urothelial carcinoma and could correlate with poor prognosis [39]. A dose escalation phase I trial is ongoing (NCT01391143) to evaluate toxicity and potential antitumor activity of the monoclonal antibody MGA271 (enoblituzumab), in patients with various refractory cancers, including urothelial cancer that express B7H3 antigen. Preliminary data were presented at the 2015 Society for Immunotherapy of Cancer (SITC) Annual Meeting. Treatment showed an optimal tolerability with few severe adverse events and a promising activity in patients with melanoma, prostate, and bladder cancer [40].

Another phase I trial (NCT02628535) is currently recruiting participants to assess safety and establish the maximum

tolerated dose (MTD) of MGD009, a humanized, Dual-Affinity Retargeting, or DART® molecule that recognizes both B7-H3 and CD3. Patients must have B7-H3 positive unresectable locally advanced or metastatic tumors, including bladder cancer.

*6.1.4. OX-40 and 4-1bb.* OX-40 and 4-1bb, also known respectively as CD134 and CD137, are both members of the Tumor Necrosis Factor receptor (TNF-r) super-family. The former is expressed on CD4 and CD8 T-cell surfaces, the later on NK and activated T-cells. The activation of both signal pathways promotes T-cell proliferation and survival. Moreover OX-40 provides a stimulatory signal to effectors and memory T-cell population, and an inhibitory signal to regulatory T-cells [41, 42].

A phase I dose escalation study (NCT02315066) is currently recruiting participants to assess safety and potential activity of an experimental OX-40 agonist alone or in combination with a 4-1bb agonist, in patients with various tumors, including urothelial bladder carcinoma.

*6.1.5. TILs.* Another promising therapeutic strategy is the infusion of externally manipulated T-cells that could be extracted from tumor tissue, the so-called tumor infiltrating lymphocytes or TILs, to give rapid immunity. TILs could be simple expanded ex vivo or selected according to recognized antigens. In a small open trial twelve patients underwent surgery for stage IV bladder cancer and TILs from lymph nodes draining metastatic tumors were collected. In six of them, lymphocytes were infused after in vitro expansion without any severe AEs. No data are available on the efficacy of the treatment [43]. A phase II trial (NCT01174121) is now recruiting patients with metastatic solid tumors, including urothelial bladder cancer, with at least one resectable lesion for TILs generation. Lymphocytes will be reinfused after conditioning chemotherapy and pembrolizumab administration. Results of another trial (NCT02863913), not yet open for participants' recruitment, will add important evidence. It is a phase I dose escalation clinical trial to assess the safety of PD-1 knockout engineered T-cells in treating metastatic advanced bladder cancer.

### 6.2. Tumor Targets

*6.2.1. HER2.* Human epidermal growth factor receptor 2 (HER2), also known as CD340, is a member of a big receptor family, encoded by a protooncogene whose alterations (almost amplification and overexpression) are common not only in breast and gastric, but also in urothelial cancer. HER2 target therapy had shown interesting activity in preclinical studies and phase I clinical trials. Unfortunately no difference in efficacy on addiction of trastuzumab to standard chemotherapy with platinum and gemcitabine was detected in advanced or metastatic urothelial carcinoma overexpressing HER2 in a phase II clinical trial [44]. At 2017 ASCO Genitourinary Cancer Symposium preliminary results of the ongoing phase IIA MyPathway trial were presented. Twelve patients with platinum-resistant HER2-positive metastatic urothelial cancer have been enrolled, and at a median follow-up of 5.4 months there were a CR, two PR, and two stabilisation of disease [45]. Other clinical trials are still ongoing in these patients, testing other HER2 inhibitors, like trastuzumab emtansine (NCT02999672) and Lapatinib (NCT00949455, NCT02342587).

Alternative strategies are under development, looking at HER-2 as a target for immunotherapy. A dendritic cell vaccine called AdHER2, created using an individual's own immune cells, has been developed to stimulate the immune system to recognize HER-2. A phase I study (NCT01730118) is now recruiting patients with various solid tumors and HER2 overexpression [46].

*6.2.2. hCG-$\beta$.* Human Chorionic Gonadotropin beta-chain (hCG-$\beta$) is an antigen frequently expressed by epithelial malignancies, including urothelial cancer. Elevated hCG-$\beta$ serum levels and/or tissue expression are associated with a more aggressive disease course. CDX-1307 is a human monoclonal antibody against the APC mannose receptor fused to hCG-$\beta$ that acts like a vaccine. Internalized by APCs, CDX-1307 is processed and hCG-$\beta$ is presented as an antigen, inducing specific cellular and humoral immune response.

A phase I trial demonstrated that CDX-1307 is well tolerated and active, inducing consistent humoral and T-cell responses when coadministrated with Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and Tall Like Receptor (TLR) agonists, in patients with advanced epithelial malignancies, including bladder cancer [47]. A phase II trial (NCT01094496) to evaluate antitumor activity before and after bladder surgery has recently been completed, but results are not available.

*6.2.3. MAGE-A.* Melanoma associated antigen A (MAGE-A) are a family of tumor specific antigens expressed in several cancer cell types, but not in normal tissue, except for the testis. MAGE-A proteins are recognized by cytotoxic T-cells and are promising targets for immunotherapy [48]. Partial or complete responses after MAGE-A target immunotherapy have been reported, also for advanced bladder cancer. Particularly three of four heavily pretreated patients with high expression of MAGE-A were enrolled in a small pilot clinical trial. They were treated with subcutaneous injections of autologous dendritic cells pulsed with MAGE-A3 epitopes peptides, showing significant reduction of tumor burden [49].

In a phase I/early II trial, patients with stage III or IV malignancies, including three with bladder cancer, all MAGE-3 positive, were randomized to three different escalation dose levels of a recombinant MAGE-3 vaccine, associated with fixed doses of an immunological adjuvant, in order to further improve its immunogenicity. One of the bladder cancer patients showed a short-term almost complete response of two months [50].

A dose escalation phase I trial (NCT02989064) is now recruiting patients with MAGE-410 positive advanced malignancies, including bladder cancer. Treatment protocol provides the administration of autologous genetically modified

MAGE A10 T-cells, and the primary endpoint is the evaluation of safety and tolerability of that treatment.

*6.2.4. NY-ESO-1.* NY-ESO-1 is one of the most immunogenic tumor antigens, expressed in cancer and testis, but not in other normal tissues (similarly to MAGE-A1). It is expressed in approximately 25–30% of bladder cancers, highly in advanced stages, and it is considered one of the best targets for T-cell receptor (TCR) based immunotherapy in solid cancers. NY-ESO-1-specific T-cell responses, induced in cancer patients using NY-ESO-1 peptides, proteins, and viruses encoding NY-ESO-1, are too weak to eradicate tumor cells [51]. So to improve clinical response TCR gene therapy is being developed. Two phase I trials (NCT02457650 and NCT02869217) are currently recruiting participants with NY-ESO-1-expressing malignancies to evaluate the safety and feasibility of the administration of anti-NY-ESO-1 TCR engineered autologous T-cells.

*6.3. Peptide Personalized Vaccination.* In the context of an increasingly personalized medicine, an open-label, randomized phase II trial evaluated safety and efficacy of peptide personalized vaccination compared to best supportive care in eighty patients with advanced urothelial bladder cancer progressing after platinum-containing chemotherapy. Vaccination consisted in subcutaneous injections of maximum four peptides chosen from a pool of thirty-one peptides according to patients HLA type and specific peptide-reactive IgG titers. PR was observed in 9 (23%) patients in the experimental arm, with mo CR. A significant improvement in OS was also noted (HR, 0.58; 95% CI, 0.34–0.99, $p = 0.049$), but not in PFS. Treatment was fairly well tolerated; almost all AEs were of grade 1 or 2; there were no grade 4 AEs or treatment-related deaths. Obviously, as the authors concluded, further large-scale, randomized trials are needed to confirm these results [52].

## 7. Discussion

The encouraging results recently obtained with several immune checkpoint inhibitors [23–25, 27, 31, 36] raise enthusiasm about the future role of this therapeutic approach for patients affected by advanced urothelial cancer. As well known, standard treatment for these patients is platinum-based chemotherapy, characterized by a difficult balance between efficacy and treatment toxicity. The availability of immune checkpoint inhibitors, both in patients who are considered medically unfit for cisplatin and in patients who have experienced disease progression after chemotherapy, represents a clinically valuable opportunity. Interestingly, a nonnegligible proportion of patients experience a durable disease control, with a chance of long-survival that has been observed, with the use of these drugs, in many types of solid tumors.

However, similar to what is occurring also in other tumors, knowledge about predictive factors of efficacy and patients' selection criteria for immunotherapy is still not ideal and, within all the clinical trials, a relevant number of patients failed to respond to the PD-1/PD-L1 checkpoint blockades. From this point of view, it would be crucial to identify a biomarker to predict the response to checkpoint blockades. In principle, a perfect positive predictive value could allow avoiding treating patients who are not going to obtain any benefit, while a perfect negative predictive value could allow not denying treatment to any of the patients who could potentially benefit. Unfortunately, at the moment, we have no biomarker with a good positive and negative predictive value. The expression of PD-L1 has been studied as a putative biomarker in many of the trials testing anti-PD-1 and anti-PD-L1 drugs, but PD-L1 staining cannot be used to accurately select patients for PD-1/PD-L1 pathway blockade due to the low prediction accuracy and dynamic changes [53]. Interestingly, tumor infiltrating immune cells and molecules in the tumor microenvironment, alone or along with PD-L1 expression, could be useful as predictive factors [53]. Furthermore, gene analysis (gene signatures, mutational landscape, and/or mismatch-repair deficiency) could be useful if interesting preliminary evidence will be confirmed and validated in further studies [23, 24].

The diffusion of immune checkpoint inhibitors in clinical practice will imply the confidence of medical oncologists with the diagnosis and management of typical side effects associated with this therapeutic approach [54].

As for the applicability of trials results in clinical practice, reasonably, we will have no direct comparisons between different anti-PD-1 and anti-PD-L1 that are currently undergoing the clinical development. In the absence of obvious differences in efficacy emerged in indirect comparison of clinical trials, we do not know which is the best treatment choice. Other issues that are not completely answered by the evidence produced in clinical trials are the dose-response relationship (recent evidence in melanoma with ipilimumab suggests that higher dose is associated with higher efficacy [55]) and the optimal duration of treatment (continuous until disease progression or with planned "stop-and-go").

Currently ongoing trials will clarify the role of immune checkpoint agents as first-line treatment, compared to platinum-based chemotherapy. Based on the results of these trials, along with the trials testing other categories of immune treatments, treatment paradigm for patients affected by advanced urothelial cancer could be soon radically changed compared to current guidelines.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

## Authors' Contributions

Clizia Zichi and Marcello Tucci equally contributed to the review.

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