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REVIEW

Neoantigen prediction and computational perspectives towards clinical benefit: recommendations from the ESMO Precision Medicine Working Group

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Background: The use of next-generation sequencing technologies has enabled the rapid identification of non-synonymous somatic mutations in cancer cells. Neoantigens are mutated peptides derived from somatic mutations not present in normal tissues that may result in the presentation of tumour-specific peptides capable of eliciting antitumour T-cell responses. Personalised neoantigen-based cancer vaccines and adoptive T-cell therapies have been shown to prime host immunity against tumour cells and are under clinical trial development. However, the optimisation and standardisation of neoantigen identification, as well as its delivery as immunotherapy are needed to increase tumour-specific T-cell responses and, thus, the clinical efficacy of current cancer immunotherapies.

Methods: In this recommendation article, launched by the European Society for Medical Oncology (ESMO), we outline and discuss the available framework for neoantigen prediction and present a systematic review of the current scientific evidence.

Results: A number of computational pipelines for neoantigen prediction are available. Most of them provide peptide major histocompatibility complex (MHC) binding affinity predictions, but more recent approaches incorporate additional features like variant allele fraction, gene expression, and clonality of mutations. Neoantigens can be predicted in all cancer types with high and low tumour mutation burden, in part by exploiting tumour-specific aberrations derived from mutational frameshifts, splice variants, gene fusions, endogenous retroelements and other tumour-specific processes that could yield more potently immunogenic tumour neoantigens. Ongoing clinical trials will highlight those cancer types and combinations of immune therapies that would derive the most benefit from neoantigen-based immunotherapies.

Conclusions: Improved identification, selection and prioritisation of tumour-specific neoantigens are needed to increase the scope of benefit from cancer vaccines and adoptive T-cell therapies. Novel pipelines are being developed to resolve the challenges posed by high-throughput sequencing and to predict immunogenic neoantigens.

Key words: neoantigen, mutation, cancer, computational, immunotherapy, personalised vaccine

INTRODUCTION

The genomes of cancer cells contain non-synonymous somatic mutations that are not present in their healthy counterparts. These mutated peptides may represent neoantigens that can be displayed on major histocompatibility complex (MHC) molecules [called human leukocyte antigen (HLA) in humans] on the surface of malignant cells and are not subjected to central or peripheral tolerance.[1,2] Neoantigens are tumour-specific and capable of inducing antitumour immune responses via T-cell-mediated cytotoxicity; thus they represent ideal targets of antitumour immunity.

Recent work has demonstrated that neoantigens hold promise for developing novel immunotherapeutic approaches.[3–10] Several groups have shown that T cells target neoantigens in patients that respond to either immune checkpoint inhibition,[11–14] adoptive cell transfer of

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tumour-infiltrating lymphocytes (TILs),[15–21] or to neoantigen-based cancer vaccines.[4–9]

Direct evidence of the therapeutic effect of targeting neoantigens came from pre-clinical studies in which vaccination with neoantigens led to tumour shrinkage in mouse models.[22–26] In human cancers, a number of studies have shown that response to immune-mediated therapies, such as immune checkpoint inhibitors, correlated with high tumour mutation burden (TMB)[11–14,20,27–31] as well as with higher numbers of predicted neoantigens.[7,32–35] Accordingly, cancers with the highest mutational burden, a proxy for the presence of immunogenic neoantigens, exhibited the highest clinical objective responses to PD-1/PD-L1 inhibitors.[11–14,27–31] Although TMB varies across different cancers,[36] strong neoantigens, defined as those eliciting strong and effective T-cell-mediated immunity, can be found in all cancer types; it is therefore plausible that patients whose cancer has a lower tumour mutation and neoantigen burdens may still derive benefit from checkpoint inhibitors[37,38] and personalised cancer immunotherapies.[5,8]

Currently, personalised neoantigen-based cancer vaccines and adoptive T-cell therapies (ACT) have been shown to prime host immunity against tumour cells and are under clinical trial development.[4–9,39] However, despite much interest, there are many technical challenges and important questions to be answered to bring personalised cancer vaccines alone or combined with immune checkpoint blockade to the forefront of cancer care. Improved identification of tumour-specific neoantigens and a better definition of its formulation to be delivered to the immune system are all needed in order to increase tumour-specific T-cell responses and the benefit from current cancer immunotherapy.

The European Society for Medical Oncology (ESMO) Translational Research and Precision Medicine Working Group (TR and PM WG) launched a collaborative project to generate, specifically in the framework of neoantigen prediction, an analysis of the current scientific evidence and consensus recommendations on the (i) most important definitions related to the concept of neoantigens and immunogenicity; (ii) current computational methods for neoantigen prediction and the role of *in vitro* approaches for identifying ligandome and T-cell reactivity; and (iii) the open challenges in the field and how this knowledge will enable the advancement of precision immunotherapies.

IMMUNOGENICITY AND NEOANTIGEN LANDSCAPE

Immunogenicity is the ability of a peptide bound to an MHC molecule to induce adaptive immune responses. Neoantigens recognised by $CD8^+$ T cells arise from somatic mutations that result in the production of novel peptides presented on the tumour cell surface by MHC-I molecules. In addition, professional antigen-presenting cells (APC) can present mutated peptides derived from extracellular proteins bound to their MHC-II molecules.[40]

Here we refer to the neoantigen landscape as the set of accurately identified *bona fide* neoantigens within a tumour. The majority of identified mutant peptides in a tumour are not recognised by T cells (i.e. are not immunogenic).[41–44] The reason probably is that not every possible expressed mutated peptide will be processed and presented on the cell surface by MHC molecules nor will all MHC-presented peptides induce T-cell reactivity and act as immunogenic T-cell epitopes. T-cell responses against the full breadth of predicted neoantigens may be limited by immunodominance, the process in which T-cell reactivity is dominated by responses to only a small subset of potential epitopes.[45] Thus, predicting which non-synonymous somatic mutations represent *bona fide* neoantigens from sequencing data[46–48] or screening methods [reviewed in Garcia-Garijo, Fajardo, and Gros (2019)[49]] represents a major challenge.

Immunogenicity depends on several factors, including the stability and binding affinity of the peptide-MHC complex, peptide competition for MHC binding, the diversity of the T-cell receptor (TCR) repertoire, the propensity of the $CD8^+$ TCR to recognise the peptide-HLA complex, and neoantigen foreignness.[2,50] Furthermore, protein expression, post-translational modification, antigen processing and transport also play an important role.

Mutations that generate neoantigens

The most commonly studied class of neoantigens are those derived from somatic non-synonymous single-nucleotide variants (SNVs), which, by definition, are not present in matched normal tissues. Neoantigens derived from SNVs, however, are not that dissimilar to their unmutated counterpart. For this reason, only a minority of these candidate neoantigens are likely to be immunogenic. In cancers with high TMB, for instance, melanoma and lung cancers, SNV-derived neoantigens are particularly relevant.

Besides SNV-derived neoantigens, high-specificity tumour antigens arising from non-SNV genomic causes have also recently been identified.[51] These include neoantigens derived from mutational frameshifts, splice variants, gene fusions, endogenous retroelements and other tumour-specific processes.[51–53] Compared with SNVs, these mutation types have the potential to alter the protein sequences in a more dramatic fashion and may yield neoantigens that are more immunogenic.

Mutational frameshifts are insertions or deletions of nucleotides that alter the reading frame of the protein. In many cases, the altered reading frame results in novel peptides that are long enough to be recognised by T cells. In fact, mutational frameshifts are predicted to generate up to nine times more neoantigens per mutation than SNVs.[53] Frameshift neoantigens are particularly relevant for microsatellite instability-high tumours and renal cell carcinomas,[53] both of which are associated with high burden of mutational insertions and deletions. Such alterations may engender entirely novel transcripts that have no wild-type, 'normal' equivalent and, as such, may be particularly immunogenic and thereby represent ideal candidates for immunotherapy.

Splice variants may result from mutations in splice sites (e.g. *MET* exon 14 skipping) or splicing factors (e.g. *SF3B1*, *U2AF1*), intron retention and a variety of other post-transcriptional modifications. It has been suggested that mutations in splice sites produce 2.5× more predicted neoantigens than missense SNVs.[54] Splice-variant derived neoantigens may be particularly relevant for cancer types with few mutations but harbouring splice-factor mutations, such as leukaemias and lymphomas.[55]

Gene fusions are a source of immunogenic neoantigens that can mediate responses to immunotherapy.[56] An exceptional responder with metastatic head and neck cancer experienced a complete response to immune checkpoint inhibitor therapy despite a low mutational load and minimal pre-treatment immune infiltration in the tumour. A novel gene fusion (*DEK—AFF2*) was identified and demonstrated to produce a neoantigen that can specifically elicit a host cytotoxic T-cell response.[56] These findings pave the way for other low mutation burden cancers and/or fusion driven cancers, including *BCR—ABL* in chronic myeloid leukaemia, *TMPRSS2—ERG* in prostate cancer, *EWS—FLI1* in Ewing's sarcoma, and *EML4—ALK* in lung adenocarcinoma, to be further explored for immunogenicity.[57] Computational tools like INTEGRATE-neo and NeoFuse can predict putative fusion antigens from tumour RNA-sequencing data.[58,59]

Unlike SNV neoantigens currently used for personalised cancer vaccines, which are rarely shared among patients (see 'Neoantigen: Towards Clinical Benefit' section), several hotspot mutations commonly occurring in multiple cancer patients and some non-SNV-derived neoantigens (fusions and frameshift) may have the advantage of being common to multiple tumours and patients with a given cancer type.[6,23,60,61] For example, the *KRAS* G12D driver mutation has been shown to be immunogenic in the context of the HLA-C*08:02 genotype.[41]

However, for most solid tumours, there may not be recurrent neoantigen peptide sequences that would predict responder patients.[12] For example, in a cohort of 10 patients with metastatic gastrointestinal cancers, there were no immunogenic epitopes shared between these patients,[41] highlighting the challenges in developing universal vaccines. By contrast, an analysis of 10 186 *The Cancer Genome Atlas* (*TCGA*) tumour samples has described a source for common neoantigens derived from frameshift mutations present in several cancers.[62] However, whether these neoantigens evoke T-cell or clinical responses has not been demonstrated to date.

A small but relevant subset of neoantigens are those generated by oncogenic driver mutations. The advantage of targeting neoantigens from putative oncogenic drivers is that they are generally clonal (i.e. present in all cancer cells) and it would be disadvantageous for tumours to reduce their expression. The immune system may negatively select tumour clones with antigenic genetic alterations. In fact, oncogenic drivers tend to be poorly presented by MHC-I[63] and MHC-II.[64] Furthermore, targeting a single driver mutation may promote the selection of resistant subclones with alternative driver alterations. Prior work in mouse models and human tumours has shown that T-cell recognised neoantigens can be lost from a tumour cell population when exposed to T-cell pressure.[45,65,66] Nevertheless, the benefit of identifying these shared neoantigens is that they could be used for off-the-shelf immunotherapies.

Clonality of neoantigens and tumour heterogeneity

Tumour heterogeneity and the distribution of clonal and subclonal (i.e. estimated to be expressed by only some of the tumour cells) neoantigens may influence their predictive role for immunotherapy response and relevance to immune evasion mechanisms.[35,67–69]

The clonality of neoantigens seems instrumental in achieving durable benefit from immune checkpoint inhibitors (i.e. PD-1/PD-L1 blockade).[35,67] Recent work has demonstrated that the predictive value of neoantigen burden, as determined by *in silico* predictions of T-cell epitopes, was improved when solely focusing on predicted neoantigens that were found to be clonally expressed by tumours in lung cancer patients treated with anti-PD-1 therapy.[35] In addition, neoantigen-specific T-cell responses were observed against clonal mutations but not subclonal antigens in a small number of patients.[35] A high clonal neoantigen burden was associated with an inflamed tumour microenvironment.[35] Consistent with this, a recent analysis of 249 patients treated with immune checkpoint therapy found that patients whose tumours exhibited a large proportion of subclonal mutations (>50%) were less likely to benefit from therapy.[67] It thus remains unclear whether subclonal neoantigens represent markers of poor prognosis or whether clonal neoantigens simply elicit a more effective immune response.

Consistent with the importance of clonal neoantigens, a recent study used syngeneic mouse models of melanoma and patients' data to investigate the effect of intratumour heterogeneity on immune response.[69] This study demonstrated that high intratumour heterogeneity of cancer neoantigens drives immune evasion and resistance to checkpoint inhibitors, suggesting the need to quantify intratumour heterogeneity to improve patient selection for those therapies.

Multiregion metastases in disseminated lethal breast cancer patients[68] demonstrated that most of the predicted neoantigens originated from mutations shared across metastases of each patient and only a small number was private (or unique) to individual metastasis or derived from driver genes. Immune selection, in terms of depletion of neoantigens, was scarcely seen across metastases, suggesting that in advanced breast cancer, most of the metastases are in the escape phase of immunoediting.[68,70]

NEOANTIGEN IDENTIFICATION AND SELECTION

With the advances of next-generation sequencing (NGS) technologies, it has become feasible to identify mutations in genes present in the exome of an individual tumour. Once these mutations are identified, one can then predict the binding affinity of the encoded mutated peptides to the patient's HLA alleles and thereby predict potential

neoantigens.[17,71] This development has enabled the implementation of neoantigen discovery pipelines (Figure 1).[46,47,72–74]

Current bioinformatic pipelines for the prediction of candidate neoantigens from somatic mutations share four main computational modules: (i) HLA typing from tumour RNA-seq, whole-genome (WGS) or whole-exome sequencing (WES) data; (ii) inference of the mutated peptides resulting from a set of somatic mutations; (iii) prediction of HLA binding and antigen presentation[75]; and (iv) candidate neoantigen prioritisation and selection.

Bioinformatic pipelines are being actively refined to improve neoantigen identification and selection. In this section, we will discuss bioinformatic tools involved in each of the four modules.

HLA typing

The tumour neoantigen repertoire that will be presented for recognition by T cells depends on the HLA alleles of the patient. Thus, the first critical step in neoantigen prediction involves determining the HLA genotypes of the patient. There are now several computational tools that can use NGS data to accomplish this task.[76,77] These tools differ in the type of data that they use as input, the types of HLA molecules that they predict (class I and/or II) as well as the HLA resolution that they can provide (supplementary Table S1, available at *Annals of Oncology* online).

Most methods use DNA-derived NGS data, either WES or WGS. Optitype[78] and Polysolver[79] only identify class I HLA alleles, whereas other methods including ArcasHLA,[80] seq2HLA,[81] and Athlates[82] can identify the alleles of both class I and class II HLA (supplementary Table S1, available at *Annals of Oncology* online). RNA-seq data can also be used for typing HLA alleles. Optitype reported that HLA typing obtained from WES has shown better results compared with those obtained from RNA-seq data.[80]

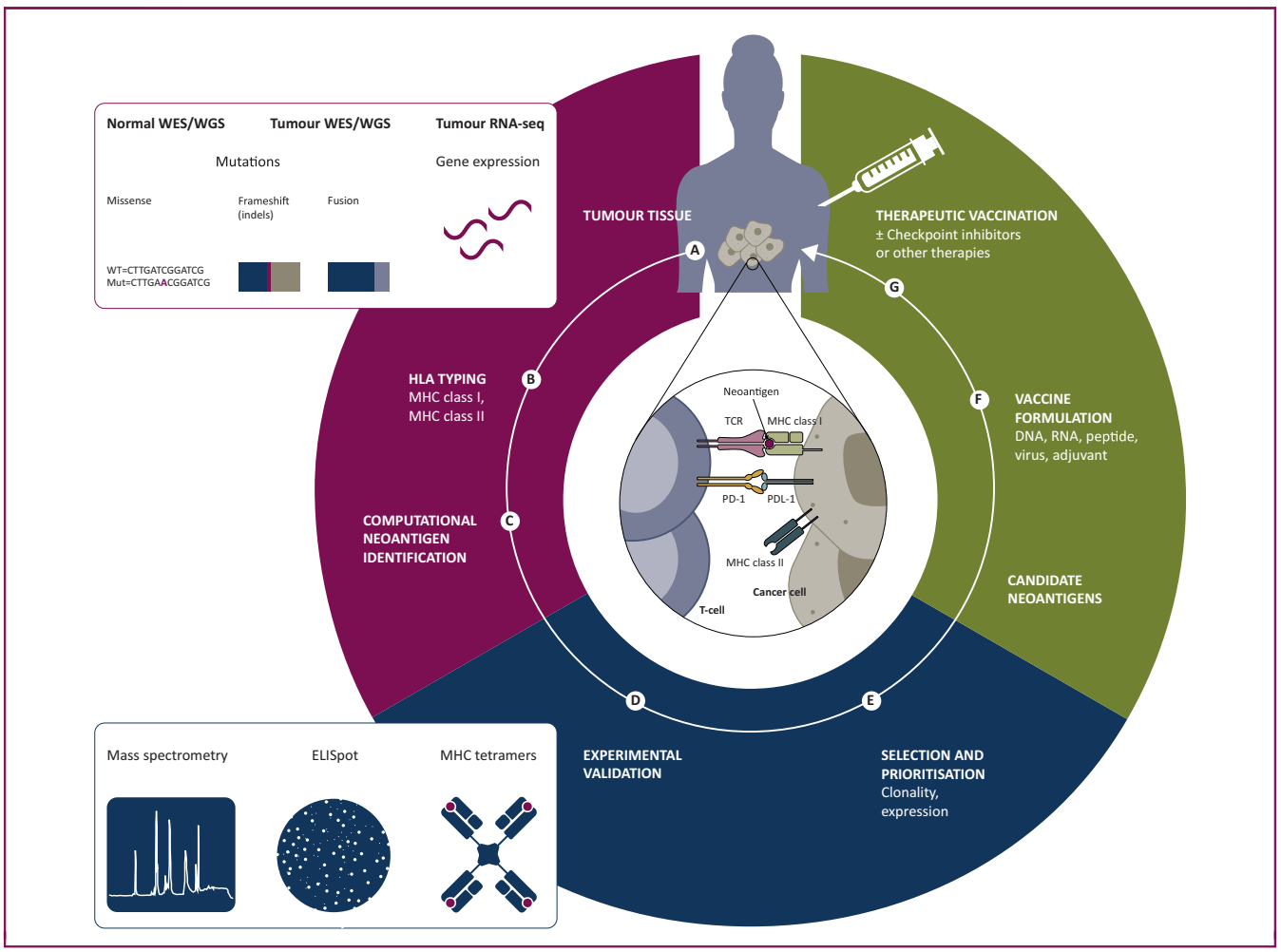

Figure 1. Neoantigen prediction workflow.
(A) Tumour tissue biopsy sampling and obtaining nucleic acids for next-generation sequencing [NGS: whole-exome sequencing (WES), whole-genome sequencing (WGS) and/or RNA-seq] and somatic variant calling; (B) Human leukocyte antigen (HLA) haplotypes of the patient inferred from NGS; (C) algorithms model the binding of mutant peptides to the major histocompatibility complex (MHC) proteins and predict candidate neoantigens; (D) validation approaches — mass spectrometry (MS) of eluted peptides; fluorescently-labelled MHC tetramers, ELISpot; (E) neoantigen selection and prioritisation; (F) vaccine formulation; (G) neoantigen cancer vaccine will be injected to enhance the strength and persistence of the patient's T-cells against the tumour.

Inference of the mutated peptides resulting from a set of somatic mutations

The present process of identifying candidate neoantigens from NGS data generally starts with the mapping of tumour-specific genetic aberrations using the WES of the tumour and normal DNA (Figure 1). In addition, RNA-seq NGS data may be integrated with WES to determine whether a mutant gene is expressed in the tumour. RNA-seq can also be used to infer the relative frequency of expression of the mutant allele (i.e. allele-specific expression) and to inform on the presence of alternative splicing events.

Prediction of HLA binding and antigen presentation

MHC binding prediction. Several computational approaches have been developed for the identification of T-cell neoantigens based on MHC class I and II processing and presentation. State-of-the-art computational methods mainly rely on machine-learning (ML) algorithms, including linear regression (LR) and artificial neural networks (ANN), trained on large experimental datasets of HLA-binding peptides (supplementary Table S2, available at *Annals of Oncology* online). Despite the observation that ANN methods provide better performance than LR, recent benchmarking studies suggest that there is no single best performing method for all HLA alleles.[72,83,84] In addition, computational methods can be further classified as allele-specific or pan-allele predictors, depending on their ability to provide predictions for MHC alleles that are not included in the training data. As no experimental binding data is available for the vast majority of known human alleles, pan-allele predictors might represent a better solution for clinical applications.

A number of MHC-I allele-specific predictors are shown in supplementary Table S2 (available at *Annals of Oncology* online). Of interest, NetMHCpan[74] is a 'panspecific' ML method for MHC-I alleles that integrates information from binding affinity data with mass spectrometry (MS) peptidome data.[85] It incorporates the notion of sequence/feature similarity between HLA alleles to allow it to make inferences about alleles for which training data does not exist based on how similar they are to the HLA alleles that do have data. It has shown an increase in predictive performance compared with state-of-the-art methods to identify cancer neoantigens.

Compared with class I, predicting MHC-II binding specificity has been more challenging.[86] The higher variability both in length and sequence composition of the recognised peptides and the diversity of the MHC-II dimer itself are usually invoked as the main reasons behind the lower performance of computational predictions. Reference methods for the prediction of peptide binding to MHC-II alleles are NetMHCII and NetMHCIIpan,[87,88] allele-specific and pan-allele, respectively.

Candidate neoantigen prioritisation and selection

The prioritisation of candidate neoantigens is usually based on the predicted binding affinities between the peptide and the MHC molecule considered alone or in combination with other features. Processes like proteasomal cleavage and peptide transport into the endoplasmic reticulum play a role in neoantigen presentation, but their modelling has brought a marginal gain in the predictive performance.[75]

Specific neoantigen prediction approaches have been described (supplementary Table S3, available at *Annals of Oncology* online).[47,48,89–92] TIminer is a user-friendly, computational framework to perform different onco-immuno-genomic analyses, including the prediction of neoantigens from somatic mutations.[92] pVACtools is a computational workflow for identification of personalised variant antigens by cancer sequencing (pVAC-Seq) that integrates tumour mutation and expression data and the determination of an optimal order of neoantigen candidates when delivered in a DNA vector.[47,48,93] It has been used to predict and prioritise neoepitopes for neoantigen studies[94,95] and several cancer vaccine clinical trials (e.g. NCT02348320 and NCT03122106).

Bulik-Sullivan et al. built a compendium of MS and RNA-seq data from human tumours and adjacent normal tissue from 74 patients across five cancer types.[46] Using these data, they trained an ML method to predict allele-specific probabilities of peptide presentation. They used these data to train an ML predictor of peptide presentation, obtaining higher positive predictive value (PPV) performance with respect to models trained only on data from HLA-binding affinity assays.

Another feature that can be considered for neoantigens prioritisation is to which HLA allele the predicted peptides bind. The HLA genes have been found to be significantly mutated in cancer[79] and recent work has revealed copy number loss of HLA alleles to be pervasive in lung cancer evolution.[96] Thus, putative neoantigens that bind to multiple HLA alleles may be more attractive targets and should be prioritised.[97] After HLA typing, mutations in HLA genes can be detected using Polysolver,[79] while to ascertain which alleles are present in a given tumour, the bioinformatics tool LOHHLA (Loss of Heterozygosity in HLA) can be used.[96] Losing the ability to present neoantigens through HLA loss appears to be a common immune evasion mechanism in cancer.[96,98,99] Discovering new approaches to counteract this will be important and combinations of cancer vaccines with agents that increase MHC expression in cancer cells should also be explored.[100,101]

THE ROLE OF IN VITRO NON-COMPUTATIONAL APPROACHES

In vitro non-computational approaches have been applied to identify the ligandome and/or neoantigen-reactive T cells.

Analysis of HLA-bound peptides by MS: MS analysis directly surveys the peptide repertoire displayed by class I and class II MHC molecules (i.e. the immunopeptidome) and also has the capability to validate *in silico* predictions.[102] Several approaches have been developed to identify the immunopeptidome. The best established methodology seems to be based on immunoprecipitation of

MHC molecules, extraction of MHC-bound peptides and analysis by high-pressure liquid chromatography coupled with MS.[103,104] One of the main limitations of MS is that this technique can only detect a relatively small fraction of the total peptides represented by MHC molecules on the cell surface and, thus, lacks sensitivity.[105]

T-cell based assays: The ultimate desired effect of neoantigen presentation by MHC is their recognition by a TCR and the activation of T cells. Measuring the magnitude of T-cell responses against tumours requires cell-based assays, which have the advantage of directly testing whether an MHC-presented neoantigen has been recognised by the T-cell repertoire of a patient.

A variety of methods that assess neoantigen-specific T-cell reactivity [e.g. directly identifying mutated antigens recognised by T cells, multicolour-labelled MHC tetramers[106,107] and the enzyme-linked immunosorbent spot (ELISpot)[108]] or T-cell repertoire profiling (e.g. ImmunoSEQ,[109] GLIPH,[110] TracerR,[111] pRESTO[112]) have been reported. They have been used either for validation or screening of neoantigen-reactive T cells.[7,108,113,114] The mutation-associated neoantigen functional expansion of specific T-cells (MANAFEST) assay was developed as a sensitive platform for monitoring antitumour immunity.[115] A broad discussion of T-cell recognition and TCR profiling are beyond the scope of this work and have been reviewed elsewhere.[49,77]

These cell-based experiments are time-consuming, labour intense, require significant numbers of cells, are difficult to standardise and should impact turnaround times for vaccine design. Therefore, high-throughput and unbiased computational strategies that use genomic information about human tumours to efficiently determine which epitopes may be recognised by either $CD4^+$ or $CD8^+$ T cells are required. Developments in this area are limited due to scarce data availability.

NEOANTIGEN: TOWARDS CLINICAL BENEFIT

Vaccines

The increased understanding of tumour-specific neoantigens as relevant targets for personalised immunotherapies has led to the development of therapeutic cancer vaccines.[116] Anticancer vaccines targeting tumour-specific neoantigens can boost pre-existing memory or effector T-cell responses and also induce new T-cell responses against potential neoantigens thus broadening antitumoural responses.[117,118]

The wide diversity of tumours and the lack of a general formulation for cancer vaccines has motivated the experimentation of a wide range of delivery approaches. The molecular nature of the antigens (i.e. peptides, DNA, RNA), the use of specific adjuvants that co-stimulate the immune system, and the route of immunisation are sources of diversity in the development of cancer vaccines.

Recent results from first-in-human clinical trials using personalised neoantigen-based vaccines to treat patients with malignant melanoma or glioblastoma patients have shown encouraging results.[4–9] Carreno et al.[7] first showed that tumour neoantigens (i.e. seven peptides representing mutations of each patient and predicted to be bound to HLA-A2), when administered as a dendritic-cell vaccine to three melanoma patients, enhanced pre-existing antitumour T-cell responses and induced responses to neoepitopes that were undetectable before vaccination.[7]

Two subsequent clinical trials evidenced the importance of neoantigen-based vaccines for the treatment of melanoma patients.[4,9] Ott et al.[9] vaccinated six patients with synthetic long peptides representing 20 MHC class I restricted neoantigens. Four out of six vaccinated patients with stage IIIB/C remained without disease recurrence. The other two patients had untreated stage IVM1b disease. Both had disease recurrence evident on restaging scans obtained after the last vaccination. Subsequently, both patients achieved complete responses after receiving immune checkpoint blockade.

In Sahin et al.[4] 13 patients received tailored RNA vaccines encoding up to 10 of their individual mutations (27-mer peptides predicted to have MHC class I and II binding). Eight patients had no radiologically detectable lesions at the time of vaccination and remained recurrence-free for the study follow-up period (12 to 23 months). Two out of five patients (all stage IV) with radiologically detectable lesions had objective responses. It should be noted that one of these patients received an immune checkpoint inhibitor before vaccination and the other patient received it after progressing to neoantigen vaccination. A third patient presented a complete response to the vaccine administered in combination with PD-1 inhibitor.

In both studies,[4,9] the majority of vaccine-induced T-cell responses were *de novo* responses, not detectable before vaccination, and the majority were mounted by $CD4^+$ or $CD4^+$ plus $CD8^+$ T cells. There was a high overall immunogenicity rate of 60%, as demonstrated by analysing T-cell reactivity against the individual mutations.

Notably, two recent clinical trials evidenced promising results of personalised neoantigen-based vaccines for patients with glioblastoma, which have, in general, low mutation burden and immunologically cold tumour microenvironments.[5,8] The studies treated 8 and 15 glioblastoma patients with personalised neoantigen-based vaccines alone or with an off-the-shelf formulation given beforehand (containing tumour-associated antigens identified from glioblastoma specimens).[5,8] The latter evidenced that non-mutated proteins drive robust $CD8^+$ T-cell responses. The majority of responses were induced by $CD4^+$ T cells, rather than $CD8^+$ T cells, against predicted neoepitopes.

In terms of objective responses, Hilf et al.[8] reported that, out of 15 vaccinated patients, one had a complete response and three had partial responses. It is important to highlight that all vaccinations were administered after surgery, chemoradiation and concomitantly with temozolomide

administered as maintenance cycles. Keskin et al.[5] immunised patients with newly diagnosed glioblastoma following surgical resection and radiotherapy. No objective responses were observed.

Therefore, studies have shown that vaccinating against neoantigens may decrease the likelihood of relapse in cancer patients that were disease-free at time of treatment.[4,9] However, T-cell recognition may not be effectively translated into clinical objective responses. Phase I studies evidenced that a single agent vaccine was safe and capable of eliciting neoantigen-specific T-cell responses.[4–9] Clinical responses were observed in a minority of cases with vaccine as the single agent therapy. Some patients received checkpoint inhibitors concomitant with vaccines; others were rescued by checkpoint inhibitors when not responding to the vaccine. Clinical trials have now proposed multi-arm designs to explore vaccine only versus vaccine plus checkpoint inhibitor.

It should be noted that a cancer vaccine immune response is not the same as an objective tumour response by RECIST, as the patterns of response and progression to immunotherapy may differ from those observed with drugs such as chemotherapy and molecularly targeted agents.[119] In addition, immune responses to a neoantigen vaccine and antitumour immunity are not equivalent conditions. Immune responses to neoantigen vaccines were reported in treated patients, but no tumour shrinkage was necessarily observed in many patients.[4–9] Finally, the in silico prediction of putative neoantigens is still characterised by a limited PPV, as only a subset of those predicted candidates results in neoantigen-specific T-cell responses.[120] Robust data demonstrating that vaccination administered as single therapy can mediate the regression of metastatic tumours is still pending.

Currently, there are dozens of ongoing clinical trials administering personalised neoantigen-based vaccines to patients' own tumour mutations or off-the-shelf vaccines to neoantigens shared among patients. In addition, disease-specific clinical trials are ongoing using tumour-associated antigens, which are, by definition, those that are overexpressed on tumour cells and expressed, instead, at low levels in normal host cells.[121–123] For instance, an HER-2/neu peptide vaccine has been clinically developed over the last few years and it is currently being evaluated in phase 3 registration trial (NCT01479244).[124] Table 1 shows selected neoantigen cancer vaccine studies that are currently underway for cancer patients.

Adoptive T-cell therapies

Another treatment modality targeting neoantigens is adoptive T-cell therapy, in that cancer patients are directly treated with their own naturally occurring or genetically modified antitumour T cells.[15,16,18,20,21] Adoptive T-cell therapies include adoptive transfer of TILs or of T cells genetically engineered to express a TCR, or a chimeric antigen receptor (CAR).[3,10]

Many of the principles of neoantigen characterisation are relevant to adoptive T-cell therapy. Seminal work using TIL-based adoptive cell transfer has shown actual tumour responses from T-cell therapy especially in melanoma, leading to objective response rates of 40%–50%.[17,19,20] Case reports in colorectal, cholangiocarcinoma and breast cancers have demonstrated actual tumour responses from T-cell recognition against tumour-specific neoantigens.[15,18,21] For example, a patient with metastatic cholangiocarcinoma was treated with a TIL product derived from *ERBB2IP* mutation-reactive T cells expressed by the cancer.[18] This resulted in a decrease in target lesions with prolonged stabilisation of the disease. After disease progression, the patient was re-treated with a >95% pure population of mutation-reactive $CD4^+$ T cells, which was translated into an objective clinical response.

Clinical trials using TIL-based strategies are currently ongoing. Most of them utilise bulk, randomly isolated TILs from the tumour tissue for *ex vivo* expansion and infusion (i.e. NCT03645928). However, there are ongoing efforts aimed at identifying clonal neoantigens, priming TILs *ex vivo* to recognise them and treating patients with their own expanded clonal neoantigen-reactive T-cell product (e.g. NCT04032847, NCT03997474). The rationale is that targeting clonal neoantigens is expected to elicit the immune system to attack all tumour cells, overcoming the problem of leaving resistant clones.

A future goal of this field is to identify neoantigens, determine the TCR that recognises specific neoantigens, and then produce a more rationally designed, personalised T-cell therapy. This could be a modification of adoptive T-cell therapy where the right T cells are purified or enriched and expanded. Alternatively, it could be used for a personalised CAR T-cell approach. These kinds of approaches could have the advantage of having high-specificity and low off-target effects because of the tumour-specific nature of the target, but have a more acute therapeutic effect by using *ex vivo* expanded T-cell populations instead of relying on stimulating their production *in vivo*.

FUTURE PERSPECTIVES

With the advancement and diffusion of NGS for clinical tumour samples, now researchers can rapidly sequence the DNA and RNA of a patient's cancer. The information gathered from these high-throughput molecular data can be used to identify cancer neoantigens resulting from tumour-specific alterations that can elicit antitumour immune responses and, thus, are instrumental for the success of personalised immunotherapies.

The field of neoantigen prediction is moving fast and new pipelines and algorithms are being developed or improved. This recommendation article aims to provide a comprehensive picture of current standards and novel approaches for neoantigen prediction, and an overview of what is coming next (Table 2).

Despite the availability of several computational and experimental approaches that are being employed for research and for the prioritisation of neoantigens in clinical trials, there is still a pressing need to further optimise these technologies. This is in part because the main purpose of the field is to discover neoepitopes, the part of the neoantigen that is recognised and bound by T cells, and current

Table 1. Selected neoantigen vaccines clinical trials

#	Trial ID	Phase	Cancer type	Institution/company sponsors	Vaccine platform	Patient recruitment status	Patient accrual target
1	NCT03568058	Phase 1	Advanced cancers	University of California San Diego	Peptide	Recruiting	10
2	NCT03199040	Phase 1	Breast	Washington University School of Medicine, MedImmune	DNA	Recruiting	24
3	NCT02348320	Phase 1	Breast	Washington University School of Medicine	DNA	Recruiting	30
4	NCT03121677	Phase 1	Follicular lymphoma	Washington University School of Medicine, Bristol-Myers Squibb	Peptide	Recruiting	20
5	NCT03122106	Phase 1	Pancreas	Washington University School of Medicine, NCI	DNA	Recruiting	15
6	NCT03532217	Phase 1	Prostate	Washington University School of Medicine, Bristol-Myers Squibb	DNA	Recruiting	20
7	NCT03598816	Phase 2	Renal cell carcinoma	Washington University School of Medicine, MedImmune	DNA	Not yet recruiting	48
8	NCT03068832	Phase 1	Paediatric brain tumour	Washington University School of Medicine	Peptide + poly-ICLC	Not yet recruiting	10
9	NCT03422094	Phase 1	GBM	Washington University School of Medicine, Bristol-Myers Squibb	Synthetic long peptide vaccine + poly-ICLC	Recruiting	30
10	NCT03289962	Phase 1	Melanoma, non-small-cell lung cancer, bladder cancer, colorectal cancer, triple negative breast cancer, renal cancer, head and neck cancer, other solid cancers	Genentech, Bio NTech RNA Pharmaceuticals GmbH	RO7198457/mRNA	Recruiting	567
11	NCT03223103	Phase 1	GBM	Icahn School of Medicine at Mount Sinai, NovoCure	Peptide + poly-ICLC	Recruiting	20
12	NCT02721043	Phase 1	Solid tumours	Icahn School of Medicine at Mount Sinai	PGV001/peptide + poly-ICLC	Recruiting	20
13	NCT03359239	Phase 1	Urothelial/bladder cancer, NOS	Icahn School of Medicine at Mount Sinai, Genentech	PGV001/peptide + poly-ICLC	Recruiting	15
14	NCT03380871	Phase 1	Carcinoma, non-small-cell lung cancer, nonsquamous non-small-cell neoplasm of lung	Neon Therapeutics, Merck Sharp & Dohme Corp.	NEO-PV-01/ peptide + poly-ICLC	Active, not yet recruiting	15
15	NCT03597282	Phase 1	Metastatic melanoma	Neon Therapeutics, Apexigen, Inc.	NEO-PV-01/ peptide + poly-ICLC	Recruiting	40
16	NCT02897765	Phase 1	Melanoma, lung cancer, bladder cancer	Neon Therapeutics, Bristol-Myers Squibb	NEO-PV-01/ peptide + poly-ICLC	Active, not yet recruiting	55
17	NCT03552718	Phase 1	Colorectal cancer, triple negative breast cancer, head and neck squamous cell carcinoma, melanoma, non-small-cell lung cancer, pancreatic cancer, liver cancer, hormone receptor positive tumour	NantBioScience	Yeast YE-NEO-001	Recruiting	16
18	NCT03633110	Phase 1/2a	Cutaneous melanoma, non-small-cell lung cancer, squamous cell carcinoma of the head and neck, urothelial carcinoma, renal cell carcinoma	Genocea Biosciences	GEN-009/syn long peptide + poly-ICLC	Recruiting	99
19	NCT03631043	Early phase 1	Smouldering plasma cell myeloma	MD Anderson Cancer Center, NCI	Peptide	Recruiting	30
20	NCT02950766	Phase 1	Kidney	Dana-Farber Cancer Institute, Bristol-Myers Squibb, Oncovir	NeoVax/peptide + poly-ICLC	Recruiting	15
21	NCT02287428	Phase 1	GBM	Dana-Farber Cancer Institute, The Ben & Catherine Ivy Foundation, Accelerate Brain Cancer Cure, Merck Sharp & Dohme Corp.	NeoVax/peptide	Active, not yet recruiting	46
22	NCT03361852	Phase 1	Follicular lymphoma	Dana-Farber Cancer Institute	NeoVax/peptide + poly-ICLC	Not yet recruiting	20

Continued

Table 1. Continued

#	Trial ID	Phase	Cancer type	Institution/company sponsors	Vaccine platform	Patient recruitment status	Patient accrual target
23	NCT03219450	Phase 1	Lymphocytic leukaemia	Dana-Farber Cancer Institute, Neon Therapeutics, Oncovir	NeoVax/peptide + poly-ICLC	Not yet recruiting	10
24	NCT03480152	Phase 1/2	Melanoma, colon cancer, gastrointestinal cancer, genitourinary cancer, hepatocellular cancer	NCI	mRNA	Recruiting	64
25	NCT03092453	Phase 1	Melanoma	University of Pennsylvania	Dendritic cell	Recruiting	12
26	NCT03300843	Phase 2	Melanoma, gastrointestinal cancer, breast cancer, ovarian cancer, pancreatic cancer	NCI	Dendritic cell	Recruiting	86
27	NCT03639714	Phase 1/2	Non-small-cell lung cancer, colorectal cancer, gastroesophageal adenocarcinoma, urothelial carcinoma	Gritstone Oncology, Bristol-Myers Squibb	GRT-C901 and GRT-R902 (viral prime and self-amplifying RNA boost)	Recruiting	214
28	NCT02992977	Phase 1	Advanced cancer	Agenus	AutoSynVax™ (heat shock proteins-based peptide) + QS-21 Stimulon® adjuvant	Active, not yet recruiting	20
29	NCT03673020	Phase 1	Solid tumour, adult	Agenus	AutoSynVax™ AGEN2017/(HSP-based peptide) + QS-21 Stimulon® adjuvant vaccine	Recruiting	3

GBM, glioblastoma; ICLC, Polyinosinic-Polycytidylic acid stabilized with polylysine and carboxymethylcellulose; NCI, National Cancer Institute; NOS, not otherwise specified.

Table 2. ESMO Expert Working Subgroup recommendations

Challenges	Recommendation
HLA presentation is not T-cell recognition	Most studies using computational analyses to infer neoantigens rely on MHC presentation and binding affinity to predict immunogenicity.[76,89,124,125] It can be unclear which neoantigen(s) will elicit immune responses using those methods only. At this point, other strategies such as T-cell reactive assays should be employed to validate or complement the identification of neoepitopes that are potential targets for cancer vaccines until accurate predictors of T-cell recognition are developed.
Computational methods for class I HLA typing	There are methods that consistently produce accurate predictions of class I HLA typing: Polysolver[81] and Optitype.[80]
Pipelines for neoantigen prediction	A number of pipelines are available that provide binding affinity prediction, mutated peptide annotation, wild-type and mutant peptide comparison, and neoantigen filtering and ranking on a variety of criteria (e.g. binding, clonality, expression, etc.). Most of them are based on netMHCpan and IEDB for binding prediction. Novel pipelines are being developed to resolve the challenges posed by high-throughput sequencing and to predict immunogenic neoepitopes.
Neoantigen prioritisation	Several features, besides predicted peptide-binding affinity, can be considered for neoantigen prioritisation, including mutation VAF, gene expression, clonality, differential agretopicity index, peptide-MHC stability, peptide foreignness, peptide length and ability to bind multiple HLA alleles. It is still unclear how to best combine these features into an optimal selection scheme and further studies are needed in order to establish the best tools and thresholds on a rational basis.
Clinical benefit of neoantigen-based anticancer vaccines in different cancer types	The selection of which cancer types, and subtypes, will derive the most benefit from a neoantigen vaccine approach has not yet been established. The first-in-human clinical trials have focused on patients whose tumours had high TMB, such as melanoma,[4,9] but also low TMB, such as GBM.[5,8] In patients with low TMB, other types of neoantigens can be leveraged such as those from fusion genes. Preliminary studies have shown that in tumours with fewer neoantigen targets, it was possible to derive an effective immune response with a vaccine. Currently, there is a variety of clinical trials for multiple solid tumours (Table 1), testing therapeutic cancer vaccines alone or combined with standard of care, checkpoint inhibitors or novel investigational drugs. Such trials are carried out in the early curative or in advanced/metastatic disease setting.
Combination of neoantigen-based vaccines with other immunotherapies	Combining cancer vaccines with checkpoint inhibitors may turn cold tumours hot, or hot into hotter, enabling the immune system to better recognise cancer cells as foreign and unleash a vigorous T-cell attack. Personalised neoantigen vaccines based on each patient's own tumour mutation make-up should guide those antitumour immune system responses. Recent evidence suggests that cancer vaccines and immune checkpoints are synergic and could be administered in a complementary fashion. Clinical trials are investigating distinct designs and whether vaccines should be given concomitantly, before or after checkpoint inhibitors. Multi-arm clinical trials should provide answers in the near future.

GBM, glioblastoma multiforme; HLA, human leukocyte antigen; MHC, major histocompatibility complex; TMB, tumour mutation burden; VAF, variant allele fraction.

approaches are focusing mostly on MHC-peptide binding. To address this outstanding need, a number of large-scale collaborative efforts are now underway to develop transformational datasets and new algorithms. For example, the Tumour Neoantigen Selection Alliance (TESLA) initiative has gathered global scientists from >40 of the leading research groups in academia, nonprofit, and industry working on neoantigen prediction to generate additional data for algorithm training and validation and to identify the best approaches for the identification of antigenic neoantigens.

Currently, there is a discrepancy regarding the abundance of candidate neoantigens derived from computational analysis and apparent low frequency of neoantigen-specific T cells among TILs. It is plausible to think that <3% of currently predicted neoantigens give rise to robust T-cell responses at the tumour site and that poor peptide immunogenicity and an immunosuppressive tumour microenvironment are major forces behind this observed discrepancy.[120] The T-cell repertoire requirement for an effective antitumour response is unclear.[39] Our knowledge of the immunogenic features of neoantigens and the tumour-associated immunosuppressive environment has considerable room for improvement. Functional assays to identify the computationally predicted neoantigens that serve as effective T-cell targets will be very informative to progress on these paths.

Furthermore, among neoantigens, the recognition of clonal ones is relevant as there is growing evidence to suggest that the success of TIL-based adoptive cell therapy is driven by neoantigen-directed T cells and the number of neoantigens that are targeted.[125] Personalised immunotherapy directed against multiple clonal neoantigens may overcome the barriers of tumour heterogeneity and immunoediting. The number of clonal neoantigens to be included in a vaccine or adoptive cell therapy is not established yet.

The development or optimisation of computation approaches for the prediction of immunogenic neoantigens along with results learned from ongoing clinical trials should make a positive impact bridging neoantigen prediction efforts to positively reach cancer patients. Once their feasibility, safety, and efficacy are proved in the clinics, the next challenge will be related to scalability and costs for application to large patient populations.

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J.B. is CEO and co-founder and J.C. is CSO and co-founder of AlbaJuna Therapeutics SL. N.M. has received consultancy fees from Achilles Therapeutics. L.D.M.A. has received honoraria for participation in a speaker's bureau/consultancy from Roche. V.G. is CTO and founder of Nostrum Biodiscovery. T.A.C is a co-founder of Gritstone Oncology and holds stock. T.A.C. has received grant support from BMS, AstraZeneca, Eisai, Illumina, An2H, and Pfizer. T.A.C has been on the scientific advisory boards of BMS, AstraZeneca, Merck, Illumina, and An2H. All remaining authors have declared no conflicts of interest.

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