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SPECIAL ARTICLE

ESMO recommendations on microsatellite instability testing for immunotherapy in cancer, and its relationship with PD-1/PD-L1 expression and tumour mutational burden: a systematic review-based approach

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Background: Cancers with a defective DNA mismatch repair (dMMR) system contain thousands of mutations most frequently located in monomorphic microsatellites and are thereby defined as having microsatellite instability (MSI). Therefore, MSI is a marker of dMMR. MSI/dMMR can be identified using immunohistochemistry to detect loss of MMR proteins and/or molecular tests to show microsatellite alterations. Together with tumour mutational burden (TMB) and PD-1/PD-L1 expression, it plays a role as a predictive biomarker for immunotherapy.

Methods: To define best practices to implement the detection of dMMR tumours in clinical practice, the ESMO Translational Research and Precision Medicine Working Group launched a collaborative project, based on a systematic review-approach, to generate consensus recommendations on the: (i) definitions related to the concept of MSI/dMMR; (ii) methods of MSI/dMMR testing and (iii) relationships between MSI, TMB and PD-1/PD-L1 expression.

Results: The MSI-related definitions, for which a consensus frame-work was used to establish definitions, included: 'microsatellites', 'MSI', 'DNA mismatch repair' and 'features of MSI tumour'. This consensus also provides recommendations on MSI testing; immunohistochemistry for the mismatch repair proteins MLH1, MSH2, MSH6 and PMS2 represents the first action to assess MSI/dMMR (consensus with strong agreement); the second method of MSI/dMMR testing is represented by polymerase chain reaction (PCR)-based assessment of microsatellite alterations using five microsatellite markers including at least BAT-25 and BAT-26 (strong agreement). Next-generation sequencing, coupling MSI and TMB analysis, may represent a decisive tool for selecting patients for immunotherapy, for common or rare cancers not belonging to the spectrum of Lynch syndrome (very strong agreement). The relationships between MSI, TMB and PD-1/PD-L1 expression are complex, and differ according to tumour types.

Conclusions: This ESMO initiative is a response to the urgent questions raised by the growing success of immunotherapy and provides also important insights on the relationships between MSI, TMB and PD-1/PD-L1.

Key words: microsatellite instability (MSI), tumour mutational burden (TMB), tumour mutational load (TML), next-generation sequencing (NGS), immunotherapy

Introduction

Cancers harbouring a defective mismatch repair (dMMR) mechanism are very often hypermutated and accumulate mutations in monomorphic microsatellites (short tandem repeats) that are particularly prone to mismatch errors. This condition is termed microsatellite instability (MSI), which can be tested using immunohistochemistry (IHC) and molecular tests, including classic [polymerase chain reaction (PCR)]-based microsatellite testing and novel next-generation sequencing (NGS) approaches.

MSI is the hallmark of Lynch syndrome and constitutional mismatch repair deficiency (CMMRD). MSI is found in a varying proportion of sporadic cancers that belong to the spectrum of cancer types that occur in this syndrome, including colorectal, endometrial, gastric, small intestine, urothelial, central nervous system and sebaceous gland neoplasms [1–3].

Recent evidence demonstrating that MSI is a predictive biomarker for immunotherapy [4–7] has increased the clinical request from oncologists for dMMR testing on many different cancer types. Therefore, there is now a pressing need to identify where testing is appropriate and how best to perform it. For this reason, standard definitions of MSI, related concepts and consensus recommendations on MSI testing are urgently required.

In addition to MSI, tumour mutational burden (TMB; also referred to as tumour mutational load—TML) and the expression of the immune checkpoint programmed cell death protein 1 (PD-1) and its ligand PD-L1 represent other extensively studied biomarkers that may predict response to immunotherapy [8–12]. However, the relationship between these biomarkers is complex and it remains unclear whether employing a combination of biomarkers is superior to relying on a single marker [13–15].

To address these issues, 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 immunotherapy, consensus recommendations on the: (i) most important definitions related to the concept of MSI; (ii) methods of MSI testing and how to measure MSI in cancer and (iii) relationships among MSI, TML and PD-1/PD-L1 expression. This consensus generating process was designed using a systematic review-based approach.

Members of the working group have been selected among professionals with high standard records on scientific activity and routine clinical work on molecular testing of cancers, and comprised three medical oncologists (FA, J-YD, RM), four senior pathologists/molecular pathologists (FB, TB, NS, AS), a radiation oncologist and associate director for immunogenomics programme (NR) and a clinical molecular geneticist (MJLL). Two junior pathologists (CL and AN) have been involved for their expertise in pathology/molecular pathology and systematic reviews.

Materials and methods

The systematic review-based approach used as a starting point to generate consensus/guidelines adhered to the PRISMA statement pre-set protocol [16]. The final degree of consensus, obtained by the mean values used by the ESMO TR and PM WG members to indicate their consensus to each statement (values range from 0: total disagreement to 10: total agreement), was judged as inconsistent if <6/10, low in the range 6.0–6.9/10, moderate in the range 7.0–7.9/10, strong from 8.0 to 8.9/10 and very strong if >9/10.

Systematic review to generate consensus recommendations for MSI definition

There are generally accepted definitions of MSI and related concepts in the literature, but consensus recommendations for standard definitions represent an important basis for further discussion. To derive the solid definitions on MSI and related concepts, we carried out a systematic review to find all existing consensus/guidelines or surveys on MSI, searching the PubMed database up to 7 September 2018. The search terms used in PubMed included combinations of the following keywords ('microsatellite' OR 'MSI') AND ('glossary' OR 'survey' OR 'guidelines'). We also considered the lists of references of all articles and major reviews included. Studies were considered eligible for this aim if they met the following inclusion criteria: (i) consensus or guidelines of survey study; (ii) providing MSI or dMMR definition. To generate standardised definitions, we adopted the words most commonly used as the definition in each study; the final definitions we provide have been obtained through consensus among members of this collaborative project and members of the ESMO TR and PM WG.

Systematic review to generate consensus recommendations on MSI testing and how to measure MSI in cancer

There are different methods to assess MSI in cancer, and they can be subdivided into two main groups: (i) defective expression of mismatch repair (MMR) proteins as determined by IHC and (ii) MSI determination with molecular tests. To derive consensus recommendations on MSI testing and how to measure MSI in cancer, we decided to perform a systematic review of all the existing MSI-based clinical trials to find all methods used to determine MSI. We focussed on clinical trials as they often utilise methods that may be employed broadly in clinical practice. We searched the clinical trials database (https://www.clinicaltrials.gov, last accessed 1 August 2018) for all MSI-related terms. In particular, search terms used in the database were 'cancer' in the box named 'condition or disease', and 'microsatellite instability', 'MSI', 'MSI high', 'mismatch repair deficiency' and 'dMMR' in the box named 'other terms'. We also used all the previously identified (see point 1) MSI-based consensus/guidelines useful for the specific aim of point 2 to complete the MSI testing scenario. The results were summarised and used for discussion in order to reach final recommendations through consensus among members of this collaborative project and members of the ESMO TR and PM WG.

Systematic review on the relationships involving MSI, PD-1/PD-L1 expression and TMB in cancer

A growing body of evidence suggests that MSI, TMB and PD-1/PD-L1 expression are three biomarkers, each of which is associated with an increased response rate to immunotherapy.

However, relationships between them are complex and may differ on the basis of tumour type and location. In order to clarify the existence of any potential association between MSI, TMB determined by NGS and PD-1/PD-L1 expression, we conducted the first systematic review on this topic. Studies were considered eligible if they met the following inclusion criteria: (i) use of NGS to determine TMB; (ii) use of standardised methods to assess MSI and PD-1 and/or PD-L1 expression; (iii) presence of matched data about TMB and MSI and PD-1/PD-L1 expression; (iv) in the case of aggregated data on PD-1 ligands (e.g. PD-L1 and PD-L2), presence also of separated data on PD-L1 expression; (v) presence of data about any potential observed association among TMB, MSI and PD-1/PD-L1 expression; (vi) unequivocal diagnosis of sporadic, single cancer; (vii) publication in a peer-reviewed journal in English language. Exclusion criteria were (i) no mention of cancer, (ii) no mention of terms NGS, TMB, MSI and PD-1/PD-L1 in the title/abstract; (iii) assessment of the investigated parameters with non-standardised methods; (iv) case report; (v) *in vitro* or animal studies. We searched the PubMed database up to 30 June 2018. The search terms used in PubMed included combinations of the following keywords: ('PD-1' OR 'PD-L1' OR 'programmed cell death protein 1' OR 'programmed cell death ligand 1' OR 'CD274' OR 'CD279' OR 'immunotherapy' OR 'checkpoint') AND ('NGS' OR 'high-throughput' OR 'sequencing' OR 'next-generation' OR 'mutational load' OR 'mutation load' OR 'mutational burden' OR 'mutation burden') AND ('microsatellite' OR 'MSI' OR 'MMR' OR 'mismatch repair' OR 'MMRd' OR 'dMMR') AND ('neoplasm' OR 'neoplasms' OR 'cancer' OR 'cancers' OR 'carcinoma' OR 'carcinomas' OR 'tumor' OR 'tumors' OR 'tumour' OR 'tumours' OR 'malignancy' OR 'malignancies' OR 'neoplasia'). We also considered the lists of references of all articles and major reviews included. Following the searches as outlined above, duplicates were removed, and a final list of articles was drawn up. For each article, we extracted information on: authors; year of publication; country; type of cancer; all available information on the methods of NGS; methods to assess MSI; methods (including clones and their thresholds) used to determine PD-1/PD-L1 expression; key findings about any potential association among TMB, MSI and PD-1/PD-L1 expression. If precise numbers on TMB, MSI and PD-1/PD-L1 expression were provided or were extractable from the selected manuscripts, they were used to create Venn diagrams to graphically summarise these findings, availing of a specific bioinformatic program (https://www.meta-chart.com, last accessed 8 August 2018). Furthermore, to provide indications for specific tumour types where MSI status could be tested with a reliable rationale, we also used all systematic reviews on MSI [searched on PubMed database up until 15 September 2018 with the following search strategy: ('MSI' OR 'microsatellite') AND ('systematic review' OR 'meta-analysis')], the MSI-ESMO factsheet (https://oncologypro.esmo.org/Education-Library/Factsheets-on-Biomarkers/Microsatellite-Instability-Defective-DNA-Mismatch-Repair, last accessed 15 September 2018) and all manuscripts selected for point 3 to build a summary table of MSI prevalence in different cancer types. Finally, we hand-searched other potential important references on this topic, setting as a limit the analysis of MSI in at least 10 000 cancer patients.

Results

Consensus recommendations for definition of MSI and MSI-related terms

The literature search identified globally 1154 unduplicated articles, 1088 of which were excluded after reviewing their title/abstract, which left 66 articles eligible for full text review. After applying our inclusion criteria, 18 articles were selected for reviewing standardised definitions regarding MSI and MSI-related terms [17–34].

Definition of microsatellites. Microsatellites, also named short tandem repeats, are repetitive DNA sequences that are distributed along the genome, in both coding and noncoding regions. Microsatellites are repetitive sequences, composed of repeats of a sequence that ranges in length from one to six bases. Although they are highly polymorphic among different subjects (number of repeats of sequence varies between individuals), microsatellites are typically the same length in the patient's germline DNA and in the somatic DNA of their tumour. Their repetitive nature renders them particularly sensitive to DNA mismatching errors, which can occur during DNA replication or iatrogenic damage [35, 36].

Definition of MSI. An MSI is a condition of genetic hypermutability resulting from defective DNA MMR. It is characterised by clustering of mutations in microsatellites typically consisting of repeat length alterations. The presence of MSI represents phenotypic evidence that MMR is not functioning normally [37].

Definition of DNA MMR. DNA MMR is a highly conserved mechanism used to restore DNA integrity after the occurrence of mismatching errors, including single base mismatches or short insertions and deletions. Four genes that play a critical role in this process include: *MLH1* (mutL homologue 1), *MSH2* (mutS homologue 2), *MSH6* (mutS homologue 6) and *PMS2* (postmeiotic segregation increased 2) [38]. The four homonym proteins MLH1, MSH2, MSH6 and PMS2 codified by these genes function in heterodimers, namely MLH1-PMS2 and MSH2-MSH6. The inactivation of one of these genes, which can occur due to germline and/or somatic mutations or to epigenetic silencing, results in a defective MMR (dMMR) mechanism.

Comments from consensus panel: MLH1 and MSH2 are obligatory partners of heterodimers. PMS2 can form a heterodimer only with MLH1, MSH6 can form a heterodimer only with MSH2. However, MLH1 and MHS2 can form heterodimers with other MMR proteins, namely MSH3, MLH3 and PMS1. In general, mutations in *MLH1* and *MSH2* result in subsequent proteolytic degradation of the mutated protein and its secondary partner, PMS2 and MSH6, respectively. Conversely, mutations in *PMS2* or *MSH6* may not result in proteolytic degradation of its primary partner, as MSH6 can be substituted in the heterodimer by MSH3, and PMS2 can be substituted in the heterodimer by PMS1 or MLH3.

Definition of MSI/dMMR tumour. A dMMR tumour is a tumour that accumulates thousands of mutations, particularly clustered in microsatellites and consisting in repeat length alterations, resulting in MSI. Therefore, MSI is a marker of dMMR, and characterises a hypermutable state of cells.

Comments from consensus panel: The use of the very same IHC and PCR tests serves to recognise dMMR/MSI in any sporadic cancer type belonging to the spectrum of cancers associated with Lynch syndrome (colorectal, endometrial, small intestine, urothelial, central nervous system and sebaceous gland) [39–42].

Consensus recommendations on MSI testing and how to measure MSI in cancer

The search in the clinical trials database (https://www.clinicaltrials.gov, last accessed 1 August 2018) identified 31 clinical trials worldwide, of which 5 are completed [7, 43–45] and 26 are ongoing [https://doi.org/10.1093/annonc/mdx367.020, last accessed 22 September 2018]. Their main characteristics are summarised in supplementary Tables S1 and S2, available at *Annals of Oncology* online. The tumour type most frequently studied (>60% of clinical trials) is metastatic colorectal cancer, but there is also a significant proportion of studies focussing on other cancer types including advanced endometrial carcinoma, metastatic prostate cancer, gastric cancer and generic solid tumour studies. Most of these clinical trials indicates the methods required to assess MSI. For the majority (>80%), both IHC and molecular tests were utilised to define MSI status. Where IHC was used, all four MMR proteins, MLH1, MSH2, MSH6 and PMS2, are always tested in these studies. When PCR-based molecular testing was employed, one of the two panels each comprising five microsatellites was utilised: one testing five microsatellites comprising two mononucleotide (BAT-25 and BAT-26) and three dinucleotide (D5S346, D2S123 and D17S250) repeats [31, 32], and the other with five poly-A mononucleotide repeats (BAT-25, BAT-26, NR-21, NR-24, NR-27) [46]. MSI was defined as loss of stability in ≥2 out of five microsatellite markers. In addition, four trials used a NGS-based approach as the molecular test (supplementary Table S1, available at *Annals of Oncology* online).

Recognising the variability of the methods used in clinical trials that highlight lack of standards, as neither IHC nor molecular tests were indicated as the preferred methods, and taking into account recommendations by existing guidelines, after a consensus among members of this collaborative project and members of the ESMO TR and PM WG, we generated the following recommendations for MSI testing. Comments from the consensus panel have been added to each recommendations as explanatory notes.

Recommendation A. The first method for MSI testing is MMR IHC. IHC is a widely available laboratory test and utilises antibodies against the four MMR proteins: MLH1, MSH2, MSH6 and PMS2. Grade of consensus: strong (8.7/10).

Comments from consensus panel: The use of IHC for the four MMR proteins is required to assess dMMR in any sporadic cancer type belonging to the spectrum of cancers found in Lynch syndrome [colorectal, endometrial, small intestine, urothelial, central nervous system (gliomas/glioblastomas) and sebaceous gland]. For tumour types not belonging to the spectrum reported above, there are insufficient data to draw a definitive recommendation.

The IHC method is based on the fact that MMR proteins are ubiquitously expressed in cell nuclei. Most mutations in MMR genes interfere with dimerisation, resulting in the proteolytic degradation of the heterodimers and consequent loss of both obligatory and secondary proteins. Thus, mutations in *MLH1* are associated with IHC loss of both MLH1 and PMS2, while mutations in *MSH2* are associated with IHC loss of both MSH2 and MSH6.

Conversely, when mutations occur in genes of the secondary proteins (i.e. *PMS2* and *MSH6*), the heterodimers may remain stable and there is no concurrent loss of the obligatory partner proteins. This is because the function of the secondary proteins may be compensated by other proteins, such as MSH3 instead of MSH6, and MLH3 or PMS1 instead of PMS2 [47]. Consequently, the PMS2 antibody detects all cases that harbour either MLH1 or PMS2 abnormalities [48], and the MSH6 antibody detects all cases that harbour either MSH2 or MSH6 abnormality. MLH1 and MSH2 alone do not recognise cases that have PMS2 or MSH6 abnormalities.

One disadvantage of IHC is that it may give rise to both positive and negative immunostaining results not reflecting the real status of MMR machinery due to technical or biological reasons. Technical factors that may result in false negative immunostainings are mainly due to pre-analytical issues, such as tissue fixation [49]. Aberrant staining patterns may also be observed, including cytoplasmic, dot-like or perinuclear staining [50]. The presence of an internal positive control, such as normal mucosa, lymphocytes or stromal cells is mandatory for interpretation of results. Biological reasons responsible for positive immunostainings that do not reflect the actual deficiency of the MMR machinery include missense mutations in any MMR gene resulting in mutant primary proteins that are catalytically inactive but antigenically intact [50]. Biological reasons for positive immunostainings that may be associated with either functional or defective activity of MMR depend on lack of PMS2 or MSH6 that are substituted by another MMR secondary partner (MSH3 replacing MSH6, MLH3 or PMS1 replacing PMS2). This reinforces our recommendation to use all four antibodies, either concurrently or sequentially, and move to MSI-PCR (recommendation B) whenever there is any doubt in IHC interpretation. The two-antibody screen approach with PMS2/MSH6 may be considered as a cost-effective alternative, pending that in the case of negative, focal/patchy or weak immunostaining particularly for MSH6 [51], also MLH1 and/or MSH2 IHC is carried out for confirmation. In fact, the use of the latter two antibodies serves to either confirm the deficiency of MMR (if one finds the absence of the corresponding partner protein) or to move to Recommendation B in the case of lack of only one member of the heterodimer subunit, i.e. use MSI-PCR as a confirmatory test. In fact, isolated loss of PMS2 immunostaining is found in 4% of tumours with MSI [52], and cases with solitary loss of PMS2 or MSH6 protein expression may carry somatic gene variants of these genes [53]. Again, all these observations strengthen our recommendation to use all four MMR proteins. Whether testing all four antibodies simultaneously or in a sequential manner, i.e. using the two-antibody screening followed by reflex IHC for the

appropriate partner protein, is a decision left to local organisational, timing and cost considerations.

IHC can be carried out on biopsies or surgical specimens if available [surgical specimen as first choice: grade of consensus: low (6.6/10)]. The main advantages of performing IHC on biopsies are: (i) the better degree of fixation of biopsies than surgical specimen and (ii) the usefulness of knowing MSI status for the first multidisciplinary tumour board, which may change subsequent management, especially in the metastatic setting. The advantages to using surgical specimens are: (i) the analysis of more cells than those present on biopsies; (ii) the possibility to select the best specimen for IHC; (iii) the presence of internal controls (e.g. normal mucosa, inflammatory cells) but which are usually present on biopsies; (iv) the possibility to overcome tumour heterogeneity (especially with MSH6 staining), due to the analysis of a larger amount of tissue, and (v) in the specific frame of immunotherapy, the possibility to also perform a reliable IHC for PD-1 and PD-L1 on the same material.

Recommendation B. MSI-PCR molecular testing is indicated in case of indeterminate IHC results, including disagreement or difficulties in interpreting IHC or in the case of loss of only one heterodimer subunit (e.g. only MLH1 or only PMS2 and not both). The traditional molecular test is based on PCR amplification of microsatellite markers with two possible panels: one using five microsatellites comprising two mononucleotide (BAT-25 and BAT-26) and three dinucleotide (D5S346, D2S123 and D17S250) repeats, and the other using five poly-A mononucleotide repeats (BAT-25, BAT-26, NR-21, NR-24, NR-27). The five poly-A panel is the recommended panel given its higher sensitivity and specificity. MSI is defined as loss of stability in $\geq$2 out of the five microsatellite markers.

Comments from consensus panel: The use of MSI-PCR molecular testing is indicated to assess dMMR in any sporadic cancer type belonging to the spectrum of cancers found in Lynch syndrome: colorectal, endometrial, small intestine, urothelial, central nervous system (gliomas/glioblastomas) and sebaceous gland. According to 'Bethesda guidelines' for colorectal cancers [31, 32] a panel of two mononucleotide repeats (BAT-25 and BAT-26) and three dinucleotide repeats (D5S346, D2S123 and D17S250) should be used for testing cancers [31, 32]. MSI is present when two or more mononucleotide markers show repeats length alteration [31, 32]. However, an alternative panel with five poly-A mononucleotide repeats (BAT-25, BAT-26, NR-21, NR-24, NR-27) is considered the current standard because of its higher specificity and sensitivity [46]. Both panels have been and are being used to assess MSI in clinical trials. For example, the panel with dinucleotides is being used in the ongoing phase II trials NCT03435107 and NCT03150706 (https://www.clinicaltrials.gov, last accessed 28 September 2018), whereas the panel with five poly-A repeats has been used in published trials Keynote-016 and Keynote-059 [7, 43]. Grade of consensus: strong (8.6/10). Molecular methods seem to guarantee the highest values of specificity and sensitivity in MSI testing [46, 54]. If only dinucleotide repeats are altered, a secondary panel with mononucleotide repeats (e.g. BAT40) should be tested [32]. The pentaplex panel of five poly-A mononucleotide repeats may obviate the need for normal tissue for comparison and may be more sensitive than other microsatellite markers [32, 46, 54]. The terms MSI-high or MSI-low should be abandoned and MSI-low tumours should be included with microsatellite stable tumours, as suggested in the revised Bethesda guidelines for colorectal cancers [32] and confirmed by subsequent studies [55, 56].

Specific comment from the consensus panel regarding recommendations A and B for colorectal cancer: A recent report on immunotherapy in metastatic colorectal cancer has shown that an unacceptable percentage of patients (almost 10%) had been enrolled in immunotherapy trials and experienced failure due to false positive dMMR or MSI-PCR results assessed by local laboratories [57]. Thus, the consensus panel recommends the use of both MMR-IHC and MSI-PCR to assess the eligibility to treatment with immune checkpoint inhibitors of metastatic colorectal cancer and other cancers of the Lynch syndrome spectrum.

Recommendation C. An NGS represents an alternative molecular test to assess MSI [56, 58]. One main advantage of this, is the opportunity to couple MSI analysis with the determination of TMB. NGS permits parallel high-throughput sequencing of a high number of microsatellites and genes, and thus it may also identify other targetable alterations suitable for treatments other than immunotherapy, e.g. *KRAS* mutations in colorectal cancer, *EGFR* mutations in non-small-cell lung cancer (NSCLC), *BRAF* mutations in melanoma, *BRCA1* and *BRCA2* mutations in breast and ovarian cancers. NGS-based MSI testing has the potential to become the method of choice for all tumour types, including rare cancers, not belonging to the spectrum of Lynch syndrome. Grade of consensus: very strong (9/10).

Comments from consensus panel: NGS should be carried out only in selected centres experienced in these techniques. Different NGS approaches for determining MSI are described and further discussed in point 3. In the future, NGS evaluation of a large set of microsatellites different from mononucleotide repeats, i.e. including two to six bases and/or composite microsatellites, may help discovery of novel DNA repair mechanism failures beyond our current knowledge of dMMR.

The core of each recommendation has been summarised in Table 1.

Relationships among MSI, PD-1/PD-L1 expression and TMB in cancer

As MSI, TMB and PD-1/PD-L1 expression are biomarkers that have been associated with an increased response rate to immunotherapy, we conducted a systematic review to clarify the existence of any potential association among these markers. The literature search identified globally 158 unduplicated articles, 126 of which were excluded after reviewing their title/abstract, which left 32 articles eligible for full text review. After applying our inclusion and exclusion criteria, 18 articles (for 17 cohorts) were selected for this systematic review [7, 59–75] (supplementary Figure S1, available at *Annals of Oncology* online).

Epidemiological and methodological data. As summarised in supplementary Table S3, available at *Annals of Oncology* online, the 17 articles included in our meta-analysis concerned 148 655

Table 1. Summary table of recommendations for MSI testing in the framework of immunotherapy and comments from the ESMO TR and PM WG consensus panel

Recommendation A: immunohistochemistry	The first test of choice is IHC, using antibodies recognising the four MMR proteins: MLH1, MSH2, MSH6 and PMS2.
Coefficient of agreement: strong (8.7)	
Main comment: MMR proteins form heterodimers; for a correct IHC interpretation, the consensus panel highlights that mutations in MLH1 are associated with IHC loss of both MLH1 and PMS2, while mutations in MSH2 are associated with IHC loss of both MSH2 and MSH6. There exist isolated losses of PMS2, MSH2 or MSH6, this strengthening the recommendation to use all four antibodies.	
Recommendation B: polymerase chain reaction	In case of doubt of IHC, confirmatory molecular analysis is mandatory. The first-line of molecular analysis is represented by PCR. It can be carried out using two possible panels: (i) a panel with two mononucleotide (BAT-25 and BAT-26) and three dinucleotide (D5S346, D2S123 and D17S250) repeats and (ii) a panel with five poly-A mononucleotide repeats (BAT-25, BAT-26, NR-21, NR-24, NR-27). The five poly-A panel is the recommended panel given its higher sensitivity and specificity.
Coefficient of agreement: strong (8.6)	
Main comment: both the suggested panels have been and are being used to assess MSI in clinical trials. Molecular tests guarantee the highest values of specificity and sensitivity in MSI testing.	
Recommendation C: next-generation sequencing	NGS represents another type of molecular tests to assess MSI. Its main advantages are represented by the possibilities of coupling MSI analysis with the determination of tumour mutational burden (TMB).
Coefficient of agreement: very strong (9.0)	
Main comment: NGS should be carried out only in selected centres devoted to these techniques.	

Coefficient of agreement ranges from 0 = totally disagree, to 10 = totally agree.
IHC, immunohistochemistry; PCR, polymerase-chain reaction; NGS, next-generation sequencing.

patients with different types of cancer; however, complete data of all the investigated parameters (TMB, MSI and PD-1/PD-L1 expression) were available for only a portion of patients in some studies [59, 62, 75]. Most of cohorts (10/17, 59%) were specifically focussed on tumours of the digestive system [7, 63, 65–69, 71–73, 75], but the presence of large-cohort studies investigating TMB, MSI and PD-1/PD-L1 expression in many tumour types (over 100 tumour types) [59, 62, 74] ensured an in-depth analysis of a wide spectrum of different cancers. The vast majority of the cohorts reported data on patients from the USA, followed by Asian and European subjects. The majority of the manuscripts (59%) investigated original cohorts of patients [7, 61, 63–70, 75], whereas data derived from external (public or private) databases were used in some papers [59, 62, 71–74].

Complete data about TMB, MSI and PD-1/PDL1 of one cohort of patients was reconstructed by merging two studies on the same tumours [68, 69].

TMB was assessed using whole-exome sequencing in 5 (29%) studies [7, 59, 60, 69, 70], and using multigene cancer panels in the remaining 12 (71%), where TMB-high is variably defined as tumours showing at least $\geq$17 or $\geq$20 mutations/Mb upon sequencing of at least 1.2–1.5 Mb [61–67, 71–75]. To assess MSI status, an approach based on both IHC to assess MMR status and/or molecular assessment of microsatellites by MSI-PCR and NGS was applied in most studies (82%) [7, 59–61, 64, 66, 68–75]. PD-L1 expression has been tested in all selected manuscripts, while PD-1 was determined in 5 (29%) studies only [59–61, 64, 68, 71]. The most used clone for immunostaining of PD-1 was NAT105 (60% of studies), and for PD-L1 was SP142 (47% of studies). The methods to assess the positivity for such markers differed among the selected studies, the most commonly applied being the simple reporting of the percentage of positive cells for PD-1 [60, 61, 68] and the use of an integrated threshold, coupling the intensity of staining and the percentage of positive neoplastic cells, for establishing PD-L1 positivity [7, 60–62, 64, 67, 68, 72–75]. To avoid reporting data affected by any bias, the quality of the studies was also evaluated using Newcastle-Ottawa scale (NOS) [76]. The median value of NOS score was 8.05 (NOS score: maximum =9, risk of bias if <6; evaluation study by study not shown), indicating a high quality of studies with a very low risk of bias.

Venn diagrams and relationships among TMB, MSI and PD-L1. Using extractable data from the selected manuscripts and also those derived from Venn diagrams, we compared the associations involving high TMB, MSI and PD-L1 expression on tumour cells (of the PD-1/PD-L1 axis, PD-L1 expression was the only variable with suitable data for this analysis). Overall, 4186 patients were positive for at least one of the analysed biomarkers. Our findings are summarised in Figure 1 and supplementary Table S4, available at *Annals of Oncology* online. Particularly, the simultaneous presence of TMB-high, MSI-high and PD-L1 expression was observed in 2.9% of all-cancers (intending those cancers that were part of the selected manuscripts) and in significantly higher ($P < 0.05$) percentages in colorectal (12.8%) and oesophagogastric adenocarcinomas (where oesophageal neoplasms were not squamous cell cancers but adenocarcinomas, related to Barrett's metaplasia) (14.6%). If we considered TMB-high and/or MSI-high, the percentage of association with PD-L1 expression became higher above all in the categories of 'all cancers', NSCLC and melanomas, rising from 2.9% to 11.9%, 0.5% to 12.7% and 0.0% to 32.0%, respectively. This was due to the low percentages of MSI-high cases, concurrently PD-L1 positive, in 'all cancers'

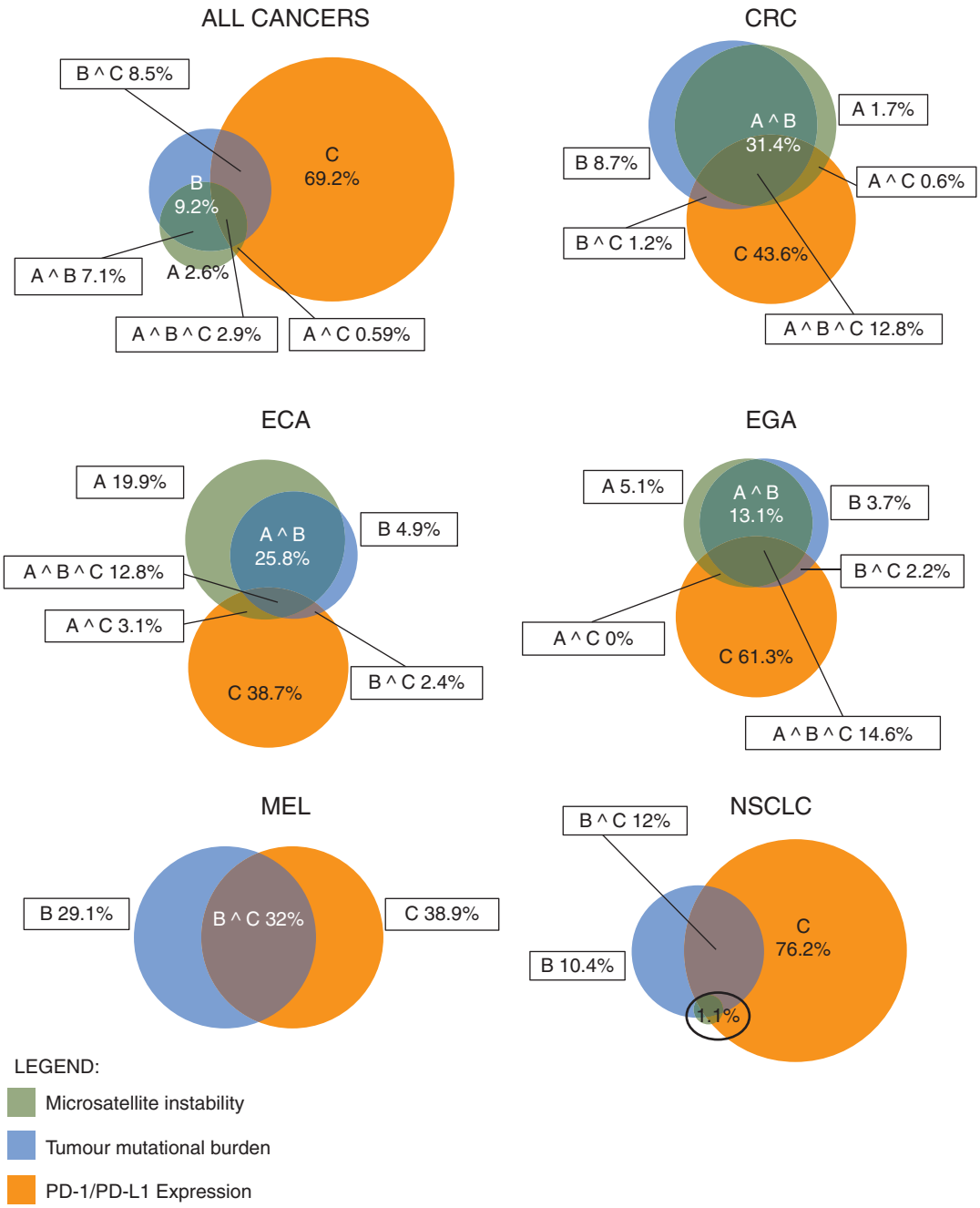

Figure 1. Relationships among microsatellite instability (MSI; letter A indicates the group with MSI), high tumour mutational burden (TMB; letter B indicates the group of high TMB) and PD-L1 expression (letter C indicates the group with PD-L1 expression) in the framework of immunotherapy. The percentages are expressed considering as total (4186 patients=100%) all cases with MSI and/or high TMB and/or PD-L1 positive. TMB-high has been defined as tumours showing at least ≥17 or ≥20 mutations/Mb upon sequencing of at least 1.2–1.5 Mb. CRC, colorectal cancers; ECA, endometrial carcinomas; EGA, oesophagogastric adenocarcinomas; MEL, melanomas; NSCLC, non-small-cell lung cancers.

(3.4%) and NSCLCs (0.7%) compared with colorectal (13.4%) and oesophagogastric adenocarcinomas (14.6%) ($P < 0.05$ for all these differences), or to the lack of MSI-high cases as observed in melanomas. Lastly, high percentages of concordance of TMB-high and MSI-high was observed in colorectal cancers (44.2%), oesophagogastric adenocarcinomas (27.7%) and endometrial cancers (31.0%) compared with 'all cancers' group, melanomas and NSCLCs ($P < 0.05$ for all these differences).

Concluding remarks of consensus panel considering tumour types

To provide further indications for the specific tumour type where MSI status could be tested with a reliable rationale, the consensus panel agreed on a summary table for MSI testing (Table 2). With the search strategy for this specific point, we initially found 205 manuscripts. After screening all the full-texts of such manuscripts,

Table 2. Recommendation for MSI testing in different cancer types and in the framework of immunotherapy

Cancer type	MSI prevalence (all stages)	Specific MSI-associated histotype, if any
Sporadic tumour types belonging to the spectrum of Lynch syndrome that can be tested using IHC and MSI-PCR or NGS (testing is indicated for stage IV cancers, whose MSI prevalence is lower than that of earlier stages)		
Colorectal	17%	Medullary, mucinous, poorly differentiated neuroendocrine
Endometrial	20%	Lower uterine segment-located, undifferentiated/dedifferentiated, mixed morphology, tumours showing high levels of tumour-infiltrating lymphocytes/lymphoid stroma
Gastric-oesophageal	13%	Adenocarcinoma (MSI up to 39% in case of carcinoma with lymphoid stroma, and absent in oesophageal squamous cell carcinoma)
Small intestine	8.3%	Including duodenum and ampulla of Vater
Ovarian	3.5%–10%	Endometrioid, clear cell
Glioblastoma	6%–13%	
All common or rare tumour types not belonging to the spectrum of Lynch syndrome with low prevalence of MSI and little data available on the reliability of IHC and MSI-PCR, to be tested using NGS		
Unknown primary	1.8%	
Cervical	4%	
Extrahepatic bile duct	3.4%	
Pancreatic	1%–7%	Medullary, IPMN-associated, periampullary (when the origin from ampulla, terminal bile duct or pancreatic duct is uncertain)
Prostate	3%	
Non-small-cell lung cancer	< 1%	
Head and neck	<1%	
Melanoma	NS	1% uveal melanoma
Sarcomas	2%	Uterine, peritoneal and retroperitoneal
Anal	NS	
Kidney	NS	

IHC, immunohistochemistry; MSI-PCR, microsatellite instability-polymerase chain reaction; NGS, next-generation sequencing; NS, no significant evidence.

6 of them were selected to build the summary table [77–82]. Another one hand-searched paper, investigating MSI status in more than 10 000 cancer patients, was selected to complete this subanalysis [6].

As indicated in Table 2, the most important cancer types where MSI testing should be carried out using IHC to assess MMR proteins status and MSI-PCR or NGS are: endometrial, intestinal (colorectal and small bowel), gastric, oesophageal (adenocarcinomas and not squamous cell carcinoma), ovarian and glioblastoma. For tumour types with low frequency of MSI and little data available on the reliability of IHC and MSI-PCR, MSI testing should be carried out using NGS. It is important to note that MMR-deficient tumours are identified more often in early-stage disease of different cancer types (defined as stage <IV), while this proportion decreases in advanced and metastatic settings [6]. Indeed, the contribution to the percentages indicated in Table 2 (which regard all stages tumours) is mainly provided by early stages tumours. At the same time, it is also true that MSI late stages derive from MSI early stages tumours, and the main aim of Table 2 is reporting the most important tumour types that may exhibit MSI and should be taken into account as potential targets for immunotherapy.

The consensus panel highlights that a decision tree on the sequential use of the different tests within the frame of immunotherapy decision-making cannot be a general one for all cancers but should be designed on the basis of the specific tumour type.

On the basis of tumour type(s) and familial history, lastly, genetic counselling should be warranted to identify patients with hereditary cancer syndromes [83]. This approach may be implemented by direct collaboration among surgeons, pathologists, oncologists and geneticists, and/or with establishing multidisciplinary tumour boards [84].

Open questions and perspectives from the consensus panel

Due to the recent successes of immunotherapy in several cancer types, clarifying the MSI landscape in cancer is becoming an urgent clinical necessity. In this consensus report, specific recommendations on MSI definition and testing in cancer are presented. Furthermore, the relationship of MSI with TMB and PD-1/PD-L1 expression in different neoplasms was explored; at the same time, it has to be highlighted that for MSI tumours further analysis for TMB or PD-1/PD-L1 expression is not required to receive immunotherapy. We have used a systematic review approach since it represents the most rigorous scientific method to explore a topic, and to generate specific recommendations; we coupled this approach with a consensus obtained among members of this collaborative project and members of the ESMO TR and PM WG.

The first consensus recommendations regarded the identification of all the most relevant terms linked to the concept of MSI.

In the literature, there are several different definitions of MSI and related variables, thus we decided to provide expert consensus-based clear definitions of all these concepts. In depth understanding of the correct meaning of all these terms is the ideal basis for approaching patients with MSI tumours. This glossary may be useful not only for oncologists/clinicians but also for pathologists, researchers and drug developers alike.

The second consensus recommendations regarded MSI testing and how to quantify MSI in cancer. Reviewing all clinical trials based on MSI and also the existing guidelines on MSI testing, we provided expert consensus-based recommendations on MSI testing. We indicated that IHC currently represents the first test to determine dMMR and its associated MSI in cancer belonging the spectrum of Lynch syndrome associated tumours, but we also provided specific comments and recommendations on the most relevant aspects that can affect IHC analysis during routine clinical practice. IHC is a widely available technique used in most laboratories around the world, and knowing its advantages and limitations is the most important aspect to correctly apply this important tool for assessing MSI. The molecular approach is mainly based on PCR, and here we provided specific recommendations also for this type of test. NGS is another potential type of test for investigating MSI, the main advantage being the possibility to also determine tumour mutation burden, along with other possibly targetable alterations. TMB, with MSI and PD-1/PD-L1 expression may be important indeed in predicting a higher rate of response to immunotherapy, and as such the last part of our study explores this interesting topic.

We found that PD-1/PD-L1 expression demonstrates great variability throughout all cancer types, but a common aspect is represented by their expression even in MSI negative and/or TMB-low cases. One of the most important aspects that merits consideration is that it is possible to have a TMB-high in absence of MSI, whereas MSI-high with TMB-low is rare (except for endometrial cancer) (Figure 1). While high percentages of concordance of TMB-high and MSI-high have been described for gliomas, colorectal and oesophagogastric adenocarcinomas, for anal cancers and oesophageal squamous cells carcinomas there is an inverse relationship [64, 66, 72, 74, 75].

These tumour types share two important risk factors: human papillomavirus (HPV) and smoking [85, 86]. Tumours positive for HPV and smoking-associated cancers have been suggested to harbour higher TMB in comparison with HPV-negative or non-smoking counterparts, independently from MSI status [72, 87, 88], although the influence of HPV on TMB is still debated [89]. In anal cancers and oesophageal squamous cell carcinoma these reasons may therefore explain, not only the lack of association between TMB and MSI, but also the considerable percentages of a high MSI-independent PD-L1 expression. Genomic analyses have shown that particular mutational signatures can contribute to high TMB independently from MSI status. These include *BRCA1/2* (breast cancer genes 1 and 2) and *APOBEC* deficiency, neoantigen load, ultraviolet rays exposure and mutations affecting *TP53* and polymerase ϵ (*POLE*) (supplementary Figure S2, available at *Annals of Oncology* online) [7, 59, 70, 90–95].

Interestingly, *MUC16* mutations may be associated with higher TMB, better survival outcomes and immune response in patients with gastric cancer [96]. Lastly other tumour intrinsic and extrinsic factors are also involved in influencing the complex interactions among MSI, TMB and PD-1/PD-L1 expression, such as tumour infiltrating-lymphocytes and the microbioma (supplementary Figure S2, available at *Annals of Oncology* online) [95, 97].

A wide range of response rates to immunotherapy has been described also in tumours with low TMB, absence of MSI and even without PD-1/PD-L1 expression, indicating that immunotherapy response is also likely driven by other biomarkers [72]. Notably, in gastric cancer Epstein-Barr virus (EBV) has demonstrated a key role in predisposing response to immunotherapy, independently from TMB and MSI [63, 71, 97]. In the variant of gastric carcinoma with lymphoid stroma, PD-L1 is expressed on tumour cells independently from MSI and even from the presence of EBV [63]. Melanomas and NSCLC also show some peculiarities. Indeed, as recently reported by Vanderwalde et al., there are no MSI cases in melanomas, but the percentage of concordance between high TMB and PD-L1 expression is significant (32%) [72]; similarly, in NSCLC, MSI cases are very rare (1.1%), but there is a very high percentage of PD-L1 positive cases (75% of cases, using as total the number of cases with TMB-high and/or MSI and/or PD-L1 positive), although in NSCLC PD-L1 and TMB-high have been demonstrated to be independent [98]. Although TMB, MSI and PD-1/PD-L1 expression represent important indicators for immune checkpoint blockade therapy, further studies are therefore needed to find new variables able to predict response to immunotherapy.

One of the most important aspects of this systematic review is represented by the usefulness of NGS that emerged in assessing not only TMB but also MSI, generating promising data that may address immune checkpoint blockade therapy. Notably, an estimation of TMB can also be made with targeted panels, avoiding the costs of whole-exome or of whole-genome sequencing [99, 100]. NGS can also determine MSI status [101–103]; a recently developed 'MSIsensor' combines NGS with biostatistics to address MSI in tumour samples [101, 102]. It has already been applied by two studies selected for this systematic review [59, 66]. In the framework of immunotherapy, NGS represents the most promising tool to test MSI in those cancers not belonging to the spectrum of Lynch syndrome and in rare cancer types, for which little is known about MSI testing with IHC and/or MSI-PCR. Interestingly, one of most promising applications of NGS regards patient monitoring during immunotherapy. In fact, Cabel et al. have applied NGS to the study of circulating tumour DNA during anti-PD-1 therapy, demonstrating that it might represent a valuable tool to assess tumour response in patients treated with immune checkpoint inhibitors [104, 105].

In conclusion, we provide expert-consensus based recommendations for MSI definition and testing. Furthermore, studying the relationships among TMB, MSI and PD-1/PD-L1 expression, we highlight that their prevalence and role may differ based on tumour type and can be affected by several factors. Although MSI, TMB and PD-1/PD-L1 expression are recognised markers for the selection of patients for immunotherapy, there is the need to standardise PD-1/PD-L1 and define cut-offs for TMB in different tumour types. To this end, ESMO TR&PM groups have been established to clarify these points. Moreover, the response rate to immune checkpoint blockade may also be high for particular tumour types or patient settings that have low TMB and lack of MSI or PD-1/PD-L1 expression. This highlights that new potential predictors of response to immunotherapy are needed. As

previously pointed out, NGS may represent a decisive tool for applying current knowledge and concurrently provide new insights on this topic. Indeed, we need to develop new models of clinical trials, not only for evaluating drug efficacy but also to test the clinical utility of different NGS approaches, and how this may improve cancer patient outcome. Along this line, our recommendations may represent a robust starting point.

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