SPECIAL ARTICLE

Germline-focussed analysis of tumour-only sequencing: recommendations from the ESMO Precision Medicine Working Group

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It is increasingly common in oncology practice to perform tumour sequencing using large cancer panels. For pathogenic sequence variants in cancer susceptibility genes identified on tumour-only sequencing, it is often unclear whether they are of somatic or constitutional (germline) origin. There is wide-spread disparity regarding both the extent to which systematic 'germline-focussed analysis' is carried out upon tumour sequencing data and for which variants follow-up analysis of a germline sample is carried out. Here we present analyses of paired sequencing data from 17 152 cancer samples, in which 1494 pathogenic sequence variants were identified across 65 cancer susceptibility genes. From these analyses, the European Society of Medical Oncology Precision Medicine Working Group Germline Subgroup has generated (i) recommendations regarding germline-focussed analyses of tumour-only sequencing data, (ii) indications for germline follow-up testing and (iii) guidance on patient information-giving and consent.

Key words: sequencing, panel, susceptibility, predisposition, gene, germline

Introduction

Tumour testing, until recently, typically comprised genotyping of specific hotspot mutations in oncogenes. These analyses seldom gave insights into patient germline status [1, 2]. Over the last 5 years, evolution of next-generation sequencing (NGS) technology, growth in molecular oncology, emergence of molecularly stratified basket/umbrella trials and vigorous marketing from commercial providers, have fuelled expansion of 'large' tumour

sequencing panels targeting the full coding region of hundreds of genes [3–5]. Whilst paired tumour-germline analysis may be carried out in major academic cancer centres, tumour-only analysis is the more typical current clinical standard [6].

As well as influencing cancer management and therapy, a substantial proportion of genes included on these large tumour panels confer heritable predisposition to cancer, the so-called cancer susceptibility genes (CSGs). A subset of variants identified in CSGs are 'pathogenic' that is when present in the constitutional

DNA, they will confer an elevated risk of developing one or more cancer types. When such a variant is detected in a tumour, it is unclear whether the variant is constitutional in origin (i.e. is present in all or most tissues, including the germline) or it has been somatically acquired (i.e. not in the germline but present in the tumour ± surrounding tissue). We refer to these as 'tumour-detected pathogenic variants of potential germline origin', or 'tumour-detected pathogenic variants'.

Having identified that clinical practice in this area is widely disparate, the European Society of Medical Oncology (ESMO) Precision Medicine Working Group (PMWG) convened a subgroup tasked with addressing germline management of tumour-detected pathogenic variants, specifically:

(i)   to identify the key issues relevant to laboratory and clinical management,
(ii) to undertake analyses of relevant tumour and germline data,
(iii) to generate consensus recommendations applicable in routine clinical-laboratory services regarding:

    a. extent of germline-focussed analyses to be carried out as routine,
    b. when follow-up testing in a germline sample should be undertaken,
    c. patient information and consent.

Key issues relevant to germline-focussed tumour analysis:

The ESMO PMWG germline subgroup identified and considered the following issues within their analyses and recommendations:

- **'On-tumour' and 'Off-tumour' associations of gene with tumour type**

CSGs have been established, through prior research, as conferring predisposition to specific tumour types; these associations we hereafter refer to as 'on-tumour'. Hence, for *BRCA1*, a pathogenic variant identified in a breast tumour would be described as on tumour, as presence of a germline *BRCA1* mutation confers elevated risk of breast cancer. A *BRCA1* pathogenic variant identified in a testicular seminoma would be defined as 'off-tumour' as presence of a germline *BRCA1* mutation does not confer elevated risk of testicular seminoma. Such a finding off-tumour would typically be termed a 'secondary' finding (if sought deliberately), or an 'incidental' finding (if happened upon by chance during data review) [7].

- **Clinical Actionability**

Quantification of 'clinical actionability' for pathogenic variants in CSGs has not been formally established but would widely be agreed to encompass the penetrance (risk) of the associated cancers, the 'severity' of the cancer and the availability of clinical management options that mitigate the increased cancer risk (screening, surgical prophylaxis, chemoprophylaxis and lifestyle modification), along with the quality of evidence underpinning each of these estimates [8].

For more frequently detected CSGs such as *BRCA1/BRCA2/ MLH1/MSH2*, evidence regarding cancer risk and the impact of screening/interventions are relatively well established. For less frequently detected CSGs, e.g. *FLCN* or *FH*, whilst robust evaluations of clinical and economic impact are lacking, clinical management

is relatively consistent. For other CSGs such as *DICER1* and *BAP1*, evidence regarding penetrance and efficacy of interventions is sparse and management varies widely between centres.

The American College of Medical Genetics (ACMG) has assembled a set of 25 CSGs of higher actionability for which they have advised analysis for and return of pathogenic variants, regardless of context of ascertainment [7, 9, 10].

An additional aspect of actionability relates to the impact on management of the current cancer of a germline pathogenic variant. For example, identification of a germline *BRCA1* pathogenic variant in the on-tumour setting (breast or ovarian cancer) may influence the choice of chemotherapy (platinum), eligibility for targeted agents (PARP inhibitors) and/or primary surgical management (bilateral mastectomy in localised unilateral disease). In genomically selected basket trials the germline status may influence eligibility for targeted drugs even in the off-tumour setting. However, for many CSGs, identification of a germline pathogenic variant has no impact on management of a cancer once diagnosed in the on- or off-tumour setting.

- **Penetrance (risk) of cancer**

Early estimates of cancer penetrance (risk) for CSGs were typically derived from linkage analysis using large multicase families. Subsequent studies based on ascertainment from more modest family clusters or unselected incident cancer cases have revealed progressively lower estimates of penetrance for many CSGs. Analyses of population data suggest penetrance may be lower still if the germline pathogenic variant is ascertained completely agnostic to phenotype [11]. Impact of preventive clinical interventions will be predicated on the estimates of penetrance used for the clinical or cost-effectiveness analyses.

- **Tumour heterogeneity and contamination with normal tissue**

A heterozygous germline pathogenic variant in a CSG would be anticipated to be present in 50% of alleles across body tissues, including a tumour. On occasion, the variant allele frequency (VAF) of a somatically-acquired mutation may exceed 50% due to Loss of Heterozygosity of the opposite allele or tumour aneuploidy. Most typically, somatic variants are detected at VAF <50% due to a combination of clonal heterogeneity, contamination with lymphocytes/non-tumour cells and/or tumour aneuploidy. It is important to note that compared with hybrid-capture-based NGS strategies such as the one used for this analysis, PCR-based NGS strategies may not yield as consistent a VAF, especially in FFPE specimens with poor DNA quality and yield.

- **Threshold for triggering germline follow-up testing**

To control the volume of germline tests triggered, we required a working threshold of 'likelihood of germline origin'. Historically, a threshold of 10% (based on personal and/or family history of cancer) has widely been adopted in the UK National Health Service for *BRCA1/2* detection on clinical testing [12].

- **Workflows, logistics and cost**

Tumour molecular genetic analysis is frequently carried out in molecular pathology laboratories geographically or administratively distinct from those offering germline testing for CSGs.

Resources in these molecular pathology laboratories are focussed on interpretation of somatic genetics and expertise in germline interpretation may be lacking [13].

• **Consent and patient education**

Explicit consent is not routinely sought ahead of molecular genetic analysis of the tumour, being a test undertaken alongside histopathological examination to characterise the malignant tissue in order to inform immediate management. Furthermore, while tumour testing for treatment selection is most often requested by oncologists, some molecular genetic tumour tests are only initiated downstream of patient contact once the pathologist has carried out histological examination.

Conversely, a germline pathogenic CSG variant is often not relevant for current cancer management, but may have implications for future health, family members, reproductive decision-making and insurance. Accordingly it remains conventional practice that explicit information and consent-taking precede such analyses [14, 15].

Methods

ESMO PMWG germline subgroup

The Germline Subgroup convened by the ESMO PMWG comprised representation from medical oncology, surgical oncology, clinical cancer genetics, molecular pathology and medical law. The Group met five times to develop these recommendations (supplementary note, available at *Annals of Oncology* online).

Case series

We utilised the largest available dataset of paired tumour-normal sequencing, comprising 17 152 unselected cancer patients who presented to Memorial Sloan Kettering Cancer Center between 2014 and 2017 in whom clinical sequencing of both germline (blood) and tumour samples had been successfully carried out using the MSK-IMPACT assay (the MSK dataset) [4, 16–18]. All patients in this cohort had consented to somatic and/or germline testing in the context of tumour-normal sequencing using an institutional review board-approved protocol. For the purpose of this analysis, all genetic data were anonymised.

Sequencing

Samples were sequenced to a median depth of 741× (tumour) and 470× (normal). Tumour samples exhibiting somatic hypermutation (defined as >95th percentile of mutational burden) were analysed separately. All patients received clinical MSK-IMPACT sequencing using either the first or generation of the panel design interrogating 341 and 410 genes, respectively. Data were extracted for 65 genes associated with germline susceptibility to invasive cancers. For 64 genes, the mode of inheritance was autosomal dominant; *MUTYH* (autosomal recessive) was also included on account of being on the 'ACMG secondary findings gene list' (supplementary Table S1, available at *Annals of Oncology* online) [7].

Calling and variant classification

Joint variant calling was carried out in the tumour and the germline samples to generate optimal somatic calls. These were summed with the germline-only calls to generate tumour variant frequencies [16, 17]. Variants retained for analysis were those predicted to cause protein truncation in genes acting via loss-of-function and/or classified as germline 4-Likely Pathogenic or 5-Pathogenic (any star rating) based on the

ClinVar variant classification resource [19]. We excluded low penetrance alleles assigned within ClinVar as 'risk-factors', such as *APC* c.3920T>A (p.Ile1307Lys) and *VHL* c.598C>T (p.Arg200Trp). Inclusion of founder mutations did not substantially alter germline conversion rates for the respective genes (*BRCA1, BRCA2, ATM*) and these founder mutations were therefore retained for subsequent analyses (supplementary Table S2, available at *Annals of Oncology* online).

Gene annotation

A panel of five clinical (medical) geneticists specialising in cancer susceptibility was convened (supplementary note, available at *Annals of Oncology* online). Annotation was carried out independently for the 65 genes for (i) association with each individual cancer type [20], (ii) actionability (high/non-high), (iii) penetrance [high (RR > 4)/intermediate (RR = 2–4)] [21] and (iv) robustness of implication pathogenic variants in cancer susceptibility (clinical—grade or not). A status was assigned to each gene based on the majority decision (supplementary Table S1, available at *Annals of Oncology* online).

High-actionability CSGs (HA-CSGs) were defined as those of a level of actionability by which return of pathogenic germline variants would be appropriate in the off-tumour as well as on-tumour context. We established as a set of HA-CSGs, the 25 genes recommended for secondary findings by the ACMG and identified five additional genes as being of equivalent actionability to these 25 (*PALB2, RAD51C, RAD51D, BRIP1, SDHA*). These genes are under current review for inclusion in the ACMG secondary findings gene list [22–26].

From the remaining 35 genes, we established a set of 27 'standard-actionability' genes (SA-CSGs), namely those of high penetrance, for which the actionability of monoallelic pathogenic variants was agreed to be 'clinical grade', for which return of pathogenic germline variants in the on-tumour context was agreed to be appropriate.

Calculation of germline conversion rate and clinical recommendations

We defined a metric of 'germline conversion rate' (number of pathogenic variants of true germline origin× 100/total number of tumour-detected pathogenic variants), which we calculated for each gene (i) for all genes and (ii) stratified by gene actionability, context (on-tumour/off-tumour) and/or patient age. We recommended for germline follow-up only genes (i) of high/standard actionability (ii) for which a total of ≥2 mutations of true germline origin were detected across relevant tumour types (iii) for which the germline conversion rate exceeded 10% for that group defined by context and patient characteristics.

Results

Gross analyses of paired tumour-germline data

In total, 17 152 tumour-germline pairs were successfully analysed of which 830 were excluded due to somatic hypermutation. The remaining 16 322 paired samples were distributed by tumour type as per supplementary Table S3, available at *Annals of Oncology* online. Across the 65 genes analysed in 16 322 tumours, in total 1 997 499 tumour variants were identified (of which 1 959 587 were of true germline origin) (Table 1). Removal of common polymorphisms [≥1% minor allele frequency (MAF)] reduced the number of tumour variants to 79 342 (of which 53 388 were of true germline origin). We then applied an automated variant 'pathogenicity filter', retaining only variants predicted to cause loss of function of the protein and/or classified in ClinVar as Likely Pathogenic (class 4) or Pathogenic (class 5). A total of 17 075

Table 1. Summary of number of (i) tumour detected and (ii) true germline variants detected with application of (A) serial filters (B) gene/context/age criteria, from data on 65 genes for 16 322 tumours (MSK dataset)

			All tumours		Associated tumours		Non-associated tumours	
			Tumour detected	True germline	Tumour detected	True germline	Tumour detected	True germline
(A) Application of serial filters to MSK data on 65 genes for 16 322 tumours: number of variants	Retained: MAF ≤0.01		1 997 499	1 959 587				
			79 342	53 388				
	Retained: LP/P/truncating		17 075	1494				
	Retained: VAF ≥0.3 (SNV) or ≥0.2 (insdel)	All	9222	1442	2904	454	6305	983
		HA-CSGs (AD)	6141	677	2259	326	3882	351
		SA-CSGs (AD)	2372	213	539	37	1820	176
		Other	709	547	106	91	603	456
(B) Application of ESMO-PWG recommendations for gene/context/age criteria based on 10% germline conversion: number of variants	HA-CSGs (AD)	all ages (18 genes)	851	615	410	300	441	315
		age <30 (APC, RB1)	63	10	37	4	26	6
		age <30, on-tumour only (TP53)	59	7	59	7	n/a	n/a
		Total	973	632	506	311	467	321
	SA-CSGs (AD)	all ages, on-tumour only (BAP1, FH, FLCN, POLE),	60	17	60	17	n/a	n/a
		age <30, on-tumour only (NF1)	9	4	9	4	n/a	n/a
		Total	69	21	69	21	n/a	n/a
	Grand total		1042	653	575	332	467	321

HA-CSGs, high actionability genes; SA-CSGs, standard actionability CSGs; AD, autosomal dominantly inherited; other, CSGs of recessive inheritance, intermediate penetrance and/or non-clinical grade actionability.

tumour-detected pathogenic variants were retained [of which 1494 (8.7%) were of true germline origin]. Having examined the distribution of tumour-detected VAF for variants of true germline origin, we then removed variants of tumour VAF < 0.3 (SNVs) or VAF <0.2 (small insertions/deletions) (Figure 1). Application of this 'VAF filter' reduced the number of tumour-detected pathogenic variants from 17 075 to 9222, and true germline pathogenic variants from 1494 to 1442 (Figure 2).

Analyses by gene: actionability and on/off tumour association

We went on to examine germline conversion rate stratified by gene actionability, context (on-tumour/off-tumour) and/or patient age. We first examined the germline conversion rate in the 30 HA-CSGs. In the off-tumour setting, 19 of 30 genes surpassed the 10% threshold (Figure 3A). In the on-tumour setting, for 14 of those 19 genes the 'germline conversion rate' was likewise >10% and for 4 genes (SDHAF2, SDHD, SDHC and RAD51D) no pathogenic sequence variants were observed in associated tumours (Figure 3B). For VHL, 67% (6 of 9) pathogenic variants observed off-tumour were of germline origin whilst in the on-tumour setting this figure

was only 6% (5 of 86). This paradoxical observation is explained by the high rate of somatic VHL mutation in renal cancers (79 of 82 tumour-detected pathogenic variants being somatic; supplementary Figure S1, available at Annals of Oncology online). Similarly, reflecting the high somatic APC mutation rate in colorectal cancer (CRC), only 2 of 637 (0.3%) tumour-detected pathogenic APC variants observed in CRC were of germline origin (supplementary Figure S2, available at Annals of Oncology online).

For the 27 standard actionability CSGs analysed, a >10% on-tumour germline conversion rate was attained for genes BAP1, FLCN, POLE and FH and a near 10% rate for CDKN2A (8.9%, 8 of 90) and NF1 (9 of 107, 8.4%). Notably intermediate penetrance genes CHEK2, ATM and HOXB13 also had a >10% germline conversion rate (Figure 4).

Analyses of TP53: the most frequently somatically mutated CSG

We went on to perform more in depth analysis of tumour-detected TP53 pathogenic variants, on account of their high frequency and the significant clinical implications of diagnosis of Li-Fraumeni syndrome. Overall, 2930 tumour-detected TP53
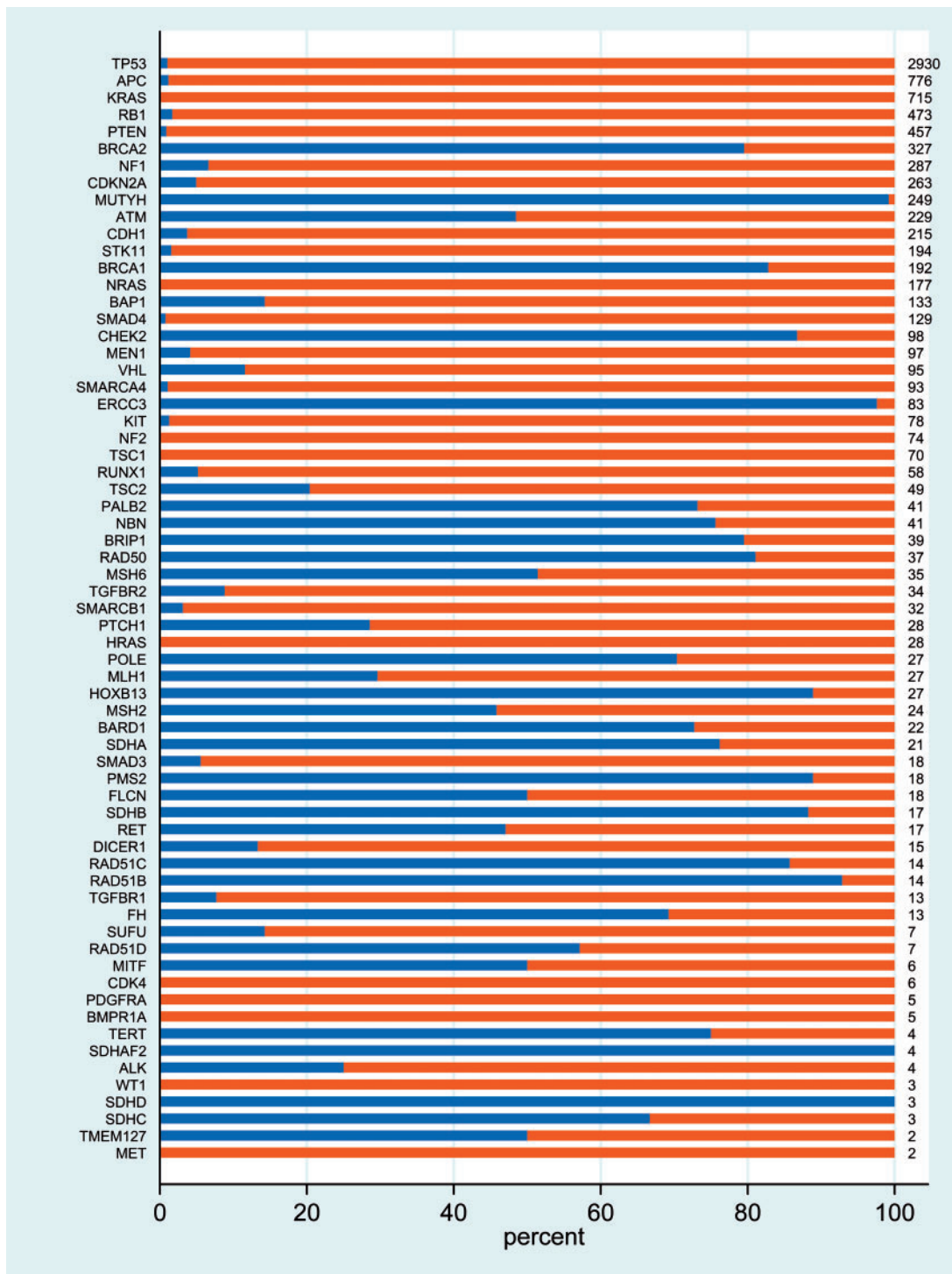
Figure 1. Distribution of variant allele frequency observed in the tumour for variants of true germline origin which were (i) small insertion/deletions (ii) SNVs.

pathogenic variants were detected across 16 322 tumours, with an overall germline conversion rate of 1% (30/2930). This rate was modestly higher in the on-tumour setting (2%, 16 of 786) than off-tumour (0.7%, 14 of 2144) (supplementary Figure S3a, available at *Annals of Oncology* online). To explore whether enrichment for germline origin was age-associated, we retained the 1273 tumours arising at age <30 years, in which there were 174 tumour-detected *TP53* pathogenic variants. The distribution of

the 11 true germline variants amongst the 174 tumour-detected *TP53* pathogenic variants included 4 of 31 (12.9%) for breast cancers, 2 of 20 (10%) for soft tissue sarcomas, 1 of 7 (14.3%) for bone tumours and 1 of 57 (1.8%) for gliomas and 3 of 60 (5%) in non-associated tumours (supplementary Figure 3b, available at *Annals of Oncology* online). The overall *TP53* germline conversation rate in the on-tumour setting age <30 years was 6.8% (8 of 117), but with the exclusion of glioma, this rate was improved to
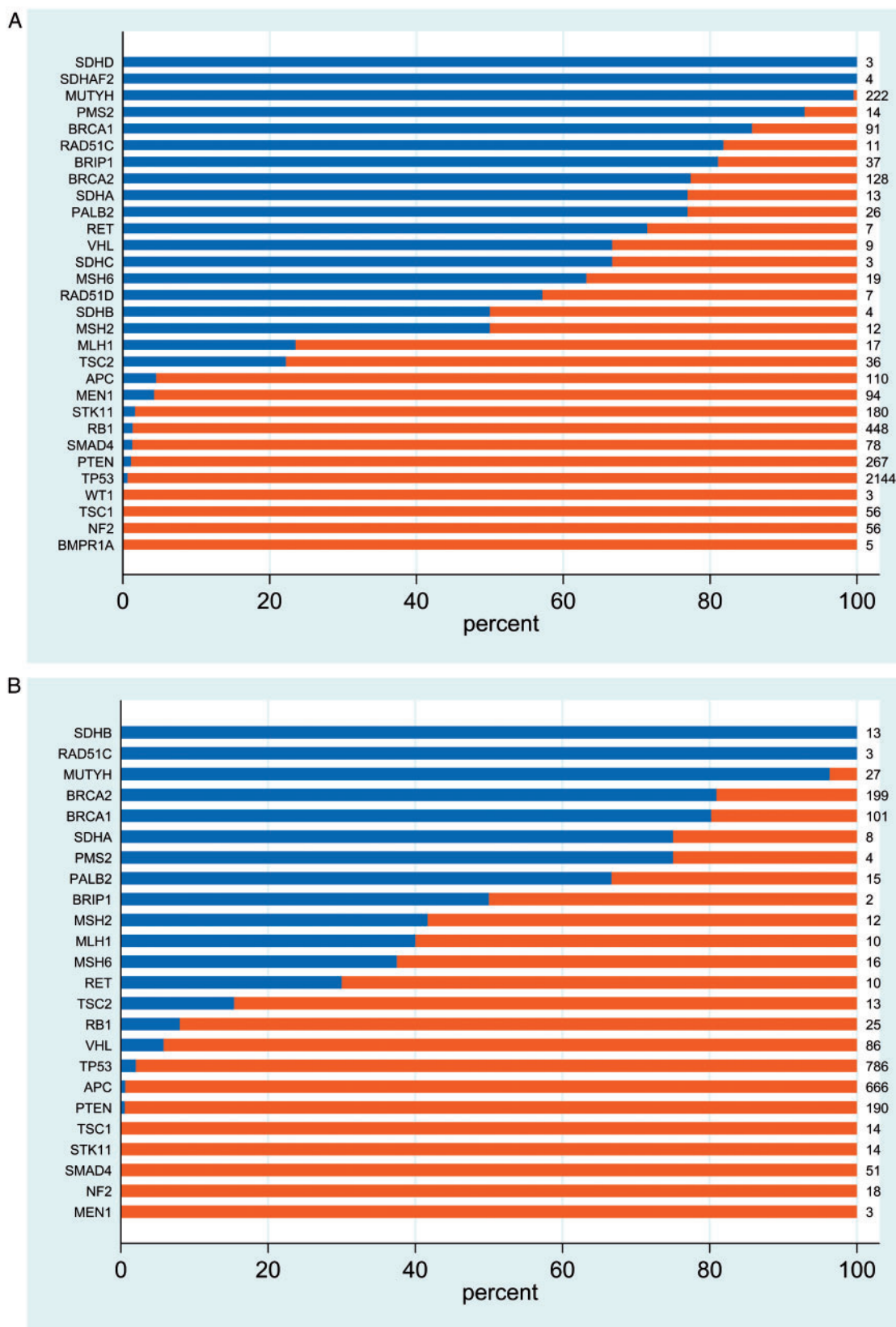
Figure 2. Distribution of germline and somatic pathogenic variants detected upon tumour analysis. Only variants classified pathogenic/likely pathogenic AND above VAF threshold are included (blue, germline origin; red, somatic origin; numbers, total number of pathogenic variants observed in tumour).
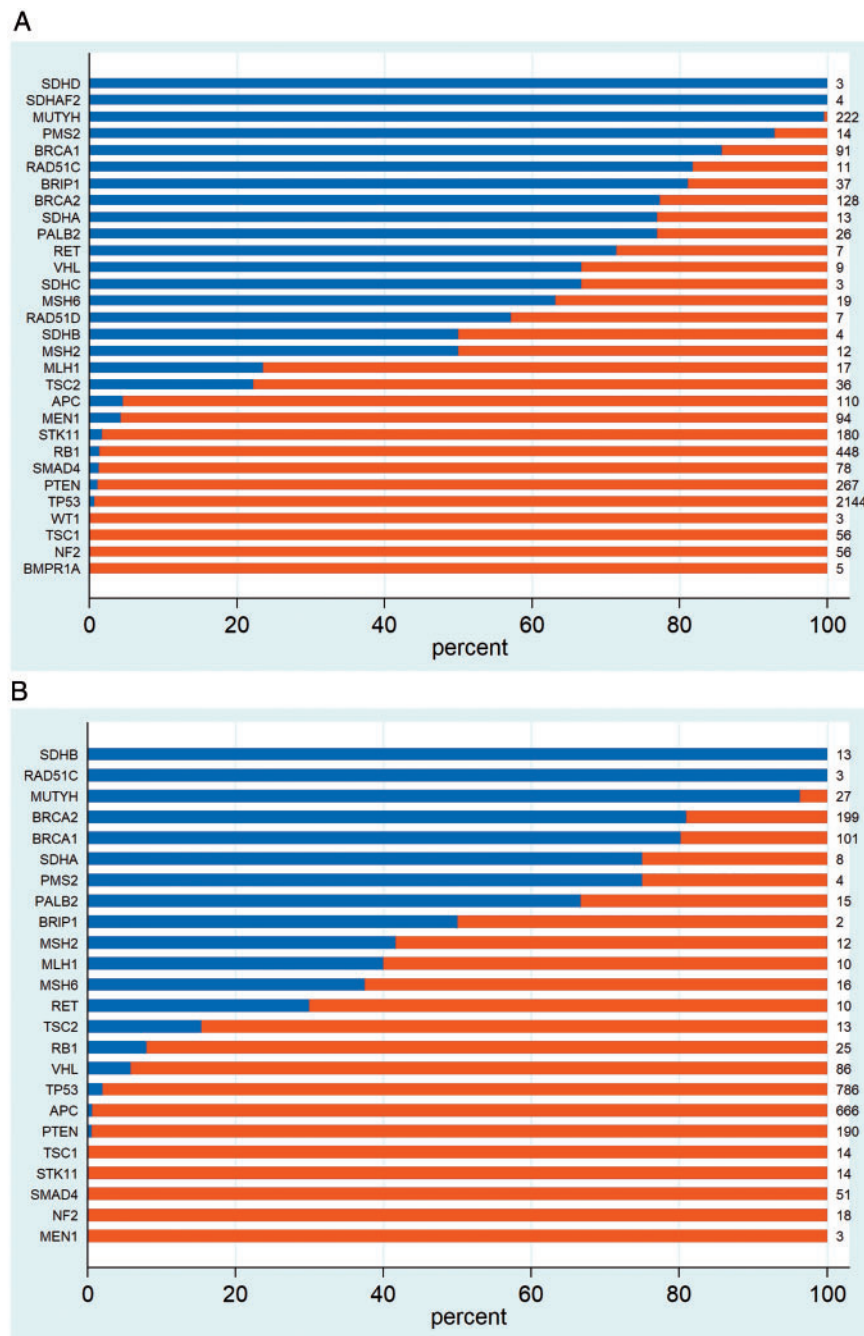
Figure 3. Distribution of germline and somatic pathogenic variants detected upon tumour analysis for 30 high-actionability CSGs. (A) Off-tumour and (B) on-tumour.

7 of 59 (11.7%). We also explored whether there was enrichment of the *TP53* germline conversation rate if only selected *TP53* mutations were included but did not find evidence for this (supplementary Table S4, available at *Annals of Oncology* online).

Analyses by patient age at tumour occurrence

Through restriction to tumours arising age <30 years, the overall germline conversion rate was improved to >10% for high-actionability genes *RB1* (7 of 35, 20%) and *APC* (3 of 28, 10.7%), and in the on-tumour context for the standard actionability gene

NF1 (4 of 9, 44.4%) (supplementary Figures S4 and S5, available at *Annals of Oncology* online).

Examining further each individual gene for specific scenarios of elevated germline conversion rate by each tumour type, and by all ages, age <30 and age <5, we did not identify any additional scenarios in which a germline conversion rate of >10% was achieved.

Analyses in hypermutated samples

In separate analysis of the 830 samples in which the mutational burden was above the 95th centile, the germline conversion rate
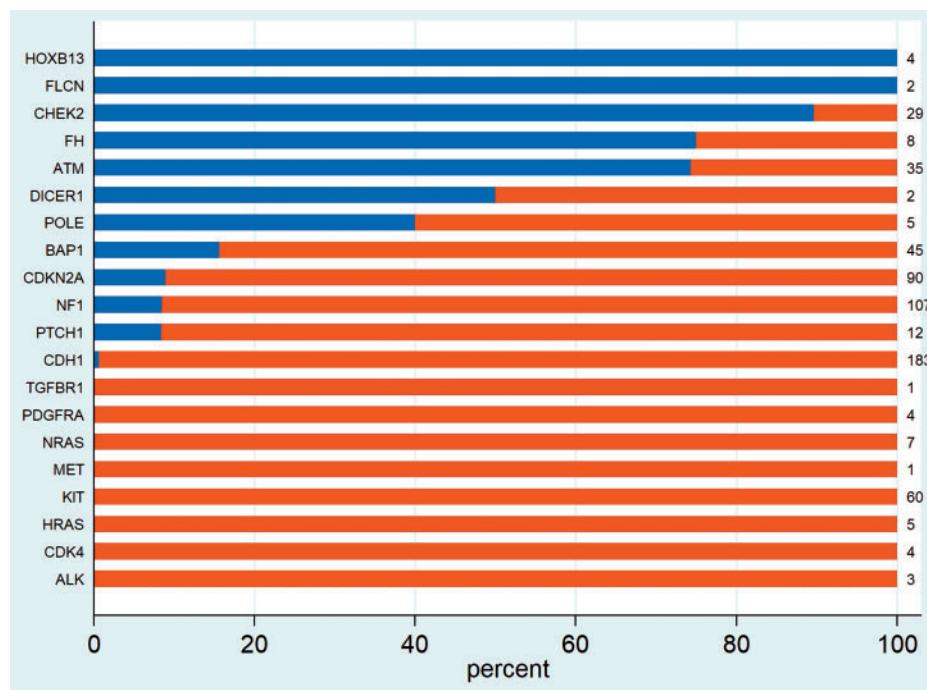
Figure 4. Distribution of germline and somatic pathogenic variants detected upon tumour analysis for non-high actionability CSGs in associated tumours (on-tumour).

was >10% for the majority of HA-CSGs and notably all four mismatch repair genes (*MLH1, MSH2, MSH6, PMS2*) (supplementary Figure S6, available at *Annals of Oncology* online). Of the 14 tumour-detected *POLE* pathogenic variants in these samples, only one was of germline origin.

Summary of rate of detection of variants of true germline origin

Overall, in our tumour-focussed germline analysis of the MSK dataset, restricting firstly by VAF and secondly to the 27 genes yielding germline conversion rate >10% as per specified gene/context/age, the number of tumour-detected pathogenic variants requiring 'germline follow-up' could be reduced by 94% from 17 075 to 1042 such that germline follow-up would only be required in 6.4% of tumours (1042/16 322) (Table 1).

Of these 1042 variants, 653 (62.7%) were of true germline origin. These 653 pathogenic germline variants comprised 615 pathogenic variants detected age-unselected in HA-CSGs (300 on-tumour, 315 off-tumour), 17 on-tumour pathogenic variants in *BAP1/FH/FLCN/POLE*, 7 on-tumour pathogenic *TP53* variants in tumours arising age <30 years and 14 pathogenic variants in *APC, RB1* and *NF1* in tumours arising age <30 years.

Of the 689 true germline variants in dominant HA-CSGs amongst the original 17 075 tumour-observed pathogenic variants, 677 remain following VAF filtering and 45 are 'thrown out' via application of the 10% germline conversion gene/context/age criteria [*MEN1*(4), *PTEN*(4), *SMAD4*(1), *STK11*(3) *VHL*(3), *TP53*(23), *APC*(6), *RB1*(1)]. Of the 37 true germline variants arising on-tumour across SA-CSG genes, 21 are detected and 16 are 'missed'. Overall, of the 789 (1442–653) true germline variants that are 'missed', 547 are in genes acting recessively (namely *MUTYH*), of intermediate penetrance and/or of non-clinical-

grade cancer association. A total of 176 variants are 'missed' because they occur off-tumour in SA-CSGs and we chose not to identify them.

Discussion

Through our large-scale analysis of paired somatic/germline data, the ESMO PMWG germline subgroup sought to develop recommendations regarding germline-focussed analysis of tumour-only sequencing data in order to optimise detection of true germline variants in genes of clinical utility, whilst avoiding excessive diversion of effort and resources towards 'germline follow-up testing' of vast numbers of variants.

The first issue we explored was restriction based on VAF. For this MSK dataset, we found that crude 'pan-tumour' VAF thresholds (20% for small insertions/deletions, 30% for SNVs) enabled reduction by almost half the number of tumour-detected variants requiring follow-up (17 075 to 9222) whilst losing only a tiny proportion of true germline variants (52 of 1494, 3.5%) and even smaller proportion of variants in dominant HA-CSGs (12 of 689, 1.7%). This filter near doubles the germline conversion rate from 8.7% (1494 of 17 075) to 15.6% (1442 of 9222). However, the VAFs used in this analysis may need to be re-evaluated by laboratories using PCR-based NGS methodologies rather than hybridisation-based methods.

Next, to reduce further the number of variants requiring follow-up, we recommend exclusion from germline-focussed tumour analysis of gene/context/age scenarios in which the germline conversion rate is <10%. Thus we highlight the 27 genes of >10% germline conversion rate (Box 1). Following filters for pathogenicity and VAF, restriction of germline-focussed tumour analysis to just these 27 genes (as per gene/context/age

Box 1. Recommendations for genes to be included for germline-focussed analysis and triggering of germline sample laboratory confirmation

	Any tumour type		Associated tumour type only
Tumour arising any age	*BRCA1*	*RAD51C*	*FLCN*
	BRCA2	*RAD51D*	*FH*
	BRIP1	*RET*	*BAP1*
	MLH1	*SDHA*	*POLE*
	MSH2	*SDHAF2*	
	MSH6	*SDHB*	
	PALB2	*SDHC*	
	PMS2	*SDHD*	
	VHL[a]	*TSC2*	
		MUTYH[b]	
Tumour arising age <30 only	*RB1*		*TP53*[c]
	APC		*NF1*

[a]Renal tumours to be excluded.
[b]*MUTYH* should be included for germline-focussed tumour analysis but reporting and germline follow-up testing should only be performed on detection of two pathogenic variants.
[c]Brain tumours to be excluded.

Recommendations, see also Box 1

1. Germline-focussed tumour analysis should be carried out in all laboratories as part of routine analysis of a large tumour panel.
2. Germline-focussed tumour analysis can be delivered via an automated pipeline so as not to add substantial additional manual work, cost or delay to tumour analysis.
3. Variants in should be flagged which are (i) predicted to result in protein truncation in genes acting through loss-of-function and/or (ii) classified as Pathogenic/Likely Pathogenic via a well-maintained, comprehensive and curated clinical resource (ClinVar is recommended).
4. Germline-focussed tumour analysis can be restricted to variants of VAF >30% (SNVs) or >20% (small insertions/deletions). Local validation will be required to confirm the accuracy of tumour VAF estimates, especially for PCR-based NGS methodologies.
5. Samples known or suspected to be hypermutated should be included for germline-focussed tumour analysis.
6. Germline-focussed tumour analysis in the off-tumour context should be restricted to 'High Actionability-CSGs' (Box 1).
7. Recessively acting 'High Actionability-CSGs' (currently *MUTYH* alone) should be included for germline-focussed tumour analysis but reporting and germline follow-up testing should be undertaken only on detection of two pathogenic variants.
8. Germline-focussed tumour analysis of 'standard actionability'-CSGs should be restricted to the on-tumour setting.
9. 'Standard actionability'-CSGs included for germline-focussed tumour analysis can be restricted to genes of high penetrance.
10. Germline-focussed tumour analysis can be restricted to gene-scenarios for which the germline conversion rate is >10%. For selected genes, it may therefore be appropriate to restrict germline-focussed tumour analysis to just those tumours arising age <30 years.
11. Formal variant review and classification should be undertaken by an experienced clinical scientist before initiation of patient re-contact and/or germline testing.
12. Before analysis of their germline sample for the pathogenic variant, adequate information should be provided to the patient regarding the implications of germline testing, along with documentation of their consent.
13. The tumour-observed pathogenic variant should be analysed in an appropriate germline sample (lymphocytes, saliva/buccal swab, normal tissue) in a laboratory accredited for germline analysis.
14. A patient in whom a germline pathogenic variant is detected should be referred to a specialist genetics service for long term follow-up and management of the family.
15. A normal/negative tumour sequencing result should not be taken as equivalent to a normal/negative germline result unless robust analysis of dosage has been carried out. This distinction is particularly important for genes such as *BRCA1* and *MSH2*, for which whole exon deletion/duplications constitute a substantial proportion of pathogenic variants.
16. Re-evaluation of this workflow, revised analyses and update of these recommendations should be undertaken at least 2-yearly. Reanalysis should include updated data regarding pathogenicity of variants and penetrance of CSGs, along with review of thresholds for 'germline conversion rates' and VAF cut-offs.

recommendations) enables reduction by 88.7% of the number of tumour observed variants requiring follow-up testing (from 9222 to 1042).

Although their germline conversion rate is high, we do not recommend germline-focussed tumour analysis for intermediate penetrance genes, such as *CHEK2* and *ATM*, as strategies are not well agreed regarding management of risk within families. We recognised nevertheless that in healthcare settings in which germline analysis for these genes is routinely offered, there would be a consistency in inclusion of these genes in germline-focussed tumour analysis. Indeed, detection of *CHEK2* c.1100delC may offer greater clinical utility to the family when ascertained in an isolated 60-year-old breast cancer case than in a multiplex breast cancer family.

We recommend germline-focussed tumour analysis in the off-tumour context is restricted to HA-CSGs, as per ACMG guidance regarding return of secondary findings. Nevertheless, for some genes on the ACMG secondary findings gene list, estimates are highly uncertain regarding penetrance outside of phenotype-driven ascertainment. Some centres may argue the merit of a narrower set of HA-CSGs [27, 28]. For the remainder of genes (of standard actionability) we recommended that germline-focussed tumour analysis be restricted to the on-tumour setting, such that germline pathogenic variants identified would be aetiologically pertinent with regard to the tumour type in which they were ascertained. For paediatric patients, special consideration will be required regarding return of either (i) a germline pathogenic variant deemed causative of the early onset cancer, (ii) an off-tumour pathogenic variant conferring risk only for adult onset cancers.

The germline conversion rates presented have been derived from the MSK dataset. When applying these filters and criteria in other settings, the frequency of germline variants detected will be predicated on (i) the distribution of different tumour types, (ii) the genes included on the panel, (iii) the purity of the tumours and (iv) the accuracy of tumour VAF estimation. Furthermore, a number of CSGs were not included on the MSK-IMPACT panel; overall, the subgroup agreed that for these more 'obscure' CSGs, whilst evaluation would be useful, these genes would at best be of standard actionability and generally (i) the frequency/contribution to overall cancer susceptibility of pathogenic germline variants is low (ii) the penetrance for cancer is poorly characterised (iii) evidence is limited regarding the efficacy of clinical interventions in carriers of pathogenic variants [20].

Local clinical workflows will need to evolve to encompass patient education, patient consent, acquisition of the germline sample and return of germline results. Some centres may elect as routine to acquire a germline sample and provide up-front consent to all individuals in whom tumour-only testing is carried out (potentially as an 'opt-out'). Alternatively, a two-stage approach may be preferred, whereby germline consultation and acquisition of the germline sample is only triggered on detection of a tumour-detected pathogenic variant. Telephone consultation, postal blood-packs and/or saliva sampling may mitigate otherwise problematic increases in clinical workload associated with this new burden of germline follow-up. If adjacent normal (or tumour-poor) tissue is available, testing in this for the tumour-detected pathogenic variant could provide a pragmatic means of triaging out variants of low germline likelihood.

In conclusion, identification of a pathogenic variant in a CSG can offer significant opportunity for the prevention and early detection of future cancers in the patient as well as their family, and may also influence management of the current cancer. Pragmatic, strategic germline-focussed tumour analysis can offer a high yield of true germline findings [63% true germline yield from follow-up of 6.4% of tumours (MSK dataset)].

The remit of the current recommendations was to guide germline-focussed tumour analysis of the tumour panels already in current use. An urgent priority for debate by clinicians and policy makers is consideration as to whether tumour panel content should be designed a priori to include genes selected for their germline utility, not just for CSGs but perhaps also those relating to pharmacogenomics.

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