

SPECIAL ARTICLE

Germline-focused analysis of tumour-detected variants in 49,264 cancer patients: ESMO Precision Medicine Working Group recommendations

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Background: The European Society for Medical Oncology Precision Medicine Working Group (ESMO PMWG) was reconvened to update its 2018/19 recommendations on follow-up of putative germline variants detected on tumour-only sequencing, which were based on an analysis of 17 152 cancers.

Methods: We analysed an expanded dataset including 49 264 paired tumour-normal samples. We applied filters to tumour-detected variants based on variant allele frequency, predicted pathogenicity and population variant frequency. For 58 cancer-susceptibility genes, we then examined the proportion of filtered tumour-detected variants of true germline origin [germline conversion rate (GCR)]. We conducted subanalyses based on the age of cancer diagnosis, specific tumour types and 'on-tumour' status (established tumour-gene association).

Results: Analysis of 45 472 nonhypermethylated solid malignancy tumour samples yielded 21 351 filtered tumour-detected variants of which 3515 were of true germline origin. 3.1% of true germline pathogenic variants were absent from the filtered tumour-detected variants. For genes such as *BRCA1*, *BRCA2* and *PALB2*, the GCR in filtered tumour-detected variants was >80%; conversely for *TP53*, *APC* and *STK11* this GCR was <2%.

Conclusion: Strategic germline-focused analysis can prioritise a subset of tumour-detected variants for which germline follow-up will produce the highest yield of most actionable true germline variants. We present updated recommendations around germline follow-up of tumour-only sequencing including (i) revision to 5% for the minimum per-gene GCR, (ii) inclusion of actionable intermediate penetrance genes *ATM* and *CHEK2*, (iii) definition of a set of seven 'most actionable' cancer-susceptibility genes (*BRCA1*, *BRCA2*, *PALB2*, *MLH1*, *MSH2*, *MSH6* and *RET*) in which germline follow-up is recommended regardless of tumour type.

Key words: tumour-only sequencing, germline, cancer-susceptibility genes, variants, germline conversion rate

INTRODUCTION

Continuing advances in sequencing technology have facilitated the rapid evolution of cost-effective tumour sequencing assays. In conjunction there has been expansion of indications for many targeted drugs and growth in molecularly stratified clinical trials, such that molecular analysis of the tumour has now become a routine component of cancer diagnosis.¹ While previously restricted to a handful of selected 'hotspots' of somatic mutation, these panels now more typically comprise the whole coding sequences of several hundred cancer-associated genes.²

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Many advocate the merits of paired tumour-normal sequencing to ensure complete detection of all germline variants, avoiding allelic loss and including exon-level deletions/duplications and intronic variants.^{3,4} Nevertheless, for reasons of cost and logistic simplicity, tumour-only sequencing remains the most frequent approach.⁵ However, tumour-only sequencing does not explicitly distinguish whether the origin of an observed variant (mutation) is constitutional (germline) or acquired (somatic).⁶ Many of the genes now included on these tumour panels are associated with inherited susceptibility to cancer. While the majority of germline variants in these cancer-susceptibility genes (CSGs) are innocuous (benign), pathogenic (disease-associated) variants in these genes may confer clinically important risk of future cancers and potentially be present in other family members.⁷ Furthermore, oncological therapeutic decision making is increasingly predicated on germline mutational status.⁸

In 2018/19 the European Society for Medical Oncology (ESMO) Translational Research and Precision Medicine Working Group first developed recommendations on germline-focused analysis and follow-up of tumour-only sequencing. These recommendations were informed by analysis of paired tumour-normal data from MSK-IMPACT available on 17 152 tumours.⁹⁻¹¹ Based on contemporaneous genetic practice, germline-focused follow-up was recommended in scenarios in which the per-gene 'germline conversion rate' (GCR; i.e. proportion of variants of true germline origin out of total number of filtered tumour-detected variants) exceeded 10%. These recommendations also differentiated according to the 'germline gene actionability', namely, the summed evidence for a given gene supporting cancer risk (penetrance), mutational spectrum and interventions for prevention/early detection. Germline follow-up for filtered tumour-detected variants regardless of tumour type (pan-tumour) was recommended for a comparatively large group of genes ($n = 21$) deemed to be of 'high' germline actionability, as based on the recommendations of the American College of Medical Genetics and Genomics (ACMG).^{12,13} For genes of lesser germline actionability, germline follow-up was only recommended when the variant was detected 'on tumour', that is where risk of that tumour type had already been formally associated with germline mutation of that gene.

Here we present MSK-IMPACT paired tumour-normal sequencing of 32 184 additional tumour-normal pairs (32k series). This has allowed us to evaluate reproducibility of findings from 17 080 samples included in the 2018/19 analysis (17k series), and then to perform better-powered analysis using the full dataset of 49 264 tumours (49k series), improving power for analysis of lower-frequency variants and/or rare tumour types. We have included in this 49k analysis (i) updated gene-tumour associations based on review of current literature, (ii) genes established as being of only intermediate penetrance (risk) of cancer, (iii) modification in per-gene GCR threshold from 10% to 5% and (iv) updated examination of germline actionability considering more and less conservative approaches for germline follow-

up. The ESMO Translational Research and Precision Medicine Working Group was reconvened to evaluate these analyses and update recommendations in the context of evolving patterns of clinical and laboratory practice.

METHODS

Assembly of expert group

The germline subgroup within the ESMO Precision Working Group (PMWG) comprised representation from medical oncology, clinical cancer genetics, molecular pathology and molecular genetics (Supplementary Note, available at <https://doi.org/10.1016/j.annonc.2022.12.003>), developing the recommendations via two meetings and subsequent communications. The Cancer Genetics Expert Group (CGEG) comprised five clinical cancer geneticists (Supplementary Note, available at <https://doi.org/10.1016/j.annonc.2022.12.003>), and convened twice to ratify gene inclusion, gene-cancer associations and gene actionability (Supplementary Figure S1, available at <https://doi.org/10.1016/j.annonc.2022.12.003>).

Curation of genes

Based on summed evidence regarding risk (penetrance), understanding of mutational spectrum and availability/efficacy of proven interventions for prevention/early detection, 58 autosomal CSGs for which analysis is offered in clinical cancer genetics to patients with a relevant personal and family history of the respective cancers were selected for inclusion by the CGEG (Supplementary Figure S1, available at <https://doi.org/10.1016/j.annonc.2022.12.003>). For these genes, the CGEG undertook literature-based review of gene-cancer associations and gene actionability. The five members of the CGEG used majority voting where consensus was not complete.

The set of 28 CSGs recommended by the ACMG for return of secondary findings (27 autosomal dominant and 1 autosomal recessive, *MUTYH*) was augmented by three ovarian CSGs deemed by the CGEG as of equivalent actionability (*RAD51C*, *RAD51D* and *BRIP1*).¹³⁻¹⁹ Among these 31/58 genes, the CGEG differentiated seven genes (*BRCA1*, *BRCA2*, *MLH1*, *MSH2*, *MSH6*, *PALB2* and *RET*) as being the most actionable CSGs (MA-CSGs), on the basis of the cancer risks being high and well-evidenced, the mutational spectrum being well characterised, the clinical interventions being well established and the GCR being appreciable. The remaining 24 genes were assigned as being high-actionability CSGs (HA-CSGs).

The other 27/58 genes (all autosomal dominant) were labelled as standard-actionability CSGs (SA-CSGs) and comprise 25 high-penetrance CSGs (relative risk >4) and 2 intermediate-penetrance CSGs (relative risk 2-4).^{20,21}

Case series

We used paired tumour-normal sequencing data from 49 264 unselected cancer patients, presenting to Memorial Sloan Kettering Cancer Center (MSKCC) between 2014 and 2021, who had clinical sequencing of both germline (blood) and

tumour samples using the MSK-IMPACT assay and, as per institutional review board-approved protocol, had consented to somatic and/or germline testing in the context of tumour-normal sequencing. All genetic data were anonymised and collapsed for the purpose of these analyses. This work was performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Sequencing

Sequencing was to a median depth of $655\times$ (tumour) and $498\times$ (normal). We analysed separately ‘hypermutated’ tumour samples, that is, with microsatellite instability or high tumour mutational burden (20 nonsynonymous mutations per megabase or higher, $n = 3393$).²² From the full MSK-IMPACT panels (comprising 341 genes or 410 genes for the 17k and 32k series, respectively), data were extracted for the 58 CSGs ratified for inclusion by the CGEG (Supplementary Figure S1, available at <https://doi.org/10.1016/j.annonc.2022.12.003>).

Variant calling, classification and filtering

Joint variant calling had been carried out in the tumour and the germline samples to generate optimal somatic calls. The somatic-only calls were summed with the germline-only calls to generate tumour-detected variant frequencies. We generated a set of filtered tumour-detected variants enriched for true germline pathogenic variants through application of three germline-focused filters: (i) variant minor allele frequency <0.01 in both gnomAD and internal variant frequencies (to remove common single-nucleotide polymorphisms and sequencing artefacts); (ii) classification in ClinVar as likely pathogenic or pathogenic (≥ 1 star) and/or truncating variants in known tumour suppressor CSGs²³ and (iii) tumour-observed variant allele frequency (VAF) >0.3 [single-nucleotide variants (SNVs)] or >0.2 [small insertions/deletions (indels)]. The VAF thresholds were based on the distribution of tumour VAF for variants of true germline origin (Supplementary Figure S2, available at <https://doi.org/10.1016/j.annonc.2022.12.003>). The threshold of ClinVar ≥ 1 star was deliberately inclusive; accordingly, it is recommended that formal classification using the ACMG framework is undertaken ahead of patient contact for any tumour-detected variants for which germline follow-up is indicated.

Analyses

The GCR was calculated for each gene: (i) pan-tumour (i.e. across all tumour types); (ii) on-tumour (i.e. across just the tumour types for which that gene is associated with germline cancer risk as per Supplementary Figure S1, available at <https://doi.org/10.1016/j.annonc.2022.12.003>); (iii) off-tumour (i.e. across just the tumour types for which that gene is not associated with germline cancer risk); (iv) for individual cancer types and subtypes and (v) for only tumours arising at young age (<30 years and <5 years). We required three or more true germline variants to be present in the tumour group under analysis for the gene-tumour pairing to

inform clinical recommendations. Two-sided Fisher’s exact tests were used to assess for heterogeneity (P_{het}).

RESULTS

Sample series and variants detected

Following application of per-sample quality control metrics, 49 264 tumours (‘49k’ series) were available for analysis, of which 17 080 had been included in the 2018/19 analyses (‘17k’ series) and 32 184 were newly analysed (‘32k’ series). The 399 cases of nonmalignant or haemato-oncological disease were excluded. In addition, 3393 tumours were excluded from the primary analysis based on somatic hypermutation. Following application of sequencing quality control, and filtering for minor allele frequency <0.01 , 161 084 tumour-detected variants were reported across the 45 472 nonhypermutated cancers, of which 109 221 (67.8%) were of germline origin and 51 863 (32.4%) were of somatic origin.¹¹ Following filtering for variants predicted to be pathogenic (P/LP in ClinVar and/or predicted to be protein truncating), 34 400 tumour-detected variants were retained, of which 3627 (10.5%) were of germline origin. Following filtering of these 34 400 tumour-detected variants for tumour-observed VAF ≥ 0.3 (SNVs) or ≥ 0.2 (indels), 37.9% of variants (13 049/34 400) were discarded and 21 351 variants were retained, of which now 3515 (16.5%) were of germline origin. Of 3627 true pathogenic germline variants, 112 (3.1%) were absent from the final set of filtered tumour-detected variants. However, loss of heterozygosity (loss of the mutant germline allele) was present in 86% of these cases and in a further 7% there was amplification of the wild-type allele (lowering the observed variant fraction of the mutant allele), suggesting that modification of the VAF filter would have modest impact on ‘retrieving’ these missed true-germline variants (Supplementary Tables S1 and S2, available at <https://doi.org/10.1016/j.annonc.2022.12.003>).

Relaxing of the VAF filters also had only modest impact on GCR. For example, raising of the VAF filter (for SNVs and indels alike) to 0.4 only increased the GCR for *TP53* from 0.9 to 1.4%, and raising the VAF filter to 0.7 only increased the GCR to 2.5%. These observations reflect the frequent somatic loss of the wild-type allele, which results in high tumour-observed VAFs for somatic *TP53* variants.

There were filtered tumour-detected variants present in 56/58 genes analysed (Figure 1). *ALK* and *PDGFRA* contained no qualifying variants in the 49 264 tumours analysed, and were excluded from subsequent analyses.

Reproducibility of the analysis in the 17k and 32k series

We observed no evidence of heterogeneity for the ‘pan-tumour’ per-gene GCR between the 17k series (included in the 2018/19 analyses)⁹ and the new 32k series (Figure 1). No gene showed significant difference on p-heterogeneity, even prior to multiple testing correction. We thereafter proceeded with subsequent analyses using the combined 49k series.

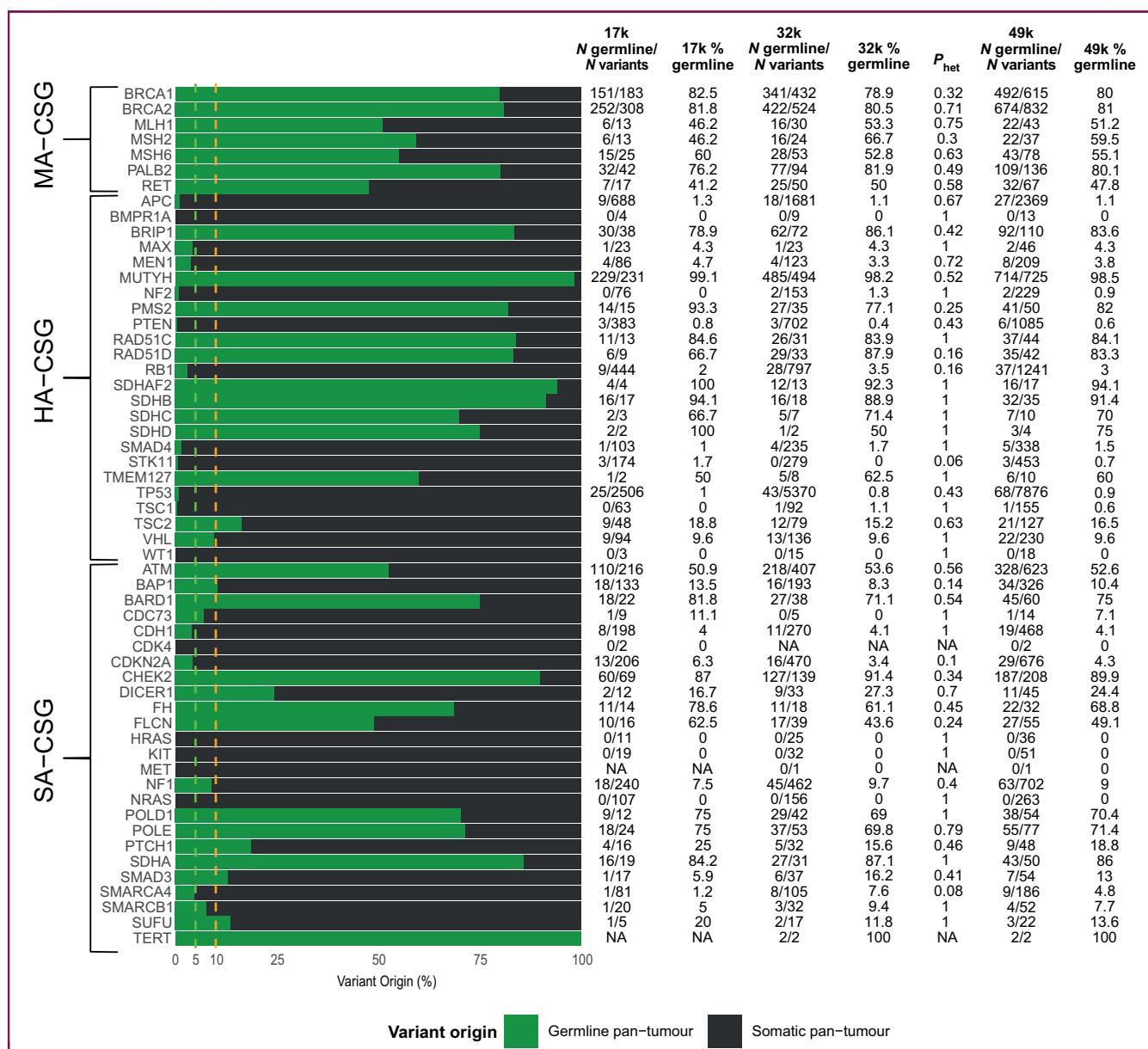


Figure 1. Proportion of tumour-detected pathogenic variants of germline versus somatic origin for all tumour types and all ages at diagnosis. Stacked bar plots showing the proportion of tumour-detected pathogenic variants that are of germline origin (green bars) versus those of somatic origin (black bars) in the combined sample series (49k series), comprising 45 472 nonhypermutated tumours across 47 cancer types combined (i.e. pan-tumour). Data are shown per gene, for 56 'actionable' cancer-susceptibility genes. The adjacent table provides the variant counts and germline proportions for the constituent 17k and 32k subsets, as well as P values (two-sided Fischer's exact test of proportions) from comparing the 17k and 32k subsets.

HA-CSG, high-actionability cancer-susceptibility gene; MA-CSG, most-actionability cancer-susceptibility gene; N germline, number of tumour-detected variants of germline origin; N variants, total number of tumour-detected variants; P_{het} , P heterogeneity; SA-CSG, standard-actionability cancer-susceptibility gene.

Per-gene analyses: by tumour association

Analysing across 45 472 nonhypermutated solid malignancies, we first examined the 'pan-tumour' GCR for each gene (Figure 1). For 13/56 of the cancer-susceptibility genes with qualifying tumour-detected variants, the pan-tumour GCR was very high [$\geq 80\%$; e.g. *BRCA1* (80%), *BRCA2* (81%), *BRIP1* (83.6%)], and for 11/56 genes the GCR was high [50%-80%; e.g. *MLH1* (51.2%), *MSH2* (59.5%), *ATM* (52.6%)]. However, for 20/56 genes the pan-tumour GCR was low [0%-5%; e.g. *TP53* (0.9%), *PTEN* (0.6%) and *APC* (1.1%)]. This includes seven genes for which the observed GCR was 0%, that is, none of the tumour-detected variants were of germline origin (*BMPR1A*, *CDK4*, *HRAS*, *KIT*, *MET*,

NRAS and *WT1*). For the remaining 12/56 genes the GCR lay in an intermediate range (5%-50%).

We then examined the GCR separately, first for the tumours with established association with germline mutation of the gene (on-tumour) and then considering tumours not associated with germline mutation of the gene (off-tumour; Supplementary Figure S3, available at <https://doi.org/10.1016/j.annonc.2022.12.003>). Of the 49/56 genes for which the filtered tumour-detected variants contained variants of true germline origin, 43/49 genes harboured variants in both the on-tumour and off-tumour contexts; 6/49 genes were only found to harbour filtered variants off-tumour (*MAX*, *SDHAF2*, *SDHD*, *SMAD3*, *TERT* and

TMEM127). The GCR in the on-tumour context was greater than or equal to the off-tumour equivalent for 27/43 genes, with the difference significant at $P_{het} < 0.05$ for *RB1*, *TP53*, *ATM*, *NF1* and *SMARCA4*. Conversely, the GCR was lower on-tumour than off-tumour for 16/43 genes, with $P_{het} < 0.05$ for *APC*, *BRCA1*, *RET* and *VHL*.

Per-gene analyses: by age of tumour diagnosis

Based on a hypothesis that individuals developing young-onset cancers would be enriched for germline-susceptibility

variants, we then examined the GCR restricting to individuals aged <30 years at tumour diagnosis (Figure 2, Supplementary Figures S4 and S5, available at <https://doi.org/10.1016/j.annonc.2022.12.003>). For 36/56 genes, the GCR was higher when restricting to those with cancers diagnosed aged <30 years, with $P_{het} < 0.05$ for 11 genes. For example, for *TP53* GCR was 0.9% all-ages and 5.1% for age <30 ($P_{het} = 4.26 \times 10^{-8}$). We also examined GCR for genes when restricting analysis to tumours aged <5 years. The GCR was significantly higher in those diagnosed <5 years compared with all ages for genes such as *RB1* (37% versus

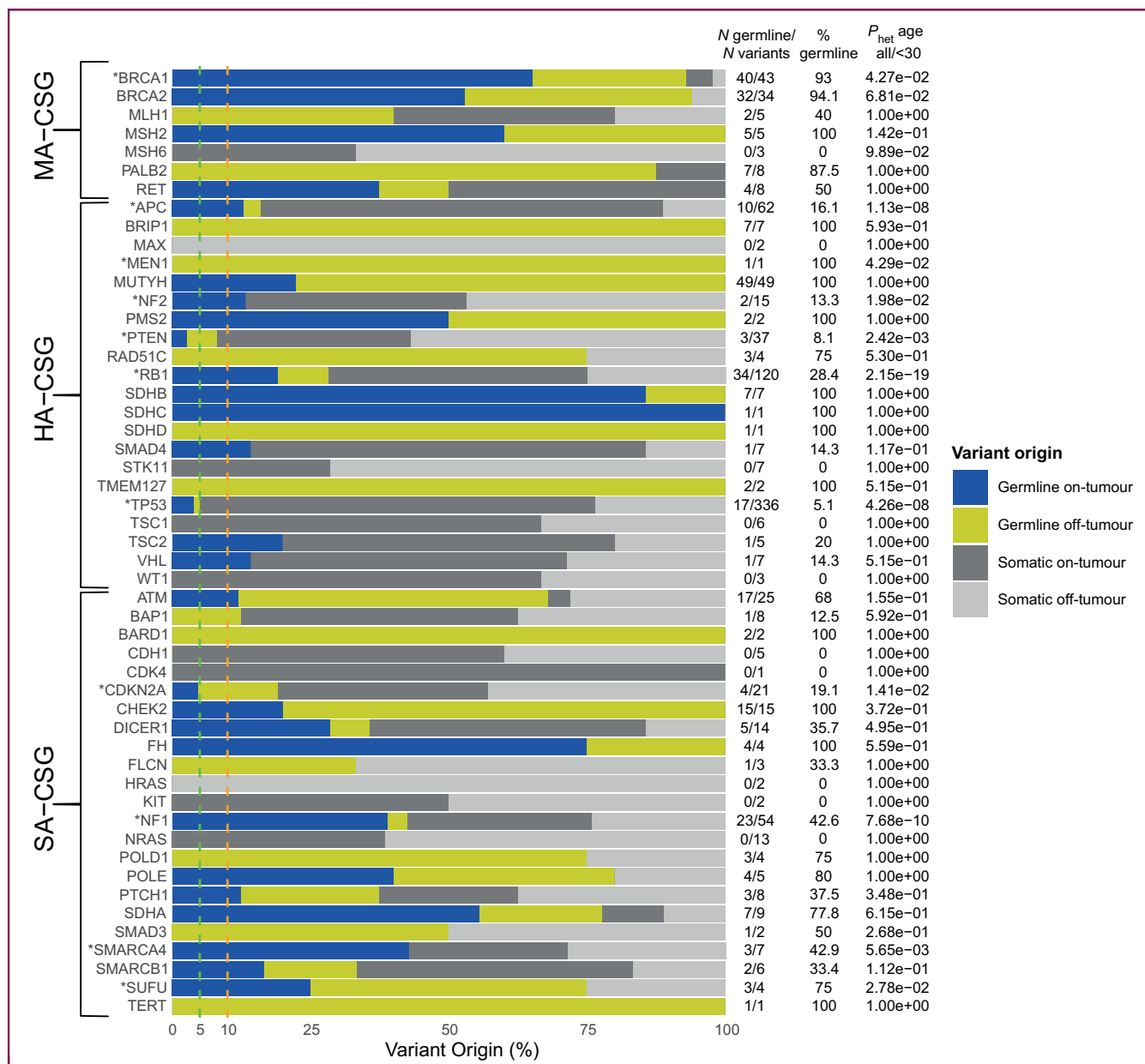


Figure 2. Proportion of tumour-detected pathogenic variants of germline versus somatic origin for all tumour types and age at diagnosis <30 years. Stacked bar plots showing the proportion of tumour-detected pathogenic variants that are of germline origin (blue bars for on-tumour and yellow bars for off-tumour) versus those of somatic origin (dark grey bars for on-tumour and light grey bars for off-tumour) in the combined sample series (49k series), comprising 45 472 non-hypermutated tumours across 47 cancer types. Data are shown per gene, for 51 ‘actionable’ cancer-susceptibility genes. The adjacent table provides the variant counts, germline proportions and the P -values (two-sided Fischer’s exact test of proportions) from comparing the GCRs between data at all ages versus age <30. The * indicates genes for which the GCR is significantly different between all ages and age <30 ($P_{het} < 0.05$). ‘On-tumour’ means across just the tumour types for which that gene is associated, ‘Off-tumour’ means across just the tumour types for which that gene is not associated.

3%, $P_{\text{het}} = 2.79 \times 10^{-21}$) and *TP53* (15.8% versus 0.9%, $P_{\text{het}} = 6.09 \times 10^{-4}$), but for most genes there were very low counts of tumours diagnosed and variants detected in the <5 years group. As anticipated, tumours arising age <5 were more somatically 'quiet', with only 58% (94/162) of tumours featuring a filtered tumour-detected variant compared with 83.5% (17 836/21 351) across the series as a whole. Thus the observation of higher GCR in younger patients is driven not only by age-related enrichment for germline variants but also by a lower denominator of somatic mutation.

Per-gene per tumour-type analyses

For each gene, we then examined the GCR broken down by tumour type (Supplementary Figure S6, available at <https://doi.org/10.1016/j.annonc.2022.12.003>). In particular, we explored whether there were specific tumour types for which GCR deviated substantially from the pan-tumour/on-tumour GCR for that gene. For example, in 1062 renal cancers there were 198 eligible *VHL* variants observed, of which only three were of germline origin (GCR = 1.5%). However, across all other cancer types (excluding renal cancer), the GCR for *VHL* was 59.4% (19/32; Figure 3A). Thus although renal cancer is a 'canonical' tumour associated with Von Hippel–Lindau heritable cancer syndrome, the common somatic *VHL* mutations in renal tumours pass germline-focused filtering and 'swamp' those of true germline origin. A similar paradoxical excess of somatic mutations is observed for *RET*, with a GCR of 22% (9/41) for thyroid cancers and 88.5% (23/26) for nonassociated cancers (Supplementary Figure S6, available at <https://doi.org/10.1016/j.annonc.2022.12.003>). Conversely, for *CDH1*, for the definitively associated tumour type (oesophageal gastric) the GCR is 36.8% (7/19). However, although breast cancer is also associated with germline *CDH1* variants, the high frequency of filtered tumour-detected variants and lower GCR (1.5%, 6/394) mean the overall on-tumour GCR is reduced to 3.8% (Figure 4B). Examining lobular breast cancers alone, where pathology grouping was available, the GCR was only 1.9% (1/52, Supplementary Figure S7, available at <https://doi.org/10.1016/j.annonc.2022.12.003>). Analysis by breast cancer subtype was also examined for other breast-cancer associated genes *BRCA1*, *BRCA2* and *PALB2* (Supplementary Figure S7, available at <https://doi.org/10.1016/j.annonc.2022.12.003>). Interestingly, for genes such as *BRCA1*, the higher observed rate of germline variants in the triple-negative tumours was offset by an elevated rate of eligible somatic mutations in this group.

Per-tumour type analysis

For each tumour type we also looked at the overall pan-gene GCR (Figure 5, Supplementary Figure S8, available at <https://doi.org/10.1016/j.annonc.2022.12.003>). This varied widely by tumour, for example, 5.8% for colorectal cancer, 21.3% for ovarian cancer and 100% for pheochromocytoma/paraganglioma. The per-tumour-type pan-gene GCR was to some extent reflective of where germline architecture is dominated by a specific gene (e.g. *BRCA1* variants drive the pan-gene GCR for ovarian cancer). However, the more

predominant influence appeared to be the highly variable frequency of somatic mutation between different tumour types. In particular, the rate of filtered tumour-detected *TP53* variants varies widely between tumour types, substantially influencing the pan-gene GCR for a tumour type. Across the 4370 colorectal cancers analysed, a filtered tumour-detected *TP53* or *APC* variant was present in 34.4% and 45.3% tumours, of which only 0.3% (4/1503) and 0.6% (11/1982), respectively, were of true germline origin.

Embryonal tumours had a high overall GCR (8/20, 40%); as for childhood tumours, embryonal tumours are somatically 'quiet' with only 20% (12/60) exhibiting a filtered tumour-detected variant.

Intermediate penetrance genes

Based on an increase in their inclusion for clinical germline testing, two intermediate penetrance genes, *CHEK2* and *ATM*, are now included as SA genes. Filtered tumour-detected variants were relatively frequent in *ATM* (a very large gene), of which 52.6% (328/623) were of germline origin. Filtered tumour-detected variants were less frequent in *CHEK2*, but a high proportion (89.9%, 187/208) were of germline origin (Figure 4).

Generation of clinical recommendations

For each gene, we examined the GCR defined by tumour association (pan-tumour/on-tumour) and/or by age (all ages, <30 years, <5 years) and compared this with a predefined GCR threshold of 10% and 5% (Supplementary Table S4, available at <https://doi.org/10.1016/j.annonc.2022.12.003>). Where a specific tumour type had a high rate of filtered tumour-detected variants but low GCR, we examined the impact on GCR of exclusion of this tumour type.

For inclusion in clinical recommendations for germline-focused analysis and germline follow-up, a gene was required to meet a GCR threshold of $\geq 5\%$, with three or more true germline variants detected; 26/56 genes (7 MA, 8 HA, 11 SA) met these criteria both pan-tumour and on-tumour for the all-age analysis. For an additional eight genes, the pan-tumour but not on-tumour criteria were met (*SDHAF2*, *SDHC*, *SDHD*, *TMEM127*, *PTCH1*, *SMAD3*, *SMARCB1* and *SUFU*). A further six genes (*APC*, *CDKN2A*, *RB1*, *PTEN*, *SMARCA4* and *TP53*) met the criteria by restricting the analysis to tumours diagnosed under 30 years of age (Box 1). This totals 40 genes (Box 1). For 34 of these the recommendation would remain the same if applying a GCR threshold of 10%, whereas for six genes (*BAP1*, *NF1*, *PTEN*, *SMARCB1*, *TP53* and *TSC2*), recommendations for germline-focused analysis/follow-up would differ if using a GCR of 10% versus 5% (Supplementary Table S4, available at <https://doi.org/10.1016/j.annonc.2022.12.003>).

Hypermutated samples

Analysis of the 3393/49 264 samples with somatic hypermutation demonstrated that the GCR per gene is almost universally lower for the hypermutated samples than for the

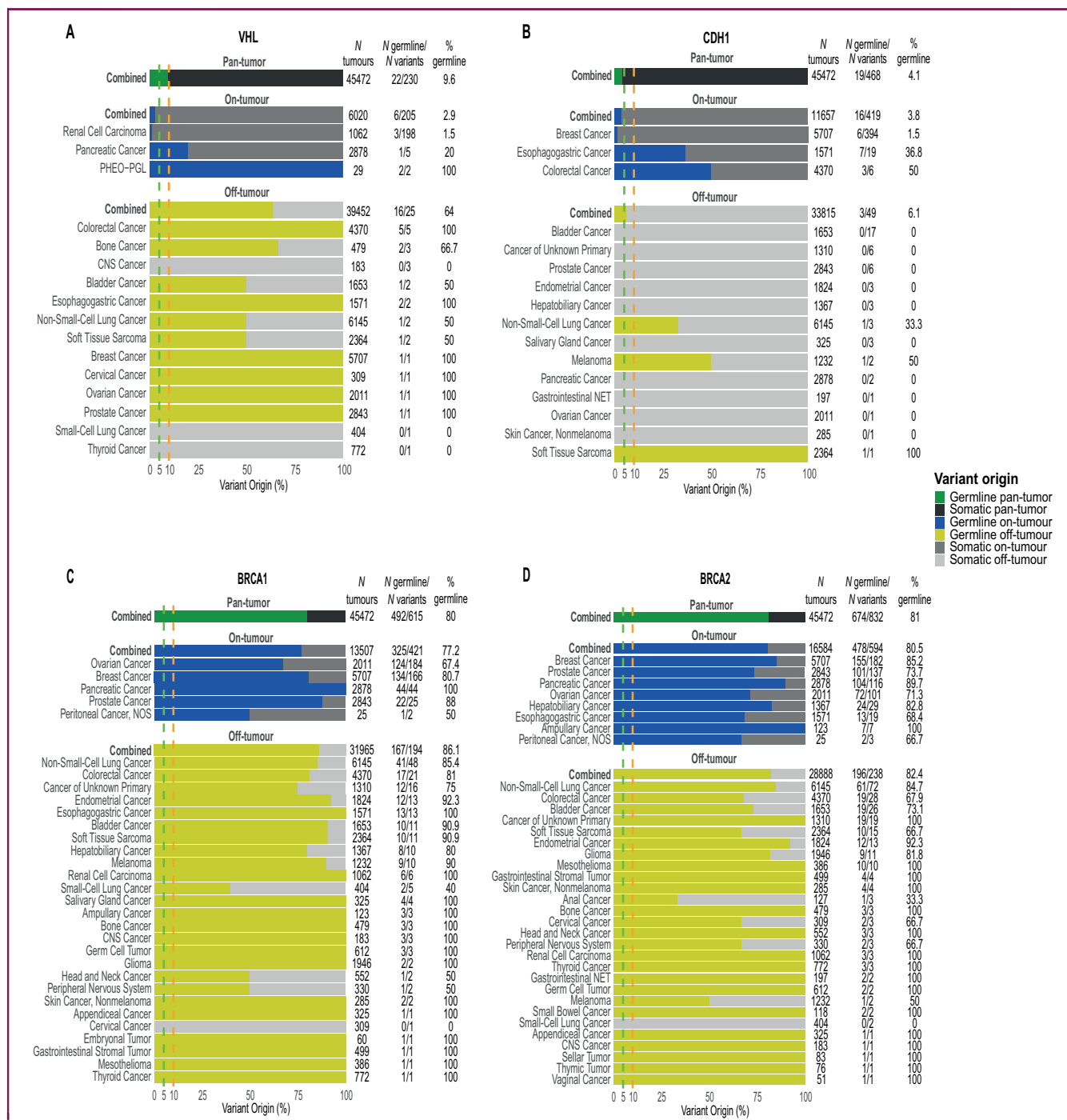


Figure 3. Proportion of tumour-detected pathogenic variants of germline versus somatic origin for individual, grouped tumour types for VHL, CDH1, BRCA1 and BRCA2. Stacked bar plots showing the proportion of tumour-detected pathogenic variants that are of germline origin (green bars for pan-tumour, blue bars for on-tumour and yellow bars for off-tumour) versus those of somatic origin (black bars for pan-tumour, dark grey bars for on-tumour and light grey bars for off-tumour) in the combined sample series (49k series), comprising 45 472 nonhypermutated tumours across 47 cancer types. Data are shown by tumour association groups and for 16, 16, 31 and 35 tumour types for (A) VHL, (B) CDH1, (C) BRCA1 and (D) BRCA2, respectively. The adjacent table provides the number of tumours, variant counts and germline proportions. Tumour types with no qualifying variants are not presented individually but are included in the combined number of tumours. ‘Pan-tumour’ means across all tumour types combined, ‘on-tumour’ means across just the tumour types for which that gene is associated, ‘off-tumour’ means across just the tumour types for which that gene is not associated.

CNS, central nervous system; NET, neuroendocrine tumour; N germline, number of tumour-detected variants of germline origin; NOS, not otherwise specified; N tumour, number of tumours; N variants, total number of tumour-detected variants; PHEO-PGL, pheochromocytoma/paraganglioma.

nonhypermutated samples. However, for 33/34 genes included for all-age analysis in Box 1, the overall GCR of >5% is maintained with the inclusion of hypermutated samples (Supplementary Tables S3 and S4, available at <https://doi.org/10.1016/j.annonc.2022.12.003>). The ESMO PMWG thus

recommends hypermutated samples be included for germline-focused analysis and follow-up identical to nonhypermutated samples. In particular, a disproportionate fraction of the true germline variants is found in the hypermutated samples for genes such as MLH1 (55/3393 in hypermutated versus 22/45

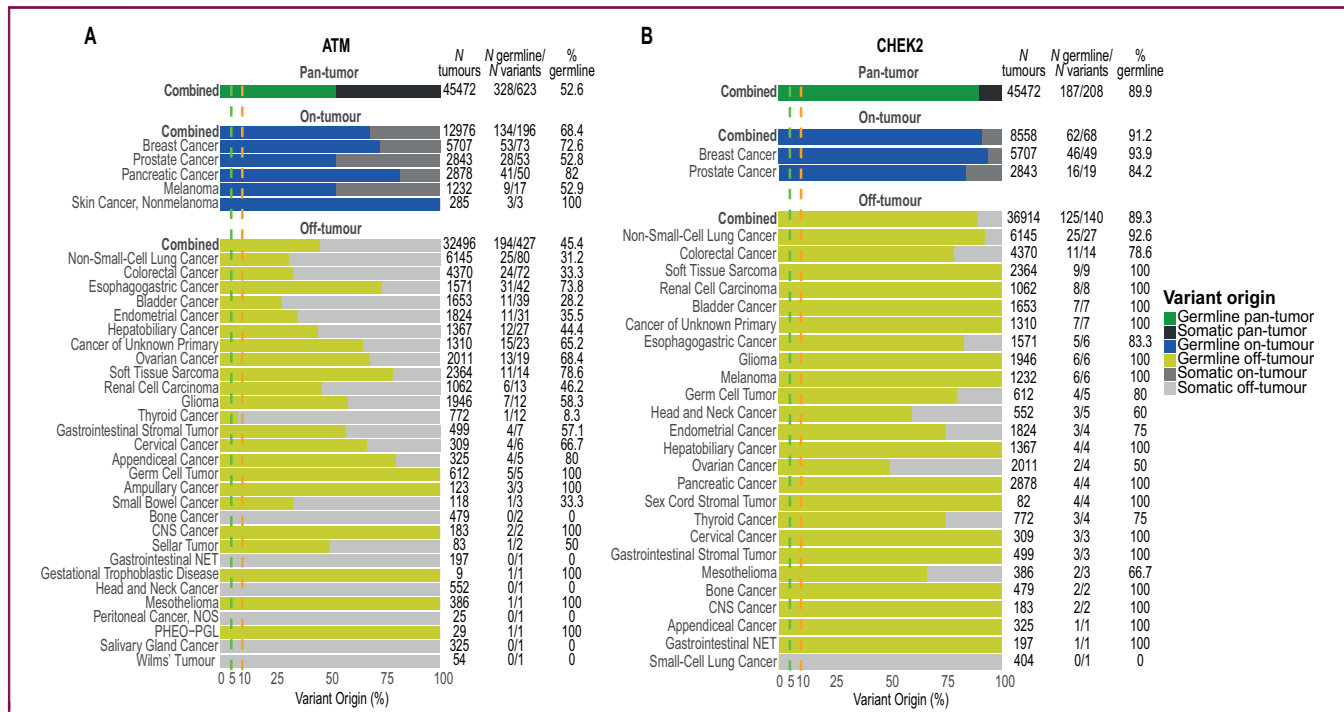


Figure 4. Proportion of tumour-detected pathogenic variants of germline versus somatic origin for individual, grouped tumour types for *ATM* and *CHEK2*. Stacked bar plots showing the proportion of tumour-detected pathogenic variants that are of germline origin (green bars for pan-tumour, blue bars for on-tumour and yellow bars for off-tumour) versus those of somatic origin (black bars for pan-tumour, dark grey bars for on-tumour and light grey bars for off-tumour) in the combined sample series (49k series), comprising 45 472 nonhypermutated tumours across 47 cancer types. Data are shown for 34 and 27 tumour types for (A) *ATM* and (B) *CHEK2*, respectively. The adjacent table provides the number of tumours, variant counts and germline proportions. Tumour types with no qualifying variants are not presented individually but are included in the combined number of tumours. ‘Pan-tumour’ means across all tumour types combined, ‘on-tumour’ means across just the tumour types for which that gene is associated, ‘off-tumour’ means across just the tumour types for which that gene is not associated. CNS, central nervous system; *N* germline, number of tumour-detected variants of germline origin; NET, neuroendocrine tumour; NOS, not otherwise specified; *N* tumour, number of tumours; *N* variants, total number of tumour-detected variants; PHEO-PGL, pheochromocytoma/paraganglioma.

472 in nonhypermutated), *MSH2* (86/3393 versus 22/45 472), *MSH6* (67/3393 versus 43/45 472) and to a lesser extent *PMS2* (13/3393 versus 41/45 472).

Clinical impact of different strategies on on-tumour/off-tumour testing

An area in which there remains wide variation in practice in germline-focused analysis and follow-up is the tumour context, that is, the on-tumour versus off-tumour status of the gene. The ESMO PMWG thus considered four levels of ‘clinical conservatism’ by which filtered tumour-detected variants in the 40 genes in Box 1 may or may not be included for germline follow-up (Tables 1 and 2):

- Permissive:** germline follow-up for all 40 genes in all tumour types.
- Intermediate-permissive:** germline follow-up for all 23 MA-CSGs/HA-CSGs in all tumour types but germline follow-up only in ‘associated’ tumour types for 17 SA-CSGs.
- Intermediate-conservative:** germline follow-up in all tumour types for the 7 MA-CSGs but germline follow-up only in ‘associated’ tumour types for the other 33 HA-CSGs/SA-CSGs.
- Conservative:** germline follow-up only in ‘associated’ tumour types for all 40 genes.

The approach for which the global GCR was highest was strategy C (intermediate-conservative) at 60.3%, while incurring germline follow-up in just 6.7% of tumours. Furthermore, this strategy yields the highest proportion of MA-CSG germline variants (76.5%). By comparison, the most permissive strategy (A) incurs germline follow-up of 11.3% of tumours (almost double), but affords detection of 824 additional variants (all HA and SA) compared with C. In the most conservative approach, germline follow-up can be reduced to 5.3% of tumours, but overall only about half as many germline variants are identified (1334) compared with 2646 in the most permissive strategy (A).

DISCUSSION

We present here paired tumour-normal data on 49 264 tumours, a dataset threefold larger than used in our 2018/19 analysis, which was the largest dataset published to date on germline-focused tumour analysis.²⁴ The new analysis has provided the greater power required to quantify patterns of tumour-detected germline variants in less frequently mutated genes such as *DICER1*, *PTCH1* and *SMARCA4*, and to undertake analyses restricted by individual tumour type and subtype, as well as age-restricted patient groups.

A limitation of these analyses is that our summary findings are necessarily predicated on the tumour type

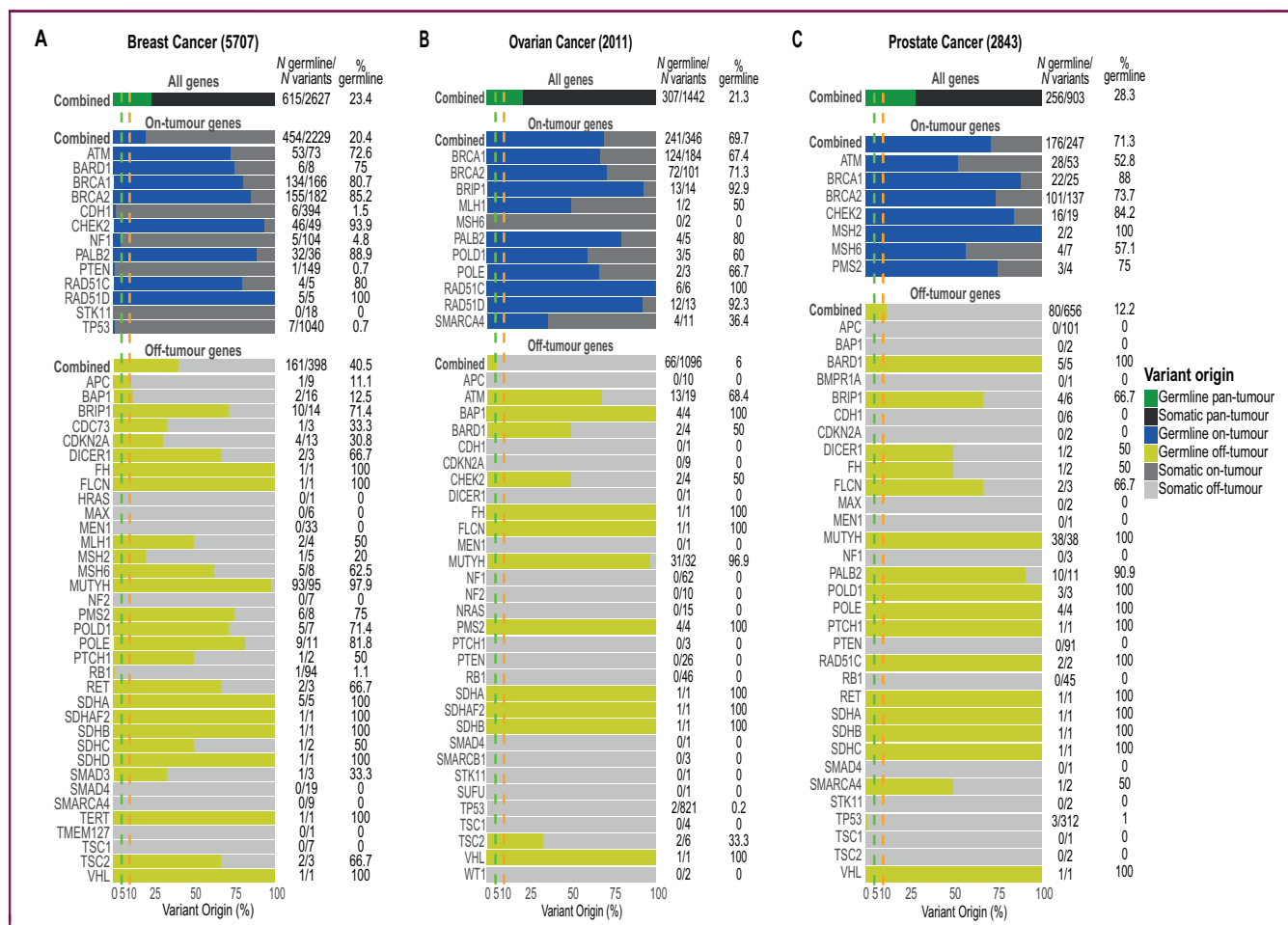


Figure 5. Proportion of tumour-detected pathogenic variants of germline versus somatic origin in breast, ovarian and prostate cancers. Stacked bar plots showing the proportion of tumour-detected pathogenic variants that are of germline origin (green bars for pan-tumour, blue bars for on-tumour and yellow bars for off-tumour) versus those of somatic origin (black bars for pan-tumour, dark grey bars for on-tumour and light grey bars for off-tumour) in the combined sample series (49k series), comprising 45 472 nonhypermutated tumours across 47 cancer types. Data are shown by gene association groups and per gene, for 48, 42 and 39 'actionable' cancer-susceptibility genes for (A) breast, (B) ovarian and (C) prostate cancers, respectively. The adjacent table provides the variant counts and germline proportions. 'Pan-tumour' means across all tumour types combined, 'on-tumour' means across just the tumour types for which that gene is associated, 'off-tumour' means across just the tumour types for which that gene is not associated. N germline, number of tumour-detected variants of germline origin; N variants, total number of tumour-detected variants.

constitution of the MSK dataset, which does not fully reflect incident cancers but also the influences of referral, accessibility for biopsy, tissue availability and engagement in sequencing for precision oncology. However, overall, the replication of per-gene GCRs between the 17k and 32k series indicates broad reproducibility of constituent tumours between the two partitions of MSK data, which thus may also be reflective of other oncology centres. Furthermore, the VAF filters by which balanced accuracy is best optimised between false-positives and false-negatives may vary according to sequencing coverage and quality control between different workflows. In addition, the constituency of the analysed gene panel is important: where the totality of genes in Box 1 are not included for tumour analysis, the overall rate of patients for germline follow-up will be lower.

As well as enlarging the series to improve power, we updated aspects of our analyses compared with the 2018/19 ESMO PMWG guidance. For example, we included additional genes such as *MAX*, a recently identified pheochromocytoma-susceptibility gene that has been

added to the ACMG secondary-findings gene set.^{12,25} We have also included in our analyses *BARD1*, for which recent large-scale breast cancer analyses have established a firm association with triple-negative breast cancer.^{26,27} The intermediate penetrance genes *ATM* and *CHEK2* are now more widely included in breast cancer-susceptibility testing; accordingly, we have now included these in our analyses as SA genes.²⁸

There have also been significant changes in variant interpretation protocols since 2019, with inception of guidance from ClinVar Variant Curation Expert Panels (VCEPs) resulting in significant variant down-classifications in genes such as *TP53*, which has impacted ClinVar germline pathogenic variant rate, thus reducing GCR.^{29,30}

We also sought to explore the impact of reducing to 5% the per-gene GCR threshold by which a gene is included for germline follow-up. Overall, because for most genes the GCR is either high (>50%) or very low (<5%), changing the per-gene GCR threshold for germline follow-up from 10% to 5% (i) resulted in only slight reduction in global GCR and (ii)

Box 1. Recommendations for genes for inclusion for germline-focused analysis and follow-up				
CSG actionability class	All ages			Age <30
Most	BRCA1 BRCA2	MLH1 MSH2	MSH6 PALB2 RET	
High	BRIP1 MUTYH ^c PMS2 RAD51C	RAD51D SDHAF2 ^d SDHB SDHC ^d	SDHD ^d TMEM127 ^d TSC2 ^f VHL ^a	APC PTEN ^{d,f} RB1 TP53 ^{b,f}
Standard	ATM BAP1 ^f BARD1 CHEK2 DICER1	FH FLCN NF1 ^f PTCH1 ^e POLD1	POLE SDHA SMAD3 ^e SMARCB1 ^{e,f} SUFU ^e	CDKN2A SMARCA4

- Germline-focused tumour analysis should be undertaken in all laboratories offering routine analysis of large tumour panels that contain cancer-susceptibility genes.
- Germline-focused analysis/follow-up is recommended for these 40 genes, on the basis of an observed per-gene GCR of $\geq 5\%$ pan-tumour and/or on-tumour. For six of the genes, germline-focused analysis/follow-up should be restricted to tumours arising in age <30.
- Filtered tumour-detected variants comprise those of (i) minor allele frequency <0.01 and (ii) predicted to result in protein truncation and/or classified as pathogenic/likely pathogenic (e.g. in ClinVar, ≥ 1 star) and (iii) of tumour-observed VAF >30% (SNVs) or >20% (small insertions/deletions). Local validation may be required to establish equivalent tumour VAF thresholds, especially for PCR-based NGS methodologies.
- Tumours exhibiting hypermutation (i.e. with microsatellite instability or high tumour mutational burden) should be equivalently included for germline-focused analysis and follow-up.
- Germline follow-up should be restricted to the 'on-tumour' context (i.e. in associated tumours only) for the genes for which risk and efficacy of interventions are less well established (see Table 1 for four strategies).
- Recessively acting 'high-actionability CSGs' (currently *MUTYH* alone) should be included for germline-focused tumour analysis but reporting and germline follow-up testing should be undertaken only on detection of two pathogenic variants.
- Germline-focused tumour analysis can be delivered via an automated pipeline. Formal variant classification using the ACMG framework is only required for the filtered tumour-detected variants for which germline follow-up is indicated [i.e. variants predicted to result in protein truncation and/or classified as pathogenic/likely pathogenic (e.g. in ClinVar, ≥ 1 star) for genes listed in Box 1]. This should be undertaken by an experienced clinical scientist prior to initiation of germline follow-up (patient recontact and/or germline testing).
- Prior to acquisition and analysis of their germline sample, patients should be appraised of the implications of germline testing with appropriate documentation of consent.
- The tumour-detected variant should be analysed in an appropriate germline sample (lymphocytes, saliva/buccal swab, normal tissue) in a laboratory accredited for germline analysis.
- Because of tumour fall-out of a small proportion of germline variants and inadequate analysis for dosage abnormalities (exon-level deletions/duplications in particular), a normal/negative germline-focused analysis of tumour-only sequencing should not be taken as equivalent to a normal/negative full clinical analysis of a germline sample.
- Re-evaluation of these recommendations should be undertaken at least every 2 years.

Data are derived from 45 472 nonhypermutated tumours across 47 cancer types. 'On-tumour' means those tumours for which that gene is judged to confer susceptibility. 'Pan-tumour' means all tumours, regardless of individual gene–tumour associations.

ACMG, American College of Medical Genetics and Genomics; CSG, cancer-susceptibility gene; GCR, germline conversion rate; HA-CSG, high-actionability cancer-susceptibility gene; NGS, next-generation sequencing; SA-CSG, standard-actionability cancer-susceptibility gene; SNV, single-nucleotide variant; VAF, variant allele frequency.

^aRenal tumours may be excluded from germline follow-up for *VHL* (very low GCR).

^bBrain tumours may be excluded from germline follow-up for *TP53* (very low GCR).

^c*MUTYH* should be included for germline-focused analysis but reporting and germline follow-up testing should only be performed on detection of two (biallelic) pathogenic variants.

^dFor five HA-CSGs (*PTEN*, *SDHAF2*, *SDHC*, *SDHD* and *TMEM127*), ≥ 3 variants are observed pan-tumour but not on-tumour, and thus should only be included for germline follow-up under the (A) most permissive approach and the (B) intermediate-permissive approach.

^eFor four SA-CSGs (*PTCH1*, *SMAD3*, *SMARCB1* and *SUFU*), ≥ 3 variants are observed pan-tumour but not on-tumour, and thus should only be included for germline follow-up under the (A) most permissive approach.

^fIf applying GCR threshold of 10%, recommendations for *PTEN*, *TP53*, *TSC2*, *BAP1*, *NF1* and *SMARCB1* may be altered (depending on the selected strategy A–D). These genes are also presented in bold characters.

yielded only a modest uplift in true germline variants detected (Table 1). As global GCR remained >50% across the four levels of conservatism examined, reduction to 5% for per-gene GCR threshold was deemed appropriate by the ESMO PMWG, being commensurate with general expansion testing thresholds. The 40 genes attaining GCR $\geq 5\%$ are summarised in Box 1, with all-age germline follow-up of filtered tumour-detected variants recommended for 34 genes and only for tumours arising age <30 years for 6 genes.

There is considerable contention as to whether the follow-up of a putative germline variant should be predicated on the tumour type in which it is detected. When routine germline testing is performed through clinical genetics services in the context of cancer phenotype and/or

family history, it is standard practice to report only a panel of genes associated with that cancer type (even where additional molecular data on other genes have been generated by the chosen assay). It has thus been argued as incongruous to offer germline follow-up for a broader set of genes in the context of a tumour analysis primarily undertaken for therapeutic rather than familial indications. Conversely, it is argued that any gene result providing information about future cancer risk or familial risk would be of interest to a cancer patient, who would not themselves ascribe importance to whether the gene result was deemed to have contributed to the aetiology of their current cancer.

For many genes our understanding of associated cancer risks is based on study of probands ascertained with the

Table 1. Summary of germline conversion rates for different germline follow-up recommendations

Parameter	(A) Permissive approach: Germline follow-up pan-tumour for all genes		(B) Intermediate-permissive approach: Germline follow-up pan-tumour for MA-CSG/HA-CSG and on-tumour for SA-CSG		(C) Intermediate-conservative approach: Germline follow-up pan-tumour for seven MA-CSGs (BRCA1, BRCA2, MLH1, MSH2, MSH6, PALB2 and RET) and on-tumour for other genes		(D) Conservative approach: Germline follow-up on-tumour only for all genes	
	10% threshold	5% threshold	10% threshold	5% threshold	10% threshold	5% threshold	10% threshold	5% threshold
Tumour-detected variants, <i>n</i>	4451	5120	3124	3471	2661	3024	2039	2402
True germline variants, <i>n</i>	2602	2646	2043	2068	1794	1822	1306	1334
Overall germline conversion rate, %	58.5	51.7	65.4	59.6	67.4	60.3	64.1	55.5
Germline variants of actionability class, <i>n</i> (%)	1394 (53.6)	1394 (52.7)	1394 (68.2)	1394 (67.4)	1394 (77.7)	1394 (76.5)	906 (69.4)	906 (67.9)
MA-CSG	356 (13.7)	368 (13.9)	356 (17.4)	368 (17.8)	107 (6.0)	122 (6.7)	107 (8.2)	122 (9.1)
HA-CSG	852 (32.7)	884 (33.4)	293 (14.3)	306 (14.8)	293 (16.3)	306 (16.8)	293 (22.4)	306 (22.9)
SA-CSG	9.8	11.3	6.9	7.6	5.9	6.7	4.5	5.3
Proportion of tumours in which a filtered tumour-detected variant is detected (i.e. germline follow-up triggered), %								

Data are derived from 45 477 nonhypermutated tumours across 47 cancer types. Data are shown for MA-CSGs, HA-CSGs, SA-CSG and all genes combined upon application of recommendations set by a 10% and 5% threshold. 'On-tumour' means germline follow-up is only undertaken in tumours for which that gene is judged to confer susceptibility (i.e. associated). 'Pan-tumour' means all tumours, regardless of whether the gene is judged to confer germline susceptibility. *MUTYH* variants are excluded from this analysis as these would only trigger germline follow-up in the (very infrequent) biallelic context.

HA-CSG, high-actionability cancer-susceptibility gene; MA-CSG, most-actionability cancer-susceptibility gene; SA-CSG, standard-actionability cancer-susceptibility gene.

relevant cancers, and risk estimates for individuals ascertained agnostic to phenotype are not well established. For example for *CDH1*, the penetrance (risk) data used clinically are derived from studies of probands/families with multiple and/or young-onset diffuse gastric cancer. In unaffected members of the family carrying the familial pathogenic variant, it is typical to advocate prophylactic gastrectomy, which results in significant morbidity. For a pathogenic variant ascertained as an 'incidental finding' in a member of the population, the penetrance for disease and consequent best management are much less certain.³¹ This variation in penetrance is largely ascribed to the concurrent distribution of other genetic modifier factors influencing risk. The ACMG maintains a list of 'HA genes' for which they advise return of pathogenic variants in the context of secondary ('additional' or 'incidental') findings.^{12,13} However, for the 28 CSGs currently included on the ACMG secondary findings list, there is wide variability in the robustness of data on penetrance and efficacy of clinical interventions. Hence, the CGEG delineated a subset of seven genes which we termed 'most actionable' in the context of germline follow-up of tumour-only sequencing (*BRCA1/BRCA2/MLH1/MSH2/MSH6/PALB2/RET*). First, there are better data for the overall penetrance of these seven genes in broader ascertainment contexts. Second efficacy of interventions for prevention/early detection are better proven, and third (importantly in this context) the off-tumour GCR is relatively high.⁷

We present detailed data underpinning strategies A–D as we recognise that the approach to germline follow-up of tumour-only sequencing adopted by a country/institution will inevitably be consistent with their wider practice of genomic 'conservatism'. In countries/institutions in which return of secondary findings is more cautious (e.g. UK and much of Europe), we recommend strategy C in which for seven key genes, germline follow-up is undertaken in all tumour types; otherwise germline follow-up is restricted to genes associated with the tumour. Strategy B is likely more consistent for countries/institutions in which reporting of long lists of 'ACMG secondary findings genes' is standard (e.g. USA); hence germline follow-up would be undertaken in any tumour type for all 23 MA-CSGs/HA-CSGs.

Uncertainty in how to manage tumour-detected variants of putative germline origin can result in unnecessary and excessive referral to clinical genetics. This can cause delays, patient anxiety and unnecessary use of clinical genetics resources. Conversely, where laboratories and services fail to undertake appropriate germline-focused analysis of tumour-only sequencing, important actionable germline findings are missed. An automated pipeline can be used for germline-focused tumour analysis, but we recommend the use of the ACMG framework for the filtered tumour-detected variants for which germline follow-up is indicated. Collaborative system design is required to optimise interaction between the oncology service, clinical genetics, the molecular tumour laboratory and germline genetics laboratory.^{32,33}

Table 2. Summary of germline conversion rates on-tumour and off-tumour

CSG actionability class	Tumour setting	Parameter	Recommendations 10% threshold	Recommendations 5% threshold
Most, n (%)	On	Tumour-detected variants	1186	1186
		True germline variants	906 (76.4%)	906 (76.4%)
	Off	Tumour-detected variants	622	622
		True germline variants	488 (78.5%)	488 (78.5%)
High, n (%)	On	Tumour-detected variants	219	425
		True germline variants	107 (48.9%)	122 (28.7%)
	Off	Tumour-detected variants	463	447
		True germline variants	249 (53.8%)	246 (55.0%)
Standard, n (%)	On	Tumour-detected variants	634	791
		True germline variants	293 (46.2%)	306 (38.7%)
	Off	Tumour-detected variants	1327	1649
		True germline variants	559 (42.1%)	578 (35.1%)

Data are derived from 45 472 nonhypermethylated tumours across 47 cancer types. Data are shown for most, high- and standard-actionability CSGs upon application of recommendations set by a 10% and 5% threshold. 'On-tumour' means across just the tumour types for which genes are associated, 'off-tumour' means across just the tumour types for which genes are not associated. *MUTYH* variants are excluded from this analysis as these would only trigger germline follow-up in the (very infrequent) biallelic context. CSG, cancer-susceptibility gene.

We thus present the largest germline-focused analyses to date using paired tumour-normal sequencing of 49 264 tumours. Alongside we present updated recommendations from the ESMO PMWG around the genes in which germline-focused analysis should be carried out, and the findings and contexts for which germline-follow-up is recommended. We believe these recommendations provide pragmatic approaches, maximising the yield of true germline findings of high clinical actionability without causing undue diversion for excessive numbers of patients.

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DISCLOSURE

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