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Original research

Opportunistic genetic screening increases the diagnostic yield and is medically valuable for care of patients and their relatives with hereditary cancer

Sara Fernández-Castillejo ,[1] Bàrbara Roig,[1] Mireia Melé,[1] Sara Serrano,[1] Mònica Salvat,[1] Montserrat Querol,[1] Joan Brunet ,[2,3] Marta Pineda ,[2] Adela Cisneros,[4] David Parada,[5] Joan Badia,[1] Joan Borràs,[1] Marta Rodríguez-Balada ,[1] Josep Gumà[1]

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For numbered affiliations see end of article.

Correspondence to
Dr Marta Rodríguez-Balada, IISPV, Reus, 43204, Spain; marta.rodriguez@salutsantjoan.cat

SF-C and BR contributed equally.

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ABSTRACT

Background Multigene panel testing by next-generation sequencing (MGP-NGS) enables the detection of germline pathogenic or likely pathogenic variants (PVs/LPVs) in genes beyond those associated with a certain cancer phenotype. Opportunistic genetic screening based on MGP-NGS in patients with suspicion of hereditary cancer reveals these incidental findings (IFs).

Methods MGP-NGS was performed in patients who fulfilled the clinical criteria to undergo genetic testing according to the Catalan Health Service guidelines. Variants were classified following the American College of Medical Genetics and Genomics-Association for Molecular Pathology guidelines and the Cancer Variant Interpretation Group UK guidelines.

Results IFs were identified in 10 (1.22%) of the 817 patients who underwent MGP-NGS. The mean age at cancer diagnosis was 49.4±9.5 years. Three IFs (30.0%) were detected in *PMS2*, two (20.0%) in *ATM* and *TP53* and one (10.0%) in *MSH6*, *NTHL1* and *VHL*. Seven (70.0%) IFs were single-nucleotide substitutions, two (20.0%) were deletions and one (10.0%) was a duplication. Three (30.0) IFs were located in intronic regions, three (30.3%) were nonsense, two (20.0%) were frameshift and two (20.0%) were missense variations. Six (60.0%) IFs were classified as PVs and four (40.0%) as LPVs.

Conclusions Opportunistic genetic screening increased the diagnostic yield by 1.22% in our cohort. Most of the identified IFs were present in clinically actionable genes (n=7; 70.0%), providing these families with an opportunity to join cancer early detection programmes, as well as secondary cancer prevention. IFs might facilitate the diagnosis of asymptomatic individuals and the early management of cancer once it develops.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Hereditary cancer syndromes are caused by inherited alterations in >200 cancer-related genes, most of which are involved in cell cycle control and DNA repair; however, in only 5%–10% of cases with a hereditary cancer suspicion, the disease-causing alteration is identified.

⇒ Opportunistic genetic screening through multigene panel testing by next-generation sequencing (MGP-NGS) can unveil unexpected pathogenic or likely pathogenic variants (PVs/LPVs) initially inconsistent with the personal and familial cancer history of the patient.

WHAT THIS STUDY ADDS

⇒ Our results confirm that MGP-NGS performed on patients with personal and/or familial histories of cancer uncovers genetic alterations inconsistent with personal and familial history of cancer, known as incidental findings.

⇒ Interestingly, we report that opportunistic genetic screening increases the diagnostic yield by 1.22% in our cohort.

⇒ In addition, 70% of the identified incidental findings in this study are present in clinically actionable genes.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The identification of incidental findings in clinically actionable genes can provide clinical benefits for patients harbouring the variant and their relatives.

⇒ Patients can benefit from personalised oncological treatments.

⇒ In the case of asymptomatic individuals, opportunistic genetic screening could yield the discovery of an as-yet clinically unrecognised disorder for its early management.

⇒ Finally, opportunistic genetic screening provides an opportunity to join cancer early detection programmes as well as to secondary cancer prevention to those healthy relatives harbouring the alteration.

INTRODUCTION

The development of next-generation sequencing (NGS) marks a turning point in the genetic diagnosis field. Compared with the gold standard technique for mutation analysis in cancer diagnosis, the Sanger sequencing method, NGS can sequence a large number of DNA regions while being time-effective and cost-effective at the same time. NGS

offers considerable benefits in clinical settings since it allows for molecular characterisation of rare diseases, individualisation of oncological treatments and population screening for disease risk, among other abilities. Moreover, these cutting-edge technologies have significantly improved assay sensitivity and enabled multigene panel (MGP) testing, that is, simultaneous sequencing of multiple genes.[1]

More than 200 genes have been associated with hereditary cancer syndromes, most of which are implicated in cell cycle control and DNA repair. However, only in 5%–10% of patients with clinical suspicion of hereditary cancer is the disease-causing variant identified.[2] Most MGPs undertaken in these patients are phenotype-driven since they include those genes associated with a certain cancer phenotype, while some of them also comprise additional genes associated with increased hereditary cancer risk. In this sense, opportunistic genetic screening through MGP-NGS testing can unveil unexpected pathogenic or likely pathogenic variants (PVs/LPVs), known as incidental findings (IFs). IFs have been defined by the American College of Medical Genetics and Genomics (ACMG) as the results of a deliberate search for PVs/LPVs in genes that are not apparently relevant to a diagnostic indication for which the sequencing test was ordered but that could nonetheless be medically valuable to the patient and the ordering clinician.[3]

The identification of germline IFs might have considerable implications for the clinician, the patient and relatives.[4–6] When a PV/LPV is identified in clinically actionable genes (online supplemental table S1), the patient can benefit from personalised treatment selection and monitoring programmes. Therefore, the role of IF in managing the patients' and their relatives' health and in correctly assessing the risks of developing pathologies is of paramount importance. However, some potential drawbacks could emerge from IFs discovery, especially in patients with no familial history of hereditary cancer. The ACMG has recently published an update of its policy statement providing recommendations for reporting IFs in clinical contexts. Despite adherence to this statement being voluntary, these recommendations provide high-quality clinical and laboratory genetic services.[7]

In the present work, we aimed to evaluate the presence of PVs/LPVs in genes that are not related to the primary clinical indications for MGP-NGS ordering through an opportunistic genetic screening approach.

MATERIALS AND METHODS

Participants and DNA obtaining

In this population-based, retrospective chart review, patients were selected from the Oncology Institute of South Catalonia (IOCS). These patients underwent genetic testing according to the Catalan Health Service guidelines (table 1, online supplemental appendix S1).[8] DNA was extracted from peripheral blood lymphocytes using the Gentra PureGene DNA Isolation Kit (Qiagen).

Next-generation sequencing

MGP-NGS library preparation was based on the Imegen Hereditary OncoKitDx kit (Health In Code), which targets coding exons and 20 bp of the flanking intronic regions of 50 genes (185.56 kb) relevant for hereditary cancer syndromes (online supplemental table S2). Products were analysed by NGS using the Illumina Platform MiSeq. Data were analysed with the Datagenomics platform (Health In Code). Only target regions with a minimum depth of 20× were considered. Variants were assumed to be of germline origin if found with a variant allele frequency (VAF) ≥20%.

DNA Sanger sequencing

PVs/LPVs detected by NGS were confirmed by Sanger sequencing with BigDye Terminator V.3.1 kit (Life Technologies). Long-range PCR was performed to discard *PMS2* pseudogene contamination.[9] Long-range PCR products were used as the template for nested *PMS2* amplification. In all cases, capillary electrophoresis was conducted on a SeqStudio sequencer (Applied Biosystems) and analysed using Sequencher V.5.0 software (Gene Codes).

Variant classification

The clinical significance of variants was examined following the ACMG and the Association for Molecular Pathology standards and guidelines,[10] the ClinGen Variant Expert Curation Panel specifications for *TP53*, *MMR* genes and *ATM*[11–13] and the Cancer Variant Interpretation Group UK guidelines (CanVIG-UK), following a 5-tier classification system.[14] In silico predictive studies were performed with those tools recommended elsewhere.[10 15] Variant frequencies were analysed through the Genome Aggregation Database (gnomAD) browser and the 1000 Genomes Project. Variants were examined in databases such as ClinVar, OMIM, Leiden Open Variation Database and BRCA Share and by reviewing updated bibliographies. The predicted consequences of splice variants were conducted mainly by Splice AI. The analyses of the consequences of missense variants were examined by REVEL and PRIORS.[16]

Fibroblasts from skin punch biopsy culture

A 5 mm skin biopsy was fragmented under sterile conditions and cultured using the explant technique.[17] DNA from cell culture was extracted manually with the Qiagen Gentra Puregene blood kit (Qiagen).

RESULTS

Participant characteristics

Between July 2020 and July 2022, a total of 817 individuals referred to the Cancer Genetic Counselling Unit of the IOCS met the Catalan Health Service clinical criteria to undergo MGP-NGS testing (table 1 and online supplemental appendix S1). Eighty-six (10.53%) of these patients harboured a PV/LPV in a gene consistently associated with their particular diagnosed cancer (online supplemental appendix S1). IFs were identified in 10 additional subjects (1.22%) and therefore comprised the study population of the present work. Among these 10 subjects, the majority were women (n=9; 90.0%), and the mean age at cancer diagnosis was 49.4±9.5 years. The most prevalent cancer type was breast cancer (BC; MIM:114480; n=7; 53.84%), followed by colorectal (MIM:114500; n=4; 30.77%), ovarian (MIM:167000; n=1; 7.69%) and cervical (MIM:603956; n=1; 7.69%) cancers (see table 2 for summarised information and online supplemental table S3 for complete information).

NGS features, confirmation of the results and general characteristics of the IFs identified

Most of the 10 IFs included in this study were detected in patients referred for hereditary breast and ovarian syndrome suspicion (MIM:PS604370; n=7; 63.63%), followed by Lynch syndrome (MIM:120435; n=4; 30.77%). The IFs identified in our study were located in six different genes: three IFs (30.0%) were detected in *PMS2*, two (20.0%) in *ATM*, two (20.0%) in *TP53* and one (10.0%) in the *MSH6*, *NTHL1* and *VHL* genes.

Table 1 Catalan Health Service clinical criteria to undergo MGP-NGS testing

Cancer phenotype	Clinical criteria for MPG-NGS testing	Genes included in the MPG-NGS panel*
HBOC	► Breast cancer diagnosed in those aged ≤40 years. ► Breast cancer diagnosed in those aged ≤50 years if non-informative family history. ► Triple-negative breast cancer diagnosed in those aged ≤60 years. ► Breast cancer in men. ► ≥3 first-degree relatives affected by breast cancer (at least one diagnosed aged ≤60 years). ► 2 cases of breast cancer diagnosed in those aged ≤50 years. ► Bilateral breast cancer (first diagnosed in those aged ≤50 years). ► Bilateral breast cancer and another breast cancer (one diagnosed aged ≤60 years). ► Metastatic HER2− breast cancer for which a treatment option with PARP inhibitors is considered. ► Invasive non-mucinous epithelial ovarian cancer (in low-grade tumours, it will be individualised according to age, family history and possible benefit to relatives).	*BRCA1, BRCA2, MLH1, MSH2, MSH6, TP53*†, *BARD1, PALB2, CHEK2, ATM, BRIP1, RAD51C, RAD51D, PTEN*‡, *CDH1*‡
Ovarian	► Invasive non-mucinous epithelial ovarian cancer (in low-grade tumours, it will be individualised according to age, family history and possible benefit to relatives) with no cases of breast cancer in the family.	*BRCA1, BRCA2, MLH1, MSH2, MSH6, BRIP1, RAD51C, RAD51D, PALB2*
LS§	► MSI or altered IHC (in the case of MLH1/PMS2 alterations, the presence of MLH1 methylation or mutations in the *BRAF* gene must be excluded) in colorectal or endometrial tumours. ► Colorectal cancer diagnosed in those aged ≤50 years or Amsterdam criteria.¶	*BRCA1, BRCA2, MLH1, MSH2, MSH6, PMS2***, *EPCAM (exons 8–9), MUTYH, POLE (exons 7–14), POLD1 (exons 6–13), TP53*††
Prostate	► Metastatic prostate cancer with Gleason score ≥7. ► Prostate cancer with Gleason score ≥7 and – diagnosed in those aged <55 years or – familial history of breast and/or ovarian cancer or ≥2 cases of prostate cancer in the same family branch. ► Prostate cancer diagnosed in those aged <55 years and familial history of ≥2 cases of prostate cancer or HBOC. ► Prostate cancer with a ductal or intraductal cribriform histological pattern. ► Prostate cancer that does not meet the previous criteria and for which an indication for PARP inhibitors is considered.	*BRCA1, BRCA2, MLH1, MSH2, MSH6, HOXB13 (G84E variant), ATM, CHEK2, PALB2*

Only those cancer phenotypes in which an IF has been found are included in this table. Clinical criteria for additional cancer phenotypes are detailed in the online supplemental appendix S1.
*In all indications, pretest genetic counselling includes offering opportunistic screening for the *BRCA1*, *BRCA2*, *MLH1*, *MSH2* and *MSH6* genes. For this reason, these genes are included in all panels and not only in screens of the related syndromes.
†Only if breast cancer is diagnosed at ≤45 years of age or meets the CHOMPRET criteria.
‡Only if a suggestive phenotype (Cowden syndrome criteria, hereditary diffuse gastric cancer criteria-lobular breast cancer).
§Screening for Lynch syndrome by IHC of DNA mismatch repair proteins and/or MSI analysis should be performed in all colorectal and endometrial cancers.
¶Amsterdam criteria: (Vasen *et al*.[54]). 4 CHOMPRET criteria: (Tinat, *et al*.[31]); (Mai, *et al*.[55]).
**If there is a loss of expression on IHC or in all cases depending on the technique available (guaranteed analytical validity).
††If Chompret criteria or colorectal cancer <50 years of age.
HBOC, hereditary breast and ovarian cancer; HER2, human epidermal growth factor receptor 2; IF, incidental finding; IHC, immunohistochemistry; LS, Lynch syndrome; MPG-NGS, multipanel gene testing by next-generation sequencing; MSI, microsatellite instability; PARP, poly(ADP-ribose) polymerase.

All IFs were identified in heterozygosis except for the variant identified in *NTHL1*. Five IF (50.0%) were classified as PV (see table 2 for summarised information and online supplemental table S3 for complete information).

IF in patients with breast and ovarian cancer

The NM_000546.6:c.743G>A variant in *TP53* was identified in patient 1 diagnosed with BC at 45–50 years (VAF=0.427; see online supplemental figure S1, table 2 for summarised information and online supplemental table S3 for complete information). This SNV in a hotspot region of the *TP53* gene leads to a missense change in the protein (p.(Arg248Gln)).[18] This particular variant was classified by the ClinGen *TP53* Variant Curation Panel as a PV based on their specific classification guidelines (PM1, PP3, PS3, PS4 and PS2).[11] This patient had a confirmed family history of BC in a first-degree relative (FDR) and in a third-degree relative (TDR), who underwent a mastectomy and died in her 30s because of cancer. None of the relatives tested were carriers of this variant. The variant was identified in the buccal swab (VAF=20%) and in tumorous and non-tumorous breast tissues (VAFs≈50%), but not in skin fibroblasts, as confirmed using Minor Variant Finder Software (Applied Biosystems).

The variant NM_000546.6:c.783-1G>A in *TP53* was identified in patient 2 (VAF=0.569), who was diagnosed with bilateral BC (aged 45–50 years), cervical cancer (aged 45–50 years) and colorectal cancer (aged 65–70 years) with conserved immunohistochemistry (IHC) for mismatch repair genes (MMR) (online supplemental figure S2). This variant is a single nucleotide substitution variant (SNV) located in a splicing consensus region of TP53 that results in aberrant transcripts and a truncated protein with compromised function, as experimental studies have confirmed.[19–21] This variant is not found in population databases, but it is reported in the National Cancer Institute of the US TP53 database in Li-Fraumeni syndrome and Li-Fraumeni syndrome-like families (last accessed October 2022). This variant has been classified as LPV according to the ClinGen TP53 Variant Curation Panel specific guidelines (PS4, PP1, PM2 and PP3).[11] Patient 2 had a long familial history of cancer: three FDRs, six second-degree relatives (SDRs) and two TDRs. Cosegregation analysis allowed us to confirm that this LPV in *TP53* has a germline origin.

The variant NM_000179.3:c.762dup in *MSH6* (VAF=0.420) was identified in patient 3, who was diagnosed with BC at the age range of 35–40 years (online supplemental figure S3). This variant is a duplication with a nonsense coding effect

Table 2 Summary of the clinicopathological characteristics of the patients and the IF detected

Patient	Gender	Personal history of cancer (age range (years) at dx)	MGP	Gene (ref sequence) and variant (*HGVS*)	VAF*	Clinical significance†	Actionability‡	Confirmed family history of cancer§
1	F	Breast (45–50)	HBOC	*TP53* (NM_000546.6) c.743G>A	0.427	PV	Yes (a, b)	FDR: 1 (breast dx 70–75)
2	F	Bilateral breast (45–50), cervix (45–50) and colon (65–70)	HBOC	*TP53* (NM_000546.6) c.783-1G>A	0.569	LPV	Yes (a, b)	FDR: 1 (leiomyosarcoma dx 40–50)
3	F	Breast (35–40)	HBOC	*MSH6* (NM_000179.3) c.762dup	0.420	PV	Yes (a, b)	FDR: 1 (breast dx 50–55+endometrial dx 50–55). SDR: 2 (mouth dx 5 5–60 ; colon dx 55–60+prostate dx 75-80)
4	F	Asynchronous breast and bilateral breast cancer (35–40; 45–50)	HBOC	*PMS2* (NM_000535.7 c.24-2A>G	0.513	LPV	Yes (a, b)	FDR: 2 (breast dx 80–85; breast dx 60–65)
5	F	Breast (50–55)	HBOC	*PMS2* (NM_000535.7) c.1579_1580del	0.480	PV	Yes (a, b)	FDR: 1 (colon dx 80–85)
6	F	Ovarian (50–55)	HBOC; LS	*PMS2* (NM_000535.7) c.989-2A>G	0.500	LPV	Yes (a, b)	FDR: 2 (colon dx 50–55; myeloma dx 50–55) SDR: 3 (gastric dx 65–70+prostate dx 65–70; liver dx 50–55; colon dx 65–70) TDR: 3 (ovarian dx 50–55+breast dx 60–65; ovarian dx 50–55; brain dx 30–35)
7	F	Breast (45–50)	HBOC	*VHL* (NM_000551.4) c.341G>A	0.501	LPV	Yes (a, b)	None
8	F	Colon (35–40)	LS	*ATM* (NM_000051.4) c.5908C>T	0.448	PV	No	FDR: 3 (breast dx 50–55; bladder dx 60–65+lung dx 70–75; myeloma dx 60–65) SDR: 4 (colon dx 70–75; prostate dx 65–70; breast dx 45–50; ovarian dx 70–75) Forth-DR: 1 (breast dx 25–30)
9	F	Colon (65–70)	LS	*ATM* (NM_000051.4) c.7670_7674del	0.442	LPV	No	FDR: 2 (colon dx 85–90; gastric dx 70–75). SDR: 3 (gastric dx 75–80; colon dx 75–80+gastric dx 80–85; colon dx 70–75)
10	M	Colon (45–50)	LS	*NTHL1* (NM_002528.7) c.244C>T	0.997	PV	No	SDR: 1 (gastrointestinal dx 45–50)

See online supplemental table S3 for complete information.
*Population allele frequency obtained from the gnomAD browser (V.2.1.1) irrespective of geographical origin.
†Clinical significance as listed in ClinVar, OMIM, Leiden Open Database and BRCA Share databases and categorised according to the ACMG-AMP and CanVIG-UK guidelines.
‡Actionability according to ACMG (a) and/or FSPPM (b).
§Additional unconfirmed cases of cancer might be present. See details in online supplemental data.
ACMG-AMP, American College of Medical Genetics-Association for Molecular Pathology; CanVIG-UK, Cancer Variant Interpretation Group UK guidelines; dx, diagnosis; F, female; FDR, first-degree relative; FSPPM, French Society of Predictive and Personalised Medicine; gnomAD, Genome Aggregation Database; HBOC, hereditary breast and ovarian cancer syndrome; HGVS, Human Genome Variation Society; LPV, likely pathogenic variant; LS, Lynch syndrome; M, male; MGP, multigene panel testing; PV, pathogenic variant; SDR, second-degree relative; SNV, single nucleotide substitution variant; TDR, third-degree relative; VAF, variant allele frequency.

(p.(Glu255*)), surely resulting in the loss of function of the MSH6 protein by premature protein truncation or nonsense-mediated mRNA decay (NMD). The variant is not found in the gnomAD database, and some authors have reported this variant as pathogenic in ClinVar. This variant has been classified as a PV according to the ClinGen ISiGHT Hereditary Colorectal Cancel/polyposis Variant Curation Expert Panel-specific classification guidelines (PVS1, PP5 and PM2).[12] The patient had a confirmed family history of breast and uterine cancer in an FDR (subject IV:2, both diagnosed at age 50–55 years, respectively) and two SDRs who suffered from mouth cancer (subject IV:8, aged 55–60 years) and both colorectal (aged 55–60 years) and prostate (aged 75–80 years) cancers (subject III:1). Moreover, posterior IHC studies performed in the endometrial tumorous tissue of subject IV:2 confirmed the lack of expression of MSH6.

The variant NM_000535.7:c.24-2A>G in *PMS2* was found in patient 4 (VAF=0.513; online supplemental figure S4), a woman diagnosed with asynchronous breast and bilateral BC (at age 35–40 and 45–50 years) and with a confirmed familial history of BC in two FDRs (subjects III:12 and II:10, diagnosed at age 60–65 and 80–85 years, respectively, the latter deceased from BC) and in an SDR (III:5, diagnosed at age 60–65 years), as detailed in online supplemental figure S4. IHC studies showed that the expression of MMR proteins was conserved in the

tumorous tissue. This intronic variant is an SNV of two nucleotides upstream from coding exon 2 in the *PMS2* gene. Despite the lack of direct evidence, splice site prediction tools predict that this variant abolishes the canonical splice acceptor site. Thus, it is expected to result in aberrant transcripts subject to NMD. However, further analyses are warranted to discern whether this variant impairs the normal splicing process and compromises protein function. Moreover, this variant is not described in population databases (gnomAD). Therefore, it is classified as an LPV according to the ClinGen ISiGHT Hereditary Colorectal Cancel/Polyposis Variant Curation Expert Panel-specific classification guidelines (PVS1 and PM2).[12]

The variant NM_000535.7:c.1579_1580del in *PMS2* was found in patient 5 (VAF=0.480; online supplemental figure S5), diagnosed with BC at age 50–55 years. IHC studies confirmed the lack of PMS2 expression in breast tumorous tissue. This two-nucleotide deletion presumably results in a frameshift coding effect (p.(Arg527Glyfs*14)) and may create a stop codon producing a disrupted or absent protein by NMD, but no functional evidence is reported. Loss-of-function variants in *PMS2* are a known mechanism of disease.[22 23] However, no functional evidence of this particular variant has been published to date. This variant has been previously found in subjects with HBOC and in population databases at a very low frequency.[24 25] Therefore, this variant is classified as a PV according to the ClinGen ISiGHT Hereditary Colorectal Cancel/Polyposis Variant Curation Expert Panel-specific classification guidelines (PM2 and PP5).[12] No blood or tissue samples of relatives were available to perform further studies.

The variant NM_000535.7:c.989-2A>G in *PMS2* (VAF=0.500) was identified in a woman affected by ovarian cancer diagnosed at age 50–55 years (patient 6; online supplemental figure S6), who underwent HBOC and Lynch syndrome MGPs-NGS. The NM_000535.7:c.989-2A>G variant in *PMS2* is an intronic SNV located two nucleotides upstream from coding exon 10 in *PMS2*, affecting an acceptor site in intron 9. This particular variant is not found in population databases (gnomAD). This variant is classified as an LPV according to the ClinGen ISiGHT Hereditary Colorectal Cancel/polyposis Variant Curation Expert Panel-specific classification guidelines (PM2 and PP5).[12] The proband had a long familial history of cancer, particularly on the paternal side. Two FDRs were affected by colorectal cancer and myeloma; four SDRs by liver, prostate, colon and gastric cancers and three TDRs by brain, ovarian and both ovarian and BC. Most of these relatives died of the oncological process. We could perform carrier studies, as well as access the clinical records, of the IHC of some of the relatives. In this sense, the IHC of subject II:2 showed that tumorous colon tissue had conserved expression of MMR proteins. In contrast, there was a lack of expression of the proteins PMS2 and MSH6 in the ovarian tissue of subject III:1. Genetic studies of subject III:10 showed that this individual harboured the same variant in *PMS2*, but no IHC analysis of this patient has been accessible. In an attempt to discern the familial origin of the variant in *PMS2*, carrier studies of available blood samples were performed. Subjects III:5 and III:3 did not harbour this variant. Interestingly, subject III:6 was diagnosed with ovarian and BC, but genetic studies showed that she carried a PV in *BRCA2* inherited from the maternal side. The ovarian IHC for the MMR proteins of subject III:6 was normal. We cannot assure the maternal or paternal origin of the variant identified in patient 6, as it was not detected in the paternal side and genetic studies on the maternal side were not possible due to the lack of contact of the proband with her maternal family. The paternal family history of cancer may lead us to think that the variant NM_000535.7:c.989-2A>G in *PMS2* was inherited from the paternal side, but we cannot guarantee it. Taking this into consideration, we can neither assure nor refuse that subjects II:2 and III:1 are carriers of the same variant in *PMS2*, as discussed in the 'Discussion' section.

The variant NM_000551.4:c.341G>A in *VHL* was found in patient 7 (VAF=0.501) (online supplemental figure S7), a woman diagnosed with BC at the age of 45–50 years. This SNV leads to a missense coding effect, resulting in a change in the amino acid in the *VHL* protein (p.(Gly114Asp)). To our knowledge, this variant has not been previously reported or described in gnomAD. This variant is located in a hotspot region where 25 PVs/LPVs, 8 variants of uncertain significance and no benign variants have been described. In addition, four pathogenic alternative changes have been reported in the same residue. The in silico tool used (Revel) predicts altered function of the VHL protein, but no functional studies have been performed to confirm it. Therefore, this variant is classified as an LPV according to the ACMG and CanVIG-UK guidelines (PM1, PM5, PP3, PM2).[10 22] Carrier studies performed in available specimens showed that subject II:4 also harboured this variant, but no cancer was diagnosed in this subject despite being aged 90–95 years. One can assume that this variant has probably a low penetrance but follow-up tests recommended to this patient showed that abdominal echography was normal, no retinal hemangioblastoma was detected and normal values of blood metanephrines were found, endorsing the low penetrance of this variant.

IFs in patients with colorectal cancer

The variant NM_000051.4:c.5908C>T in *ATM* was found in patient 8, diagnosed with colorectal cancer at age 35–40 years (VAF=0.448) (see online supplemental figure S8, table 2 for summarised information and online supplemental table S3 for complete information). Microsatellite instability analysis showed no evidence of MMR deficiency. This SNV in exon 39 has been predicted to have a nonsense coding effect (p.(Gln1970*)). The predicted creation of a premature stop codon could result in a truncated or absent ATM protein due to NMD. This variant has a low frequency in gnomAD and has been classified as a PV according to the ClinGen Hereditary Breast, Ovarian and Pancreatic Cancer Expert Panel (PVS1, PP5 and PM2).[10 13] On the maternal side of patient 8, a large deletion of exon 14 of *BRCA1* was identified in several relatives, but the ATM variant was absent on this maternal side. On the paternal side, the proband had one FRD diagnosed with bladder and lung cancers and one SDR diagnosed with colorectal cancer. According to this long history of familial cancer, the proband underwent HBOC MGP-NGS analysis, but no additional PVs/LPVs were identified. Carrier studies confirmed that the proband did not harbour the deletion in *BRCA1*. No conclusive results were obtained in the cosegregation studies performed.

The variant NM_000051.4:c.7670_7674del was identified in *ATM* (VAF=0.442) in patient 9 diagnosed with colorectal cancer at age 65–70 years (online supplemental figure S9). This deletion in exon 52 leads to a frameshift coding effect (p.(Leu2557Tyrfs*12)), in turn, resulting in the creation of a premature stop codon, and it is predicted that it causes a loss of function of the ATM protein. In addition, this variant is not reported in gnomAD and it is classified as LPV according to the ClinGen Hereditary Breast, Ovarian and Pancreatic Cancer Expert Panel (PVS1 and PM2).[10 13] Several digestive cancers are

present in the familial history of patient 9: one FDR and three SDRs. Patient 9 had conserved IHC for the MMR proteins. Similarly, medical records revealed that the IHC of the two SDRs diagnosed with colorectal cancer were also normal. Cosegregation studies were also performed (online supplemental figure S9).

We have identified the variant NM_002528.7:c.244C>T in *NTHL1* in homozygosis (VAF=0.997) in patient 10, diagnosed with colorectal cancer at age 45–50 years (online supplemental figure S10). This nonsense single base substitution in exon 2 of the *NTHL1* gene results in a premature stop codon in the protein, which could lead to a truncated or absent protein by NMD ((p.Gln82*)). This variant is classified as a PV according to the guidelines (PVS1, PP5 and PM2).[10 23] Patient 10 was diagnosed with colorectal cancer with conserved IHC for MMR proteins. Gastroscopy and colonoscopy revealed that this patient presented 6–7 gastric polyps and 10 colonic polyps. The familial history of cancer of the proband includes only one SDR. Carrier studies are being performed on the siblings and sons.

Actionability of the identified IFs

Seven (70.0%) IFs were located in genes considered clinically actionable according to ACMG[3] and the French Society of Predictive and Personalised Medicine (FSPPM)[26] (online supplemental tables S1 and S4).

DISCUSSION

We report here that 10.53% of our population harbours a PV/LPV in a gene consistently associated with their diagnosed cancer (online supplemental appendix S1, table 1). Interestingly, we report that opportunistic genetic screening has increased the diagnostic yield by 1.22% in our cohort. In addition, 70.20% of the identified IFs are present in clinically actionable genes providing these families with an opportunity to join cancer early detection programmes, as well as secondary cancer prevention. IFs might facilitate the diagnosis of asymptomatic individuals and the early management of cancer once it develops.

Conventionally, in up to 50% of families with HBOC a deleterious germline alteration is found in high-penetrance genes (*BRCA1, BRCA2* and *PALB2*) or moderate-penetrance genes (*ATM* and *CHEK2* in BC and *RAD51C*, *RAD51D* and *BRIP1* in ovarian cancer).[27] However, the development of NGS tools has allowed for the identification of PVs/LPVs in other genes also related to BC. In the present work, we identified IFs in the *TP53, MSH6, PMS2* and *VHL* genes in patients undergoing HBOC panel testing. Our group has previously reported that 8% of patients with HBOC criteria not harbouring PVs/LPVs in the *BRCA* genes were carriers of PVs/LPVs in *BARD1*, *BRIP1*, *CDH1*, *CHEK2, PALB2*, *RAD50* or *TP53*.[28] Hauke *et al* studied a cohort of non-carriers in the *BRCA*s genes with a familial or personal history of BC and found that the diagnostic yield increased by 1.66%, mainly in the *ATM* gene.[29] Maani *et al* found 24 IFs in 6060 MGP-NGS performed (0.39%) in patients on suspicion of a hereditary cancer syndrome harbouring multiple PVs with a low allele fraction.[1] In HBOC, hereditary non-polyposis colorectal cancer, Li-Fraumeni syndrome or adenomatous polyposis, extending the genetic analysis to 24 genes beyond the patients' phenotype increased the diagnostic yield by 2.07%. Interestingly, these authors observed that opportunistic screening of *BRCA1*, *BRCA2*, *MSH1, MSH2* and *MSH6* increased the diagnostic yield by only a modest 0.58% compared with 2.07% when the full 24-gene panel was analysed.[30]

TP53 is included in panel testing when BC is diagnosed in patients aged ≤45 years or those fulfilling the Chompret criteria,[31] which was not the case for either of patients 1 or 2 in our study. The variant NM_000546.6:c.783-1G>A in *TP53* identified in our study has previously been found in patients with adrenocortical carcinoma, ovarian cancer and BC,[32] agreeing with our results. However, no evidence has been published regarding the role of NM_000546.6:c.743G>A variant identified in our study in HBOC patients. As reviewed by Batalini *et al*, distinguishing a germline from a somatic PV/LPV in *TP53* has enormous clinical implications.[33] Clonal haematopoiesis (CH) is a common phenomenon in *TP53* consisting of abnormal expansion of a haemopoietic stem cell clone harbouring a somatic variant that provides these cells with survival advantages. In contrast, in classic mosaicism, the mutation occurs at a postzygotic stage so it is present only in those cells derived from this affected cell.[33] The presence of NM_000546.6:c.783-1G>A in a relative of patient two allowed us to confirm its germline origin. However, the absence of the variant NM_000546.6:c.743G>A in the relatives of patient 1 (online supplemental figure S1) allowed us to confirm that this PV in *TP53* does not have a germline origin. The presence of a PV in non-tumorous tissue (such as fibroblasts from skin punch biopsy and hair follicles from eyebrows) may discard the diagnosis of CH and confirm the diagnosis of classic mosaicism. We found that the variant NM_000546.6:c.743G>A was present in the buccal swab (VAF≈20%), breast tumorous and non-tumorous paraffined tissue (VAF≈50%) but not in fibroblasts from skin punch biopsy, allowing us to confirm the diagnosis of classic mosaicism in patient 1.

PVs/LPVs in the *MSH6* and *PMS2* genes confer an increased risk throughout the life of Lynch syndrome and BC.[34 35] Dominguez-Valentin *et al* found that BC risks were similar across all MMR genes.[35] Interestingly, Roberts *et al* reported that PV in only *MSH6* and *PMS2* increased the cumulative incidence of BC.[34] This evidence endorses the inclusion of these *MMR* genes in genetic tests undertaken in patients with a personal or familial history of BC. Concordantly, the Catalan Health Service recommends offering opportunistic screening for the *MSH6* gene, among others, in all hereditary MGP-NGS tests performed.[8] The variant NM_000535.7:c.24-2A>G in *PMS2* identified in our cohort has been previously reported in homozygosis in a Spanish woman diagnosed with lymphoma, endometrial cancer and colorectal cancer.[35] The variant NM_000535.7:c.1579_1580del in *PMS2* has previously been identified in patients with BC and ovarian cancer.[24 25] The variant NM_000535.7:c.989-2A>G in *PMS2* has been previously identified in patients with Lynch syndrome[36] and in a subject affected by two BC.[19] Conflicting results aroused when IHC and genetic studies of family members of patient 6 were investigated. Although the family history of cancer on the paternal side may lead us to think that the variant NM_000535.7:c.989-2A>G in *PMS2* was inherited from the paternal side, we cannot assure it. Taking this into consideration, we can neither assure nor refuse that subjects II:2 and III:1 are carriers of the same variant in *PMS2*. Some explanations for the discordances found in these subjects include that subject III:1 could be a carrier of a different MMR variant of somatic origin or germline origin, inherited from her paternal side and, therefore, not familiarly connected with pour proband (III:9). Sanger sequencing of the tumour or blood sample of subject III:1 was not possible because we had no access to the sample, but only to the electronic medical records stating the lack of expression of PMS2 and MSH6 proteins. Regarding the subject II:2, we ignore if she was carrier of the variant in *PMS2*. If so, one could expect an IHC with lack of expression of the proteins PMS2 and/or

MSH6 in the colorectal tissue, which was not the case. IHC screening of Lynch syndrome is usually done for only MSH6 and PMS2 proteins (two-stains method) to reduce costs. However, as reported by Pearlman *et al*[37] and recently reviewed by Leclerc *et al*,[38] it is recommended to test the four MMR proteins to diagnose Lynch syndrome, since the two-stain immunohistochemical screening may fail to detect mismatch repair deficiency in some Lynch syndrome tumours. Screening for MMR protein expression by IHC is a standard clinical practice to identify patients with Lynch syndrome but not in those diagnosed with BC. However, several authors have demonstrated that BC in women with Lynch syndrome is more prone to exhibit loss of MMR protein expression compared with sporadic BC.[34] Patient 4 with BC harbouring a PV/LPV in *PMS2* presented conserved MMR protein expression in the tumorous tissue, while patient 5 showed a lack of PMS2 protein expression.

Patients with Von Hippel-Lindau disease develop hemangioblastomas in the central nervous system and retina, renal clear cell carcinomas, renal and pancreatic cysts and pheochromocytomas, and it is caused by PVs/LPVs in the *VHL* gene.[39] To our knowledge, this is the first time that the variant NM_000551.4:c.341G>A in *VHL* has been reported (subject 7, diagnosed with BC).

Three IFs (two in *ATM* and one in *NTHL1*) were found in patients with a personal history of colorectal cancer, all of whom underwent Lynch syndrome MGP-NGS testing. Lynch syndrome predisposes patients to colorectal and endometrial cancer, among others.[40] It is mainly caused by germline PVs/LPVs in the MMR genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*) and the *EPCAM* gene and, as a consequence, tumours from patients with Lynch syndrome display loss of expression of MMR proteins, microsatellite instability and increased hypermutation phenotype.[38 40 41] With this evidence, screening for Lynch syndrome includes tumour IHC of MMR proteins and/or microsatellite instability analysis in all colorectal and endometrial cancers. According to the Spanish Society Of Medical Oncology (SEOM) guidelines, genetic studies must be undertaken when MSH2, MSH6 or PMS2 protein expression is affected. Moreover, if IHC analysis shows an absence of MLH1 staining, and *BRAF*/*MLH1* methylation testing is normal, germline MMR testing is recommended.[8 40] The variant NM_000051.4:c.5908C>T in *ATM* (patient 8) has been identified in patients with ataxia-telangiectasia in a homozygous and compound heterozygous state,[42] but the variant NM_000051.4:c.7670_7674del 9 in *ATM* (patient 9) has never been reported. Carrier studies performed in this family led us to believe that this latter variant does not segregate with the disease and thus this variant has low penetrance. We also identified the variant NM_002528.7:c.244C>T in *NTHL1* in homozygosis in a patient suffering from colorectal cancer. In the literature, this mutation has been reported in homozygosis or compound heterozygosity in several individuals affected with colorectal cancer, colorectal adenomas or polyposis.[38 43 44]

The identification of IFs can provide clinical benefits for patients harbouring the variant and their relatives. However, a major concern is how to integrate the identified IFs into routine clinical settings, as variants non-amenable to medical interventions could emerge.[4–6] The European Society of Human Genetics recommends focusing on the identification of the underlying cause of a particular disease excluding deliberate searches for additional variants.[45] In contrast, the ACMG endorses the intended and routine searching for variants not consistent with personal and family histories in cancer-related genes with known clinical actionability (online supplemental table S2).[3 7] Nevertheless, the ACMG states that the option 'not to know' must be offered to those subjects willing to be informed only of those findings associated with the initial indication, according to the principle of autonomy. Some other societies have adhered to these ACMG recommendations, with few modifications. The FSPPM compiled a curated list of 60 cancer-related genes (online supplemental table S2) according to their risk and clinical actionability. Remarkably, the FSPPM considers a two-step informed consent: a first one about opportunistic genetic screening during the initial medical procedure and a second one once primary findings are disclosed to the patient, when the patient is more likely to differentiate the extent of the information to be revealed from opportunistic genetic screening.[26]

In the present work, 8 of the 11 IFs were identified in genes considered actionable from a clinical perspective by the ACMG and the FSPPM statements (*MSH6*, *PMS2*, *TP53* and *VHL*). Nevertheless, the SEOM, the European Society for Medical Oncology (ESMO) and the National Comprehensive Cancer Network (NCCN) have published guidelines for the screening and management of *ATM* carriers and the SEOM has also published actionability recommendations for *NTHL1* carriers (online supplemental table S4).[27 40 46–50]

MSH6 and *PMS2* have similar actionability according to the SEOM, ESMO and NCNN guidelines, which include colonoscopies on an annual/biennial basis (online supplemental table S4).[48] No available evidence exists regarding prophylactic colectomy in healthy individuals diagnosed with Lynch syndrome.[40] Regarding gastric cancer surveillance in these patients, the ESMO recommends the surveillance with upper endoscopy in families with a history of gastric neoplasms and testing for *Helicobacter pylori*.[51] The NCCN recommends upper gastrointestinal surveillance with esophagogastroduodenoscopy and colonoscopy, and biopsy to assess for *H. pylori*.[51] Transvaginal ultrasound and endometrial aspirate are also recommended by the SEOM in ovarian and endometrial surveillance,[47] in contrast to the NCCN guidelines, which recommend endometrial biopsy.[48] Risk-reducing hysterectomy and salpingo-oophorectomy should be contemplated according to the SEOM, ESMO and NCCN recommendations.[27 40 47]

In subjects harbouring a PV/LPV in *TP53*, clinical breast examination (NCCN) and breast MRI (ESMO and NCCN) and screening for additional cancers should be performed.[27 51] According to the ESMO and NCCN, risk-reducing mastectomy counselling should consider the degree of protection, reconstruction options, family history and residual BC risk with age and life expectancy.[47 49] The European Reference Network-Genetic Tumour Risk Syndromes (GENTURIS) published a surveillance protocol specific for carriers of germline disease-causing *TP53* variants, as detailed in online supplemental table S4.[52]

The Danish Coordination Group for *VHL* published guidelines for the diagnosis and surveillance of *VHL* carriers, which include annual clinical examination by a paediatrician of those aged 0–14 years, among others.[53]

In conclusion, our study showed that 1.22% of our cohort harboured PVs/LPVs in genes beyond those specifically related to the diagnosed cancer. Despite few of these IFs being present in low or moderate penetrance, we found that the majority are considered clinically actionable, providing these families with an opportunity to join cancer early detection programmes, as well as secondary cancer prevention. Moreover, these findings could facilitate the diagnosis of asymptomatic individuals and the early management of cancer once it develops. The actionability of these genes and their implications for the subjects' and relatives' care reinforce opportunistic genetic screening in

all subjects undergoing genetic testing within the framework of genetic counselling.

Author affiliations
[1]Institut d'Oncologia de la Catalunya Sud (IOCS), Hospital Universitari Sant Joan de Reus (HUSJR), Institut d'Investigació Sanitària Pere Virgili (IISPV), Reus, Spain. Universitat Rovira i Virgili (URV), Reus, Spain
[2]Hereditary Cancer Program, Catalan Institute of Oncology, IDIBELL and Biomedical Research Centre Network for Oncology (CIBERONC), L'Hospitalet de Llobregat, Spain
[3]Hereditary Cancer Program, Catalan Institute of Oncology-IDIBGI, Girona, Spain
[4]Hematology Department, ICO and Hospital Germans Trias i Pujol, Josep Carreras Leukaemia Research Institute, Badalona, Spain
[5]Pathology Molecular Unit, Department of Pathology, Hospital Universitari Sant Joan de Reus (HUSJR), Spain. Institut d'Investigació Sanitària Pere Virgili (IISPV), Reus, Spain. Universitat Rovira i Virgili (URV), Reus, Spain

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ORCID iDs
Sara Fernández-Castillejo http://orcid.org/0000-0002-8109-9713
Joan Brunet http://orcid.org/0000-0003-1945-3512
Marta Pineda http://orcid.org/0000-0002-5403-5845
Marta Rodríguez-Balada http://orcid.org/0000-0002-2691-8819

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