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Profiling of the genetic features of patients with breast, ovarian, colorectal and extracolonic cancers: Association to *CHEK2* and *PALB2* germline mutations

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ABSTRACT

Background and aims: Cancer predisposition goes beyond BRCA and DNA Mismatch Repair (MMR) genes since multi-gene panel testing has become the routine diagnostic tool for hereditary cancer suspicion (HCS) cases. *CHEK2* and *PALB2* are some of the foremost-mutated non-BRCA/MMR actionable genes in families with a significant familial aggregation. Therefore, the purpose of this work is to unravel which tumours other than breast, ovary or colorectal display the patients.

Materials and methods: We have analysed 528 probands that meet the inclusion criteria for Hereditary Breast and Ovarian Cancer and Lynch Syndrome established by our Hereditary Cancer Regional Program with a customized 35 genes-panel by using Ion Torrent™ Technology.

Results: We have identified pathogenic variants (PVs) in 61 families (1.55%), of which more than half (31 probands) harboured PVs in *CHEK2* and *PALB2* genes. Ours results reveal that not only were PVs *CHEK2* and *PALB2* carriers more likely to have family history of cancer not limited to breast, ovarian or colorectal cancers, but also they are prone to other extracolonic cancers, noteworthy endometrial and gastric cancers.

Conclusions: Multigene panel testing improves the chance of finding PVs in actionable genes in families with HCS. In addition, the coexistence of variants should be recorded to implement a polygenic risk algorithm that might explain the missing heritability in the aforementioned families.

1. Introduction

Lynch Syndrome (LS) together with Hereditary Breast and Ovarian Cancer Syndrome (HBOC) are the more frequent causes for cancer development in the context of the same family. Although a familial clustering is observed in 15–20% of the patients, barely up to 10% may be due to an inherited mutation in cancer predisposing genes (CPGs). Germline defects in the DNA mismatch repair genes (MMR) lead to an increased risk for colorectal cancer development as well as other extracolonic locations, such as endometrium, ovary, gastrointestinal tract or kidney. In addition, germline mutations in *BRCA1* and *BRCA2* genes, that significantly elevated lifetime risk of breast and ovarian cancer, contributes to the development of other malignancies. A significant amount of cases showing familial aggregation are not explained by pathogenic variants (PVs) in BRCA or MMR genes (NCCN Guidelines Version 3.2023) [1]. Therefore, other HR repair genes such as *CHEK2*, *PALB2*, *ATM*, *RAD51C*, *RAD51D* and *BRIP1* have emerged as triggers in the aforementioned hereditary cancer syndromes; being a challenge to perform a properly clinical management in the patients and their families. The widespread use of next-generation technology (NGS) enables the analysis of numerous CPGs in a fast and economical way, enabling an affordable study in those families with overlapping cancer phenotypes [2,3]. Using panel testing is helpful because the combination of several variants in multiple medium to low-penetrance CPG alleles would allow establishing a polygenic risk that might affect to the personal risk and with significant repercussions for the clinical

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management, due to their prognostic and therapeutic implications [4,5].

In a previous attempt to define which genes, beyond the BRCA and MMR genes, could play a noteworthy role in the development of breast, ovarian, colorectal and other associated cancers, our lab designed a 35 genes-Ondemand panel [6]; especially intended for those families with overlapping phenotypes for HBOC and LS. In that paper, we observed an increase in diagnostic yield for HBOC families in terms of other mutated CPGs, but not in LS families, where the most frequently mutated genes are MMR genes and *CHEK2*. Interestingly, we discovered a trend of PVs or likely pathogenic variants (LP),-hereinafter PVs- in genes HBOC-related in LS families and vice-versa. In addition, the coexistence of PVs and Variants of Unknown Significance (VUS) could be observed in several genes that could cause the cancer in some families, supporting a model of polygenic risk in some families [5].

CHEK2 and *PALB2* are genes that contribute largely to inherited cancer in our target population beyond the BRCA and MMR. Particularly, *CHEK2* is one of the most frequently mutated in numerous hereditary cancer predispositions [7–9]. Its association with breast cancer (BC) and prostate cancer (PrC) is well known [7,8,10] and likely triggers the development other tumours [7]. As for *PALB2*, carriers of PVs have an increased risk of suffering BC, gastric cancer, and mainly pancreas cancer (PaC) whereas ovarian (OC) [11] or colorectal cancers (CRC) associations have been suggested, but with weaker evidence [12].

Therefore, the aim of this study is to describe the molecular and clinical features of our cohort with germline PVs in *CHEK2* and *PALB2* to shed light into the genetic contribution of these genes to hereditary cancer. In addition, we will delve into which tumours other than breast, ovarian or colorectal display the patients to establish genotype-phenotype correlations that might modify clinical management and cascade testing in at-risk relatives.

2. Materials and methods

2.1. Patient recruitment

Index cases (ICs) were selected according to the selection criteria for HBOC or LS -hereinafter with hereditary cancer suspicion (HCS)- defined in the SEOM (Spanish Society of Medical Oncology): clinical guidelines for HBOC [13] or LS [14]. The Cancer Genetic Counseling Units of the Hereditary Cancer Program of Castilla and León (Spain) selected the cancer cases and relatives who fulfilled the aforementioned criteria. Consequently, a cohort of 528 probands resulted eligible for genetic testing. The clinical features and family history of cancer, as well as the blood samples and informed consent were sent for genetic testing. The Ethical Committee of Clinical Research of the Health Areas of Burgos and Soria approved this study.

2.2. Genetic testing

Genomic DNA was extracted from peripheral blood leukocytes by a Roche MagNaPure® Compact Robot by using the "MagNA Pure Compact Nucleic Acid Isolation Kit I—Large Volume" according to the manufacturer's instructions.

Next Generation Sequencing was performed with a custom 35 genes-panel"Ion Ampliseq On-Demand" on the Ion S5™ System. The Library and template preparation was automatically carried out with the Ion Chef™ System and loaded in an Ion 520 Chip according to manufacturer's instructions (the information about the list of genes and the whole procedure has been described elsewhere [6]. The Ion Reporter software (Version 5.10) was used for filtering and variant annotation. The mean percent target coverage at 50 × was 88.6% with a coverage uniformity greater than 90% in all tested samples [6].

Variants were considered as PVs if they causes loss-of-function (LoF) or if they are reported as PV or LP in ClinVar or LOVD databases or reputable sources. In this report, we consider as LP those missense mutations with conflicting interpretations of pathogenicity (CIP) in ClinVar towards LP (VUS or LP), due to management of these families in our Regional Genetic Counseling Program. All the Variants were numbered according to Human Genome Variation Society (HGVS) nomenclature.

Multigene panel testing results yielding VUS or PVs were confirmed on a second DNA sample by Sanger Sequencing with the BigDye Terminator v3.1 Sequencing Kit (in an ABI3130XL DNA Sequencer and was also used for verify the segregation in relatives.

In those samples suspected of having large rearrangements (LGRs) after NGS analysis, a comprehensive study was performed HBOC and SL families with Multiplex Ligation Dependent Probe Amplification (MLPA) (MRC Holland), with the following MLPA probe mixes: P002-BRCA1, P045-BRCA2, P260-PALB2-RAD50-RAD51C-RAD51D, P003-MLH1/MSH2, P008-PMS2, and P072-MSH6/MUTYH.

3. Results

3.1. Prevalence of germline variants in our cohort

We have analysed 528 samples with HCS with our customized 35 genes-panel of which, 240 agreed with HBOC criteria and 288 matched LS criteria. Sixty-one families were found to be PVs carriers in 16 different genes (BRCAs or MMRs with PVs are not included). Remarkably, *CHEK2* and *PALB2* are the genes that explain more than a half of the positive families (Fig. 1a). *CHEK2* is by far, the gene with the highest rate of PV (21/61), followed by *PALB2* (10/61), mainly in families that meet the HBOC criteria (Fig. 1b).

3.2. Mutations in CHEK2 and PALB2. Clinical features of the patients

3.2.1. CHEK2 variants

Genetic testing disclosed 27 different PVs or VUS variants in 42 families, a half of them were missense variants classified as VUS or LP according to the ClinVar database. Overall, 69% of the families met the HBOC criteria, three patients developed a contralateral BC and two were male breast cancer (MBC) patients. The ages of onset of first BC ranged from 32 to 76 years old (five cases before 40 years old). Besides, carriers developed other cancer types (Table 1), such as CRC, PrC and kidney cancer, and some combined phenotypes (BC, OC and EndC in a patient or PrC and CRC in another one).

The more frequent mutation among PVs was c.1100delC that segregates into four families (overall eight cancer cases). Two of the ICs were MBC cases with familial history of breast, gastric or gynaecologic malignancies, and one of them harboured the mutation in homozygosis. Protein truncating variants (PTV) c.409C>T and c.478delA were found each in two different families. Regarding c.409C>T, one of the families involved two affected generations with BC and CRC and the second case was a double carrier of PVs in *CHEK2* and a LGR in *PALB2* gene (Table 1). The IC is a woman that developed bilateral BC, endometrial cancer (EndC) and OC, and with relatives with breast, colorectal, and lung malignancies in both genealogic branches. One of the c.478delA families displayed five BC cases, identifying the mutation in three sisters with BC (onset at ages 44, 58 and 72), and the second IC is a 38 years-old healthy man whose mother had CRC. The rest of inactivating mutations were only identified in one family each: c.593-1G>T and c.793-1G>A, c.467dupT and c.1209_1233del24 (Table 1).

Furthermore, among the 30 families carrying missense mutations, nine of the families with CIP in the ClinVar database were included as disease triggering. The ICs with the c.190G>A mutation were a 32 years-old woman familial antecedents of PrC and PaC and a 37 years-old CRC male with familial antecedents of BC and CRC. The c.349A>G variant was detected in two unrelated families with a high familial history of BC, ascertaining the segregation in one of them. The variant c.433C>T is present in a three affected members family (Table 1). Variant c.499G>A is present in a woman who developed BC at 34, without ascertain the segregation in her mother. Variant c.917G>C was found in two sisters

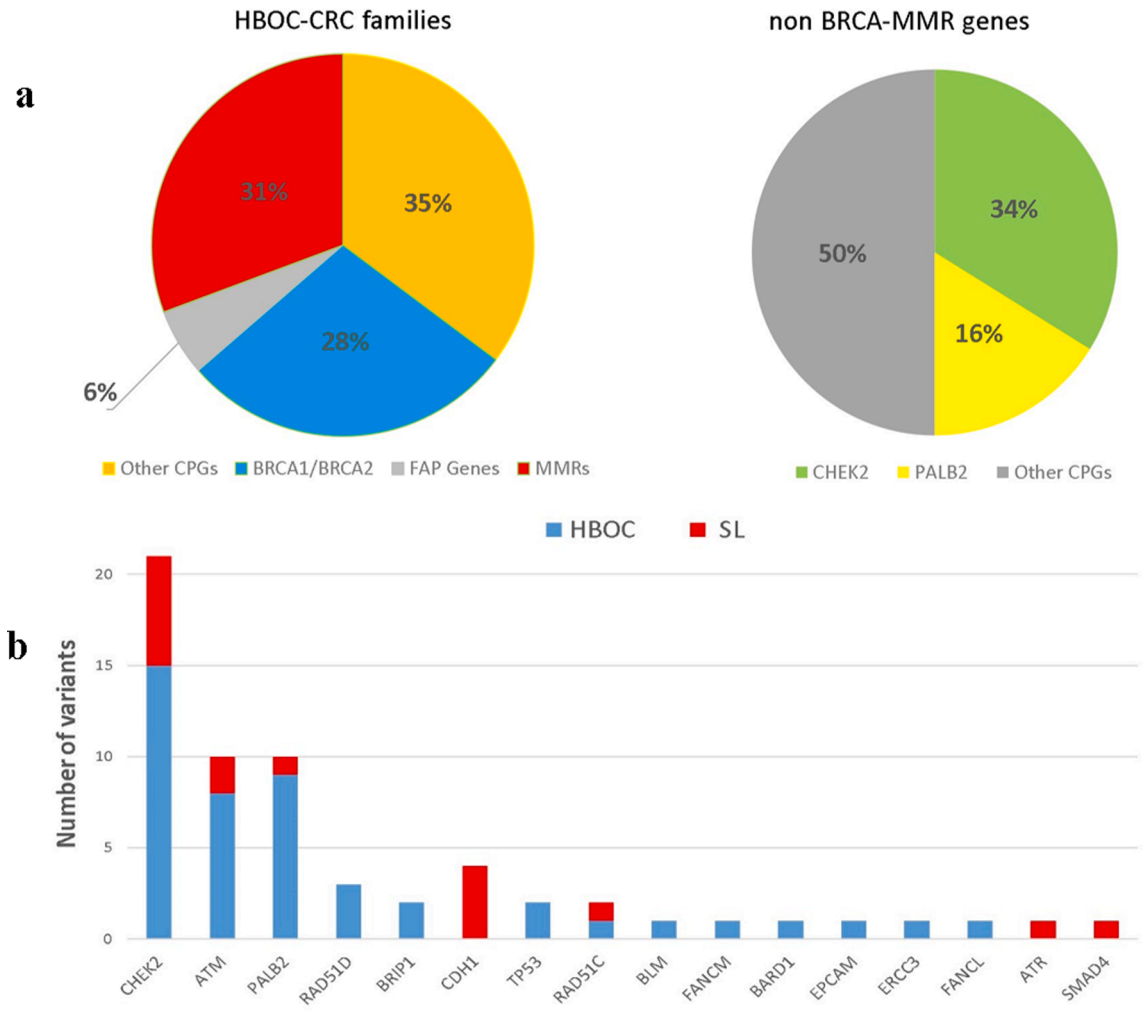

Fig. 1. Distribution and frequencies of germline Pathogenic Variants (PV) or Likely Pathogenic Variants (LPV) identified in the Hereditary Cancer Suspicion cohort. A. Germline PV or LPV in 528 Hereditary Breast and Ovarian Cancer (HBOC) and Colorectal families (CRC) in BRCA and MMR genes and in non-BRCA-MMR genes analysed by the Ampliseq On-demand 35 genes-panel. B. Number and distribution of PV or LPV found in non-BRCA-MMR genes by gene according to familial selection criteria (HBOC or SL).

that suffered from BC at 58 and 42 years old respectively, but it was absent in another BC sister aged 79, other untested relatives were her mother who had EndC at age 54. Finally, two families carried the c.1427C>T mutation, the former IC suffered from kidney cancer at age 72 (verifying segregation in two female cousins with BC), the latter IC with CRC at age 62, without cancer ancestors.

Additionally, 16 different VUS were detected in 21 HCS families, 15 of which best fit the HBOC selection criteria. Remarkably, a third of the families had a personal or family history of extracolonic cancers (mainly EndC or gastric) and a fourth of the carriers were triple negative BC (Table 2). Taken together, gastric cancer, endometrial, and colorectal were the most prominent cancer types among the family history of *CHEK2* carriers (Fig. 2).

3.2.2. *PALB2 variants*

Eight different *PALB2* PVs were found in 10 families (Table 1). The mutations c.1857delT and c.2257C>T were carried by two unrelated families each. Noticeably, whereas both c.2257C>T ICs are BC cases, the families with c.1857delT showed dissimilar phenotypes, for instance, family BC-1479, with two sisters displaying BC and OC at a very young ages (previously reported in [11]) and, in family BC-1543 a multiple location cancer woman (bilateral BC, CRC and PaC) with numerous cancer antecedents. Furthermore, three families have LGRs. The first was a deletion involving exons 1 to 3 that segregates in the IC (a woman diagnosed from BC at 39 years-old) and her father with PrC at 65 years-old. Secondly, a deletion of exon 12 of *PALB2* gene was found concurrently with a *CHEK2* PV as described above. Lastly, an exon 11 duplication was discovered in a 43 years-old OC patient, with familial history of BC, CRC, PrC and EndC. The three remaining PVs (c.212-2A>G, c.1349delA, and c.2748+1G>T) were identified in BC probands with antecedents of OC, PrC and gastric cancer.

In addition, thirteen different VUS accounted for 14 unrelated ICs, six of which were BC cases, three gastric cancer, three OC and two PrC cases (Table 3). Particularly, the carrier of c.995T>C was a male with multifocal (melanoma, PrC and Bladder) cancers. Besides, gastric cancer, PrC, OC and CRC were the most outstanding reported antecedents among the VUS carriers (Fig. 2).

4. Discussion

In this work, we focused on the prevalence of PVs in a cohort of 528 individuals selected for gathering high-risk criteria for HBOC and/or LS. The ICs were analysed by a custom panel of 35 cancer predisposing genes, excluding families harbouring mutations in BRCA or MMR genes. Although *CHEK2, ATM* and *PALB2* emerged as the foremost mutated genes in the 528 ICs, we have disregarded *ATM* results from this work due to the homogeneity of phenotypes (chiefly BC cases with clear antecedents fulfilling HBOC or Lynch syndromes), thus prevailing *CHEK2*

Table 1
Pathogenic or likely pathogenic variants in CHEK2 and PALB2 in 528 hereditary cancer suspicion families.

Gene	c.DNA variant	Protein change	Family ID	Index Case/ Other cases	Tumor (subtype)	Age at diagnosis	Other relatives without genetic testing (n° of cases) onset age
CHEK2	c.190G>A	p.E64K	BC-1148	1799	BC (IDC)	32	PrC (1)78, PaC (1) 78,Leu (1) n.d.
			CRC-1100	1791	CRC	37	BC (1) 50; CRC (3) n.d.
	c.349A>G	p.R117G	BC-810	1274	BC	37	BC (3) 52, 60, 67
			BC-2496	3945[a]	BC (luminal IDC)	49	BC (3) 29, 55
				3946[a]	BC (luminal IDC)	48	
	c.409C>T	p.R137*	BC-3204	5159r	BC	60	BC (1) 50; CRC (1) n.d.
			CRC-1640	2542[¥]	bBC,OC, EndC	42-55, 47, 47	BC (1) 68; CRC (1) 73; LC (3) 50, 50, 60; CUP (5) 40, 45, 65, 70, n.d.
	c.433C>T	p.R145W	BC-3580	5756r	BC	34	GC (1) 70; CUP (1) n.d.
				5756	PrC	66	
				6171	bBC	53-63	
	c.467dupT	p.Y156	CRC-1670	2580[b]	AFAP	64	BC (1) <50
	c.478delA	p.I160	BC-1378	2150	BC (luminal IDC)	58	BC (2) 45, 75
				2151	BC	44	
				2150r	BC	72	
			CRC-1682	2689[b]	healthy		CRC (1) n.d
	c.499G>A	p.G167R	BC-1083	1699	BC (luminal IDC)	34	BC (1) 50
	c.593-1G>T		CRC-1099	1788	PrC + CRC	76	GC (2) n.d.; PrC (1) 73, PaC (1) 75, CUP (4) n.d.
	c.793-1G>A		BC-3550	5716	BC (luminal IDC)	42	bBC (1) 55-74; OC (1) 39
	c.917G>C	p.G306A	BC-2902	4585	BC	58	BC (1) 50; EndC (54)
				5167	BC	76	
	c.1100delC	p. T367Mfs*15	BC-1331	2084	MBC	69	BC (1) 50; bBC (1) 71; GC (2) 58, n.d.; EndC (1) 72
			BC-2766	4352	BC (luminal IDC)	59	BC (1) 60; CRC (1) 65;LC (1) n.d.; Mel (1) n.d.
				4352r	EndC + BC	45 - 64	
				4352r1	BC	40	
			BC-4015	6447	BC (luminal IDC)	65	BC (3) 40,65,n.d.; bBC (1); BC-OC (1) n.d.; CRC (1) 58;GC (1) 61; BrC (1) 34
				6447r[c]	MBC	n.d.	
			BC-4069	6656	BC IDC	53	
				6656r	BC (luminal IDC)	49	
	c.1209_1233del24	p.Y404Vfs*2	BC-4048	6487r	bBC	45	BC (3) 50,65,71; CRC (1) 47.; EndC (1) 45
	c.1427C>T	p.T476M	BC-3789	6181	Kidney Cancer	72	BC (1) 40; PrC (4) 63,65,65,80
				6181r1	BC	38	
				6181r2	BC	43	
				6181r3	BC	44	
			CRC-1102	1793	CRC	62	
PALB2	g.(?_23649161)_(23652488_?)del		BC-3148	5135	PrC	65	PaC (1) 59; BlC (1) n .d. GC (1) n.d.; CUP (3) n.d.
				5135r	BC	39	
	c.212-2A>G		BC-4059	6505	BC (luminal IDC)	69	TNBC+OC (1) 35-44; OC (1) 91, Lym (1) 60
	c.1349delA	p.N450Ifs*2	BC-2733	5542	BC (IDC TNBC)	53	BC (4) 42,48,55,64; GC (1) 64
	c.1857delT	p.F619Lfs*8	BC-1479	2333	OC (HGSC)	33	EndC (1) 38; LiC (1) 78; LC (1) 62 Leu (1) n.d.
				3680	BC	36	
			BC-1543	2445	bBC,CRC + PaC	72-75, 80+80	BC (2) 39,64; CRC+OC (1) 51; GC (1) 68; EndC (1) 70; PrC (1) 70; CUP (1) 54
	c.2257C>T	p.Arg753X	BC-2063	5691	BC (luminal IDC)	67	BC (1) 49
			BC-4109	6593	BC (luminal IDC)	26	BC (3) 35,44, 76; bBC (1) 76-83; BC-OC (2) 50-83; CRC (1) 82; Mel (1) 65
	c.2748+1G>T		BC-3177	5114	bBC	44-57	OC (1) 48; PrC (1) 84; GC (1) 81
	c.3114-?_3201+?dup88		BC-2835	4465	OC (HGSC)	43	bBC (1) 44-57; PrC (1) 72; CRC (1) 74 GC (1) 65; EndC (2) 50, 61
	c.(3201 + 1_3202-1)_(3350 + 1_3351-1)del		CRC-1640	2542[¥]	bBC,OC, EndC	42-55, 47, 47	BC (1) 68; CRC (1) 73; LC (3) 50, 50, 60; CUP (5) 40, 45, 65, 70, n.d.

Abbreviations: BC, breast cancer; bBC, bilateral breast cancer; MBC, male breast cancer; IDC, Invasive ductal carcinoma; TNBC, triple negative breast cancer; OC, ovarian cancer; HGSC, high grade serous carcinoma; CRC, colorectal cancer; EndC endometrial cancer; GC, gastric cancer; PaC, pancreas cancer; PrC prostate cancer; AFAP, attenuated familial adenomatous polyposis; Mel, melanoma; Leu, leukaemia; BlC, bladder cancer; LiC, Liver cancer; LC, lung cancer; CUP, Carcinoma of unknown primary; n.d. not determined. [¥]Double carrier for *CHEK2*and *PALB2*PVs [a]The ICs is also carrier of VUS in *BRCA2*[b]The ICs is also carrier of a heterozygous PV in *MUTYH*[c]The ICs is also carrier of PV in *BRCA2*.

Table 2
Variants of Unknown Significance (VUS) in *CHEK2* gene.

Gene	c.DNA variant	Protein change	Family ID	Index Case/ Other cases	Tumor (subtype)	Age at diagnosis	Other relatives without genetic testing (n° of cases) onset age	ClinVar Classification
CHEK2	c.254C>G	p.P85R	BC-3235	5209	bBC (luminal IDC), EndC	58–70, 73	BC (1) 63; GC (1) 82	VUS
	c.338A>G	p.Y113C	BC-3729	5978	BC (IDC)	36		VUS
	c.442A>G	p.R148G	BC-0345	471	MBC	47	BC (2) 40, 50; PrC. (1) 75; Thyroid cancer(1) 50	VUS
				6239	BC (IDC)	44		
				471r	BC	59		
			BC-2443	3877	healthy		BC (2) 42, 66; OC (2) 36, 45; PrC (1) 70, PaC (1) 55, CRC (1) 45	VUS
			BC-3974	6373	bBC (TNBC-luminal IDC)	40–40	BC (2) 44, 53; PrC (1) 83; Leu (1) 18	VUS
	c.455C>T	p.P152L	CRC-1523	2395	EndC, BC (mucinous)	58, 66	GC (1); CRC (1), LC (2), CUP (2)	VUS
	c.569C>T	p.A190V	BC-3093	4938r	BC	39	PrC (2) 60, 65; CRC (1) 67	VUS
	c.598G>A	p.V200I	BC-3811	6093	BC (luminal IDC)	47	BC (1) 45, bBC (1)65; LC (1) 74	VUS
	c.715G>A	p.E239K	BC-3245	5225	BC (luminal IDC)	48	BC (1) 60; GC (3) 55, 62, 74; CRC (3) 46, 51; PrC (1) 59; Leu	VUS
	c.904G>A	p.E302K	CRC-1209	1966	EndC	79		VUS
			BC-4084	6646[b]	BC (luminal IDC)	44	BC (4) 42, 44, 45, 50; CRC (1) 50; GC (1) 50	VUS
	c.910A>G	p.M304V	BC-3741	5996	BC (TNBC)	58	BC (1) n.d.; CRC (1) n.d.; BlC(1) n.d.	VUS
			CRC-1136	1840	BC (TNBC), CRC	37, 63	PrC (1) 67, LC (1) 50	VUS
	c.934A>G	p.K312E	BC-3984	6390	healthy		BC, OC (1) 48, 49; EndC (1) 49, CRC (1) 78; PrC (3) 47, 49, 55	VUS
	c.1008G>A	p.Q336=	BC-4084	6646[b]	BC (luminal IDC)	44	BC (4) 42, 44, 45, 50; CRC (1) 50; GC (1) 50	CIP (VUS/LB)
	c.1216C>T	p.R406C	BC-3945	6322	BC (TNBC)	46	BC (2) 46, 55;; EndC (1) 46; PaC (81) 82; GC (1) 80	CIP (VUS/LB)
			CRC-1260	2029	CRC	67		
			CRC-1410	2251	healthy		CRC (3) 37, 41, 41; GC (1) n.d.	
	c.1337A>G	p.N446S	CRC-1392	2226	CRC	27	Leu (1)	VUS
	c.1450C>A	p.P484T	CRC-1154	1878[a]	AFAP	66	GC (3) 61, 77, 81	VUS
	c.1489G>A	p.D497N	BC-2719	4273	BC (luminal IDC)	67	BC (3) 45, 60, 78; OC(1) 74; PrC(2) 61, 79	CIP (VUS/LB)
	c.1522C>G	p.L508V	CRC-1490	2355	healthy		BC (1) 81;CRC (4) 55, 70, 71, 70; BlC(1) n.d.	VUS

Abbreviations: BC, breast cancer; bBC, bilateral breast cancer; MBC, male breast cancer; TNBC, triple negative breast cancer; IDC, Invasive ductal carcinoma; OC, ovarian cancer; CRC, colorectal cancer; EndC endometrial cancer; PaC, pancreas cancer; PrC prostate cancer; GC, gastric cancer; AFAP, attenuated familial adenomatous polyposis; Leu, leukaemia; BlC, bladder cancer; LC, lung cancer; CUP, Carcinoma of unknown primary. CIP, Conflicting Interpretation of Pathogenicity; VUS, variants of unknown significance; LB, Likely Benign; B, Benign. [a] The ICs is also carrier of a heterozygous PV in *MUTYH*. [b]The ICs is also carrier of VUS in *CHEK2*.

and *PALB2* in families that do not meet the commonly established inclusion criteria for HBOC and LS. Therefore, we focused on the prevalence and phenotypes of PVs carriers that open to the possibility for treatment with targeted therapies and properly genetic counselling for them and their close relatives.

Regarding to *CHEK2*, even though there are contradictory studies on the different risks conferred by some rare missense compared to LoF variants [8,9,15], we have considered as PVs all those variants that have conflicting interpretations P/LP vs VUS in ClinVar. Taking this into account, nearly a half of mutations (6/13) are missense changes against seven PTV, but they did not underwent worse phenotypes. Although females with BC prevails among *CHEK2* PVs carriers (24/33; 72%), our ICs suffered from other cancer types, such as colorectal (3/33; 9%), prostate, kidney, or gynaecologic and two of them had more than two primary tumours (Fig. 2). Our results subscribe those reported by others [7,16],where BC and PrC are the more frequent phenotypes in *CHEK2* carriers, followed to a lesser extent by MBC, CRC and thyroid cancers. However, the associations showed by most of these studies are grounded in the more frequent mutations such as c.1100delC and p.Ile157Thr that could not be extrapolated to a population like ours. The former is present in 4/528 (0.75%) of the cohort tested by NGS and the latter is actually absent. Consequently, our spectra of mutations are rather comparable to other Spanish publications [8,17], evidencing the differences among diverse population's origins.

In fact, c.190G>A, c.409C>T, c.478delA and c.1427C>T *CHEK2* mutations have been detected in both HBOC and CRC families. It has been reported that *CHEK2* PTV carriers have younger onset of the disease [8,16,18] but in our cohort, six ICs are under 40 years-old, and particularly, five of them (four BC and one CRC) are missense variants carriers.

CHEK2 c.1100delC mutation is the more studied variant in *CHEK2* worldwide [7]. It has been suggested to be enriched among BC female patients and increases the risk of MBC [19] and some studies also reports that can cause a worse prognosis [20] although its credibility as PV has been called into question due to its incomplete penetrance and its frequency among controls [7]. In our cohort, four unrelated HBOC families harbour the mutation, two are MBC cases with familial antecedents of gastric and colorectal cancers and one of them is a homozygosis carrier, the rest of ICs are female BC patients. Furthermore, c.1100delC segregates within the relatives in two families. It is relevant to point out that although the association of *CHEK2* with gynaecologic malignancies including non-high-grade OC is not so clear [19], one of the two carriers in family BC-2766, developed a gynaecological cancer prior to the BC (Table 1), and a first-degree relative developed CRC.

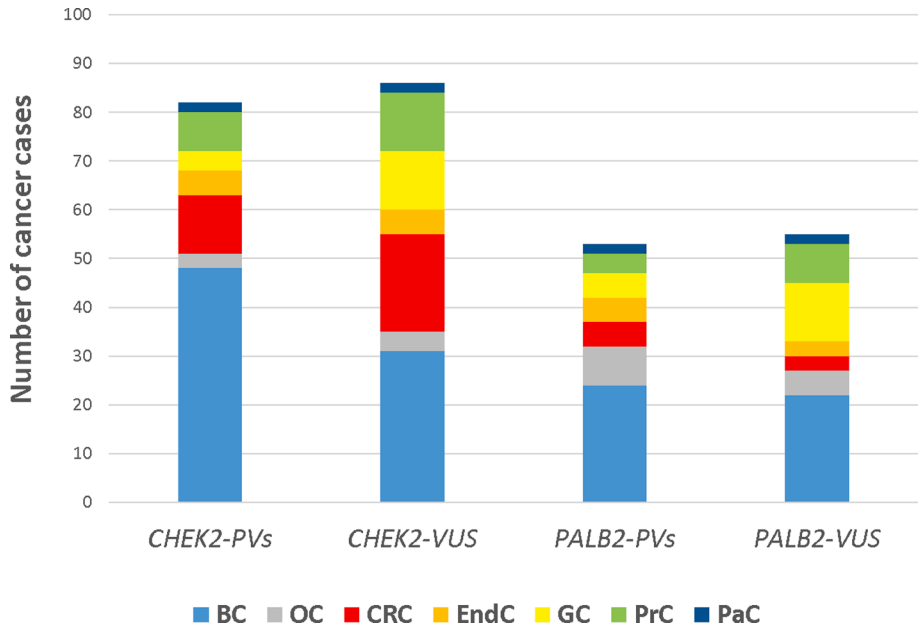

Fig. 2. Types of cancer in *CHEK2* or PALB2 families with germline variants (PVs and VUS). Abbreviations: BC, breast cancer; OC, ovarian cancer; CRC, colorectal cancer; EndC endometrial cancer; GC, gastric cancer; PrC prostate cancer; PaC, pancreas cancer.

Table 3
Variants of Unknown Significance (VUS) in *PALB2* gene.

Gene	c.DNA variant	Protein change	Family ID	Index Case/ Other cases	Tumor (subtype)	age at diagnosis	Other relatives without genetic testing (n° of cases) onset age	ClinVar Classification
PALB2	c.100C>T	p.R34C	BC-3581	5757	BC	34	BC (3) 35, 52, 54	VUS
	c.229T>C	p.C77R	BC-3977	6376	PrC	65	PrC (2) 67, 68;CRC (1) 70; GC (1) 87	VUS
	c.629C>T	p.P210L	BC-3457	5572	BC (luminal IDC)	30	GC (4) 28, 35, 53, n.d.; PaC (1) n.d.; BlC(1) 60; Leu (1) 58	CIP (VUS/LB/B)
	c.814G >A	p.E272K	BC-196	275	OC	35	BC (1) 45	VUS
	c.995T>C	p.L332P	BC-3765	6032	Mel, PrC, BlC	51, 53, 67	BC (2) 40, 52; OC (1) 72; CRC (1) 52; PrC (1) 62	VUS
	c.1194G>A	p.V398=	CRC-1427	2273[a]	GC	73	GC (1) 45	CIP (VUS/LB/B)
	c.1222T>C	p.Y408H	BC-2938	4634	OC (HGSC)	47	BC (2) 53, 60; PrC (2) 62, 67; GC (1) 84; EndC (2) 30,45	VUS
	c.1544A>G	p.K515R	BC-0368	505	BC	46	BC (1) 38	CIP (VUS/LB)
			BC-3774	6047	OC (HGSC)	74	EndC (1) 72; CRC (2) 41, 53	
	c.2201C>A	p.T734N	BC-796	5885	BC	49	BC (2) 42, 66	CIP (VUS/LB)
	c.2816T>G	p.L939T	CRC-1427	2273[a]	GC	73	GC (1) 45	CIP (VUS/LB/B)
	c.2869A>C	p.K957Q	BC-3903	6247	healthy		BC (1) 45; OC (1)70; PaC (1) 60; PrC (1) 79	VUS
	c.3152T>C	p.I1051T	BC-3820	6110	BC	50	BC (4) 31, 36, 38, 45	VUS
	c.3472C>T	p.H1158Y	CRC-1451	C-2301	BC, GC	53, 71	GC (1) 77	VUS

Abbreviations: BC, breast cancer; bBC, bilateral breast cancer; TNBC, triple negative breast cancer; IDC, Invasive ductal carcinoma; OC, ovarian cancer; HGSC, high grade serous carcinoma; CRC, colorectal cancer; EndC endometrial cancer; PaC, pancreas cancer; PrC prostate cancer; GC, gastric cancer; AFAP, attenuated familial adenomatous polyposis; Mel, Melanoma; Leu, leukemia; BlC, bladder cancer. CIP, Conflicting Interpretation of Pathogenicity; VUS, variants of unknown significance; LB, Likely Benign; B, Benign. [a] The ICs is also carrier of VUS in *PALB2*.

Concerning the rest of families with LoF variants, the mutation c.409C>T was harboured by two families reporting BC, OC and CRC as personal and familiar antecedents, and has been linked to BC and OC predisposition [21] despite low allele frequencies in the population control are reported. This mutation segregates with the disease in BC-3204 family and, remarkably, the IC of CRC-1640, which developed multiple cancers (Table 1), also carries a LGR in *PALB2* gene, an occurrence that is increasing mainly due to the routine use of NGS [22]. To our knowledge, c.478delA mutation has been reported neither in affected individuals with hereditary cancer nor in controls such as gnomAD database. In our cohort, two families present the mutation, one of them segregating in three sisters with BC and the other is a healthy man who inherited the variant from his mother that suffered from CRC (Table 1).

Some authors have shown their doubts whether missense mutations in *CHEK2* can be considered as increasing the risk of hereditary cancer [8], consequently, they proposed a score based on a Bayesian model and, after a literature review have adjusted ACMG criteria, being conservative with some missense variants such as c.190G>A and c.1427C>T and maintaining them as VUS. In particular, our reasons for considering c.190G>A as LP are the younger onset age of our ICs (BC at 32 and CRC at 37) and the different phenotypes showed by both families (Table 1). Furthermore, this variant had been associated with a significant breast cancer risk despite having shown somewhat functional impact in a mouse embryonic stem cell-based assay [23]. In contrast, they still maintain it as VUS [8] because DNA repair assays are discordant

[24,25]. According to databases (ClinVar, LOVD) and based on the results of functional assays, in which kinase activity [10,19] and growth after DNA damage are impaired [24,25], we keep c.1427C>T as LP. What is more, c.1427C>T segregates in the BC-3789 family (Table 1). Conversely, there seems to be no doubt about the LP of the missense variants c.349A>G, c.433C>T, c.499G>A and c.917G>C, since different studies demonstrate an impairment in their functional activity [8,23–25]. Nevertheless, it is the changes they imply in clinical management that are the most powerful reason to rule them out as VUS.

Notwithstanding, the scenario changes if we focus on the 19 carriers of VUS in *CHEK2*, we have found four triple negative subtype cases among the 12 BC carriers, and two ICs with BC and endometrial (Table 2). The association of *CHEK2* with OC or EndC cannot be discarded according to several studies [7,15,17], and triple negative is not a frequent phenotype. In addition, almost all of these families have antecedents of a broad spectrum of cancer types (Fig. 2), being endometrial and gastric cancers declared as a common cancer type, which deserves attention from a clinical oncology point of view to offer a proper management in such families. Even more so if we take into account that around 60% of *CHEK2* missense variants confers an increased risk for BC and the frequency of these variants is comparable to PTV ones [9] and despite the efforts of depict the risk of *CHEK2* in larger sample sizes.

Therefore, our results agree with some preceding studies [7,19], that attribute to *CHEK2* an increased risk to BC, including MBC and PrC and proposed that germline PV carriers are likely to suffer from a second primary cancer [19]. Conversely, our results were more suggestive of a susceptibility to multi-organ primary cancer, particularly gastric cancer, EndC, and CRC, which were the most prominent cancer types among the personal and family histories of *CHEK2* P/LP carriers.

Less controversy arises regarding *PALB2* mutations given that barely six rare missense variants have CIP results in ClinVar (accessed in June 2023). It has been reported that PTVs carriers in this gene have up to 30 times increased risk of develop BC [26] and most of the missense variants do not seem to confer an increased risk for BC [9]. Overall, the most frequent phenotype in PTVs carriers is, as expected, BC (eight cases) one of them triple negative, and three bilateral BC, and the series is completed by three OC and one PrC. Interestingly, two cases present multiple primary tumours, being the double heterozygote *PALB2/CHEK2* one of them. The familial antecedents mainly includes CRC, gastric and EndC cases (Fig. 2). *PALB2* association with gastric cancer is well documented [12,27], but conversely, an increased risk was not set up for CRC, PrC or EndC [12]. In addition, they conclude [12] that OC predisposition is at odds, suggesting that other genetic and non-genetic factors could modify the risks for a determined phenotype. In fact, the link between *PALB2* mutations and cancer types such as CRC, PrC, EndC and OC is more outstanding in the ICs with VUS (Fig. 2), since several relatives with CRC and PrC were reported among the familial pedigrees (Table 3). Nevertheless, although these differences might be likely due to our small sample size, if compared to a larger international cohort [12], preventive surveillance for these cancer types should be carefully considered for healthy individuals.

The main hurdles in our study are firstly, the relative small size and the founder effects in BRCAs and MMR genes in our cohort [28,29]. However, the mutations identified are in line to those reported in other Spanish regions [17]. Secondly, the pedigrees are built based on the ICs-reported data, thus, they might be under- or over-reported due to unknown or inaccurate kin-cohort antecedents of cancer, and segregation within the cancer relatives cannot have always been accomplished. Other issue is whether splicing is impaired, so we performed bioinformatics analysis by using SpliceAI delta (https://spliceailookup.broadinstitute.org/) and MaxEntScan (http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html) in VUS variants. None of the variants is likely to disrupt splicing. To validate or disprove an effect on mRNA splicing, several experimental approaches are used, such as RT-PCR with subsequent Sanger sequencing from RNA carriers [30], hybrid minigenes [31] or targeted RNA-seq [30,32]. Traditional RT-PCR sequencing methods, while effective, are limited to provide comprehensive information on alternative splicing patterns. RNA-seq, on the other hand, offers a high-throughput approach that simultaneously quantifies transcript levels and identifies alternative splice variants; therefore, the power of RNA-seq lies in the ability to classify accurately variants based on their splicing effects, which could significantly reduce the burden of VUS.

Reports of double heterozygosity are likely to increase with the use of NGS indeed, which will act as risk modifiers or cumulatively, thus supporting a polygenic risk that should be considered in risk prediction [6]. In our cohort, and disregarding the *PALB2/CHEK2*double heterozygote, up to eight families harbour a second VUS mutation. Another issue that could underestimate our data is that LGRs in *CHEK2* gene has been untested, which could bias its contribution, since some authors identified a high frequency of LGRs in this gene mainly in populations with founder effects [19].

In contrast, considering as P/LP those VUS that are likely to be at-risk-variants constitutes one of the strengths of our study, even more when *CHEK2* and *PALB2* are actionable genes, that involves clinical management and cascade testing in at-risk relatives. In that way, reviewing which of non-classic phenotypes are identified in the context of HBOC and LS will help to estimate the cancer risks in mutation carriers. It is worth mentioning that the elevated number of VUS detected in *PALB2* and *CHEK2* not only constitutes a challenge, but also that reporting the coexistence of VUS (in the same or different CPGs) is outright essential to implement a predictive model for polygenic risk that prompt an explanation of the cause of multiple cancer' types in high-risk families. Otherwise, a significant amount of patients with clinically actionable germline variants may be missed. The present work makes a difference because not only encompassed HBOC cases, as most studies do, but also families suspected of LS.

Future directions are to ensemble all these genetic factors in HCS families without mutations in known high-risk genes to implement a polygenic inheritance model to upgrade the cancer risk accuracy. This polygenic risk score will prompt an optimal management of the families to anticipate to the disease and to harness targeted treatments, that is, tackling personalized medicine.

5. Conclusions

Beyond BRCA and MMR genes, *CHEK2* and *PALB2* are the most frequently mutated genes in HCS in our region. Overall, ours results reveal that not only were PV *CHEK2* and *PALB2* carriers more likely to have family history of cancer not limited to breast, ovarian or colorectal cancer, but also they are prone to other extracolonic cancers. The most featured cancers in the families harbouring *CHEK2* and *PALB2* mutations are endometrial and gastric cancers that will utmost have been unnoticed if we do not consider families that do not exactly fit the inclusion criteria marked by SEOM guidelines.

The coexistence of variants either in the same or different CPGs is a common event since NGS has become the routine technique for genetic testing and should be recorded to implement a polygenic risk algorithm, which might explain the different phenotypes in families that do not adjust to a single hereditary cancer syndrome.

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CRediT authorship contribution statement

Mar Infante: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review and editing. **Mónica Arranz-Ledo:** Data curation, Formal analysis, Methodology, Software, Validation, Visualization. **Enrique Lastra:** Data curation, Funding acquisition, Project administration, Resources. **Amaya Olaverri:** Data curation, Resources. **Raquel Ferreira:** Data curation, Resources. **Marta Orozco:** Data curation, Resources. **Lara Hernández:** Formal analysis, Methodology, Software, Validation, Visualization. **Noemí Martínez:** Formal analysis, Methodology, Software, Validation, Visualization. **Mercedes Durán:** Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. All authors have read and agreed their individual contributions prior to submission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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