# Genetic evaluation of young adults with cancer

by

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## Abstract

The oligogenic coinheritance of heterozygous pathogenic variants (PVs) in cancer-related genes is a poorly studied event. Currently, due to the increment of cancer survivors and the the wide-spreading of next generation sequencing (NGS) methods, the probability of presenting multiple primary cancers (MPCs) and uncommon cancer-gene associations is higher.

This study included young MPCs patients and patients with single or MPCs and multiple PVs. NGS sequencing techniques of germline and tumoral DNA were used.

Ten patients with MPC were included in the study and presented a mean of three cancers per patient. Whole exome sequencing (WES) of the germline DNA identified 1-3 variants possibly related to the disease in each patient, and most of them were classified as variants of uncertain significance. Additionally, six patients from five MPCs families who coinherited PVs in two cancer predisposition genes, and three patients with metastatic colorectal cancer that were heterozygous for a previously known *BRCA1* nonsense variant were described.

The sequencing of patients with early cancers, family history and multiple tumors is already a standard of care. However, the growing evidence suggests that patient´s assessment should not stop at the identification of one PV in a cancer predisposition gene.

# Résumé

La co-hérédité oligogénique de variants pathogènes (VP) hétérozygotes dans les gènes liés au cancer est un événement peu étudié. Actuellement, en raison de l'augmentation du nombre de survivants du cancer et de la large diffusion des méthodes de séquençage de nouvelle génération (NGS), la probabilité de présenter plusieurs cancers primaires (PCP) et des associations rares de gènes de cancer est plus élevée.

Cette étude a inclus de jeunes patients atteints de PCP et des patients présentant un ou plusieurs PCP et plusieurs VP. Des techniques de séquençage NGS de l'ADN germinal et tumoral ont été utilisées.

Dix patients atteints de PCP ont été inclus dans l'étude et présentaient en moyenne 3 cancers par patient. Le WES de l'ADN germinal a identifié 1 à 3 variants possiblement liés à la maladie chez chaque patient, et la plupart d'entre eux ont été classés comme variants de signification incertaine. De plus, six patients de cinq familles PCP qui ont co-hérité de VP dans deux gènes de prédisposition au cancer, et trois patients atteints d'un cancer colorectal métastatique qui étaient hétérozygotes pour un variant non-sens *BRCA1* précédemment connu ont été décrits.

Le séquençage des patients présentant des cancers précoces, des antécédents familiaux et des tumeurs multiples constitue déjà une norme de soins. Cependant, de plus en plus de preuves suggèrent que l'évaluation du patient ne devrait pas s'arrêter à l'identification d'un variant pathogène dans un gène de prédisposition au cancer.

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On a personal note, I would like to express my deepest gratitude to my parents, Fausto Freire and Olga Chadrina, whose example inspired me to pursue a PhD. I owe my strength and perseverance to them.

I also wish to thank my best friend, Francisco Yanez, for his constant moral support throughout this journey, and my husband, Dimitri Astashka, for his patience, love, and unwavering support. Their belief in me has been my greatest source of motivation.

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## List of Abbreviations

| | |
|---|---|
| ACMG | American College of Medical Genetics |
| AD | Autosomal Dominant |
| AP | Apurinic/Apyrimidinic |
| AR | Autosomal Recessive |
| BCC | Basal Cell Carcinoma |
| BER | Base Excision Repair |
| BHD | Birt–Hogg–Dubé |
| CMA | Chromosomal Microarray Analysis |
| CNVs | Copy Number Variants |
| CPGs | Cancer Predisposition Genes |
| CPS | Cancer Predisposition Syndromes |
| CRCs | Colorectal Cancers |
| DDR | DNA Damage Response |
| DR | Direct Repair |
| DSBs | Double-strand Breaks |
| dx | Diagnosis |
| ExAC | Exome Aggregation Consortium |
| FA | Fanconi Anemia |
| FA/BRCA | Fanconi Anemia/Breast Cancer |
| FAP | Familial Adenomatous Polyposis |
| FFPE | Formalin-Fixed Paraffin-Embedded |
| FISH | Fluorescence in situ Hybridization |
| GG | Global Genome |
| GIST | Familial Gastrointestinal Stromal Tumor |
| GWAS | Genome-Wide Association Studies |
| HBOC | Hereditary Breast-Ovarian Cancer |
| HDGC | Hereditary Diffuse Gastric Cancer |
| HDR | Homology-Directed Repair |
| HLRCC | Hereditary Leiomyomatosis and Renal Cell Cancer |
| HR | Homologous Recombination |
| HRD | Homologous Recombination Deficiency |
| IARC | International Agency for Research on Cancer |
| ICL | Interstrand Crosslinking |
| LOH | Loss of Heterozygosity |
| MAP | *MUTYH*-Associated Polyposis |
| MEN1 | Multiple Endocrine Neoplasia Type 1 |
| MEN2 | Multiple Endocrine Neoplasia Type 2 |
| MINAS | Multilocus Inherited Neoplasia Alleles Syndrome |
| MMEJ | Microhomology Mediated End Joining |
| MMR | Mismatch Repair |
| MPCs | Multiple Primary Cancers |
| MPM | Multiple Primary Melanoma |
| MSI | Microsatellite Instability |
| NCCN | National Comprehensive Cancer Network |
| NER | Nucleotide Excision Repair |
| NF1 | Neurofibromatosis Type 1 |
| NGS | Next Generation Sequencing |
| NHEJ | Nonhomologous End Joining |
| OGM | Optical Genome Mapping |
| P/LP | Pathogenic or Likely Pathogenic |
| PARPi | Poly (ADP-ribose) Polymerase Inhibitor |
| PET-CT | Positron Emission Tomography-Computed Tomography |
| PGL/PCC | Hereditary Paraganglioma-Pheochromocytoma |
| PJS | Peutz-Jeghers Syndrome |
| PKcs | Protein Kinase catalytic subunit |
| PPAP | Polymerase Proofreading-Associated Polyposis |
| PRS | Polygenic Risk Scores |
| PVs | Pathogenic Variants |
| RCC | Renal Cell Carcinoma |

| | |
|---|---|
| ROS | Reactive Oxygen Species |
| RTK | Receptor Tyrosine Kinases |
| SEER | National Cancer Institute Surveillance, Epidemiology, and End Results Program |
| SNP | Single Nucleotide Polymorphism |
| SNVs | Single Nucleotide Variants |
| SSA | Single-Strand Annealing |
| SSBs | Single-Strand DNA Breaks |
| TC | Transcription-Coupled |
| TSC | Tuberous Sclerosis Complex |
| US | United States of America |
| UV-DDB | UV-Damaged DNA-Binding Protein |
| VHL | Von Hippel-Lindau |
| VUS | Variants of Uncertain Significance |
| WES | Whole Exome Sequencing |
| WGS | Whole-Genome Sequencing |
| XL | X-linked |

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# Introduction

## Multiple primary cancers

Cancer is a leading cause of premature death worldwide, and the global cancer burden is expected to exceed 27 million new cancer cases per year by 2040 (1). In recent years, advances in the diagnosis and treatment of cancer have increased the probability of survival of the patients, making the development of a subsequent cancer a growing concern (2).

### Definition

Multiple primary cancers (MPCs) are generally defined as two or more malignant tumors that are histologically distinct and arise in the same or a different organ (3). Historically, Renaud and Rokitansky reported the first MPC patient in 1855 (4). However, it was Theodor Billroth in 1889 who proposed the first diagnostic criteria for MPCs (5,6), followed by Warren and Gates (7) in 1932. Moertel considered Billroth's criteria to be too rigid, while Warren's criteria were subjected to an excessively liberal interpretation. Consequently, in 1977 Moertel published his classification (6) with examples of each category (see Table 1).

**Table 1.** Multiple primary cancers definition and classification according to different authors

**Billroth criteria, 1889**

1. Each tumor must have a distinct microscopic morphology.
2. Each tumor should generate its own metastases.

**Warren and Gates Criteria, 1932**

1. Each tumor must be histologically proven as malignant.
2. Each tumor must be anatomically distinct.
3. The probability of one being a metastatic lesion originating from the other must be ruled out.

**Moertel criteria, 1977**

I. Multiple primary malignant neoplasms of multicentric origin.
   A. The same tissue and organ (for example, multiple epitheliomas of the skin).

B. A common, contiguous tissue shared by different organs (for example, a squamous cell carcinoma of the pharynx and a squamous cell carcinoma of the larynx).
C. The same tissue in bilaterally paired organs (for example, bilateral breast cancer).

II. Multiple primary malignant neoplasms of different tissues or organs (for example, an adenocarcinoma of the breast and an osteogenic sarcoma, or a squamous cell carcinoma of the mouth and a squamous cell carcinoma of the cervix).

III. Multiple primary malignant neoplasms of multicentric origin plus a lesion(s) of a different tissue or organ (it is a combination of I and II).

The American Cancer Society in 2009 proposed a more detailed list of criteria (2) for considering each tumor as a separate primary. These conditions include:

- A cancer in a different site and histologic type from the original is considered a separate primary.
- Same-site cancers of different histological types are considered separate primaries regardless of the time of diagnosis.
- The threshold for considering a cancer of the same histology in the same site as a separate tumor is 2 months unless a recurrent or metastatic disease is specifically stated in the medical record.
- In paired organs, each one is considered a separate site.
- Most histological types of prostate and urinary bladder cancer are exceptions – multiple tumors are reported as a single primary in these cases.
- The rules to define MPCs in the lymphatic and hematopoietic systems are different.

The development of epidemiological cancer surveillance and the creation of cancer registries created a need for more strictly defined criteria for MPC. Currently, for reporting purposes two main sets of criteria are used: the National Cancer Institute Surveillance, Epidemiology, and End Results Program (SEER) (8) mainly in the United States of America (US) and the International Agency for Research on Cancer (IARC) (9) in the rest of the world. Both classifications use different rules for solid tumors and hematological malignancies.

The SEER generally defines as MPCs separate/non-contiguous tumors in different primary sites excluding metastasis and separate/non-contiguous tumors in the same primary site but of different histology regardless of time. The SEER and IARC classifications have some differences which are further explained in Table 2.

**Table 2.** The main differences between the SEER and IARC MPCs classification of solid tumors

| Criteria | National Cancer Institute Surveillance, Epidemiology, and End Results Program (SEER)(8) | International Agency for Research on Cancer (IARC)(9) |
|---|---|---|
| Topography codes of the International Classification of Diseases for Oncology | Each group is considered one site (e.g. tumors of different parts of the colon are considered MPCs) | Several groups are considered one site (e.g. the whole colon is considered one site) |
| The time between the original diagnosis/last recurrence and the new tumor | 5 years for breast, head, and neck cancer<br>3 years for urinary tissue, lung, and kidney cancer<br>1 year for colon and other cancers | Time is not a defining criterion |
| Paired organs or tissues | Separate/non-contiguous tumors in paired organs are considered MPCs | Only one tumor is recognized in each paired organ or tissue |
| Multifocal tumors | Can be considered MPCs depending on histology* | Are always considered one tumor |
| Skin cancer | Multiple skin tumors are considered MPCs (even with the same histology) | Only the first tumor of a specific histological type is considered an incident tumor during a person's lifetime |

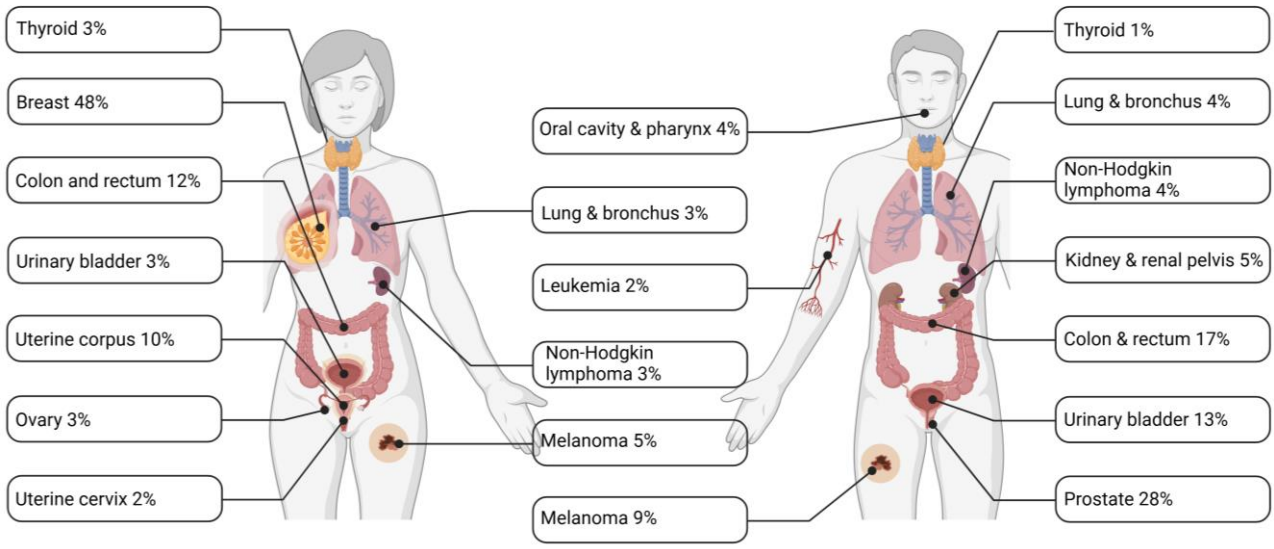
MPCs – multiple primary cancers. *Specific histological subtypes that are considered MPCs: micropapillary urothelial carcinoma and urothelial carcinoma of the bladder; at least one small cell carcinoma (or its subtypes/variants) combined with a non-small cell carcinoma (or its subtypes/variants) irrelevant of laterality; the combination of a first glial tumor followed by a glioblastoma multiforme; subsequent small cell carcinoma of the prostate >1 year following a diagnosis of acinar adenocarcinoma or its subtypes; follicular and papillary tumors in the thyroid gland diagnosed >60 days; anaplastic thyroid gland carcinoma combined with a tumor of any other histology.

MPCs can also be divided into synchronous and metachronous according to the time between the diagnosis of the tumors. SEER defines synchronous cancers as those present within 2 months since the diagnosis of the first tumor (8), while the IARC sets this threshold at 6 months (9).

## Epidemiology

The increased cancer survival due to improvements in early detection, supportive care, and treatment augmented over time the probability of developing more than one cancer (10,11). As of January 2005, 880 300 of the 11 million cancer survivors in the US were diagnosed with more than one cancer (2). Furthermore, the risk of developing a second primary malignancy is different in each cancer site (see Figure 1).

**Figure 1.** Ten most common primary sites among men and women diagnosed with more than one cancer alive as of January 1, 2005




*Note*: Adapted from (2) and created with BioRender.com.

The frequency of MPCs in epidemiological studies varies between 2-17% (12–16). The variation in the reported numbers may stem from differences in the applied definition of MPCs, the duration of follow-up (where longer observation periods are associated with an increased likelihood of subsequent cancer development), and the characteristics of the studied population (11).

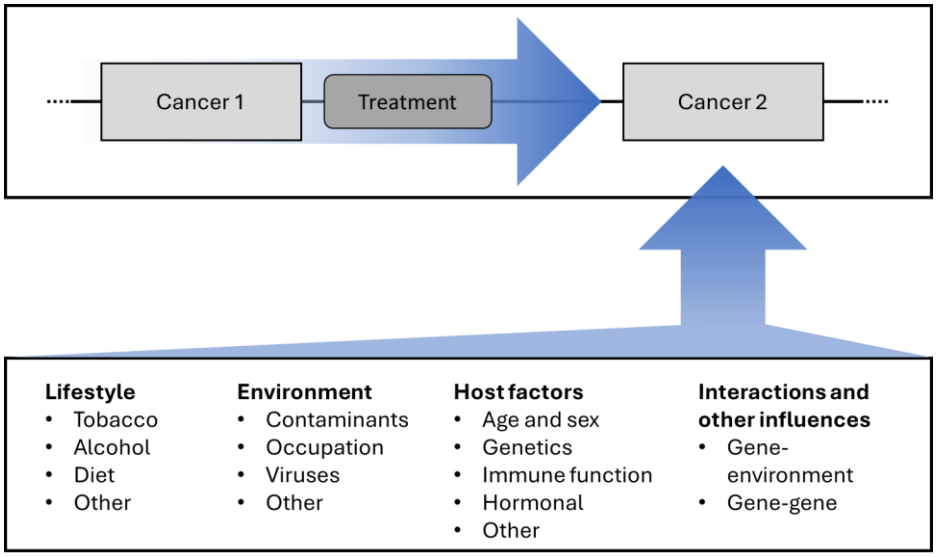
In the period 2004-2017, MPCs accounted for 12.2% of cases in Belgium. About 90% of these MPCs were a second primary cancer, while 9% were a third primary cancer. The fourth or higher primary cancer represented less than 1% of all the MPCs. In the same period, 67 344 (8.3%) of patients had at least two primary cancers, and 5 804 patients (8.6% of patients with at least two primary cancers) had the first

and second primary cancer diagnosed on the same day. The majority (70.2%) of MPC patients in Belgium had the same cancer site for their first and second cancer, primarily breast and colorectal cancer (CRC) for women and CRC and head and neck cancers for men (17).

## Risk factors

The probability of developing MPCs in the cancer patient population is higher than the incidence of primary malignancies in the general population. In most of the patients from the National Cancer Institute of the US registry who survived their first cancer, a second cancer was expected even if the cancer survivors had the same risk of disease as the general population. The likelihood of a person developing more than one cancer depends on the initial cancer type (the first primary site), treatment, lifestyle factors, environment, host factors, and gene-gene or gene-environment interactions (2) (See Figure 2).

**Figure 2.** Multiple primary cancer risk factors




The previous cancer diagnosis and treatment along with other risk factors affect the development of subsequent cancers. *Note*: Adapted from (18).

### Lifestyle factors

Lifestyle risk factors for the development of MPCs include smoking status, alcohol consumption, and dietary patterns, among others. Tobacco and tobacco smoke contain over 9500 chemical compounds, 83 of which are known carcinogens (19). Exposure to these substances over time can trigger field cancerization, leading to the formation of multiple patches of transformed cells, mainly in the respiratory and urinary systems. These cells can later evolve into second or subsequent cancers (20).

Smoking is a well-established risk factor for developing new malignancies and cancer recurrence (21). Both former and current smokers face elevated risks of developing MPCs compared to those who have

never smoked (22). Furthermore, individuals who continued smoking after their initial cancer diagnosis exhibited a 35% increased risk of developing smoking-related cancers (23).

Similar to tobacco, alcoholic beverages contain many carcinogens, with ethanol serving as their primary carcinogenic compound. The IARC classified alcohol consumption and ethanol in alcoholic beverages as carcinogenic (24). Alcohol consumption was linked to various cancers, including those of the oral cavity, oro- and hypopharynx, larynx, esophagus, colon, rectum, liver, intrahepatic bile duct, and female breast (1). Alcohol intake contributes to 8% of cancer incidence in France (25), 3% in Australia (26), and 4% in the UK (27).

**Environmental influences**

Environmental factors such as geography, contamination, pathogens, and occupation play significant roles in cancer development. Areas of radon exposure have shown an increased risk of lung cancer. Additionally, infections caused by pathogens such as human papillomavirus and Epstein-Barr virus have been linked to cancer development. Certain professions, like those involving asbestos handling, are linked to cancers such as mesothelioma (28). In France, infections ranked fifth (accounting for 4.0% of cancer incidence), while environmental exposures ranked sixth (3.6% of cancer incidence) among the leading causes of cancer in 2015 (25).

**Host factors**

Host risk factors associated with developing MPCs encompass age, sex, genetics, immune function, hormonal profile, and other factors. Age is a non-modifiable risk factor for MPC development. Moreover, among individuals who have survived cancer, one of the leading causes of morbidity and mortality was the emergence of a second cancer (29). Studies have revealed that people who survived childhood cancer had a six-fold increased risk of developing cancer compared to the general population (30). In the US, those diagnosed with cancer before the age of 18 had 9.4% higher odds of having two or more cancers ($p = 0.0057$) compared to the reference age group (18-29 years). In the same study, the individuals who developed their first cancer at 30-49 years and 50-64 years had 20.5% and 13.7% higher odds of developing more than one cancer ($p < 0.0001$ in both cases). On the contrary, people who developed their first cancer after 65 years old had 11.6% lower odds of having MPCs than those 18-29 years old ($p < 0.0001$) (22).

Gender is another non-modifiable host risk factor significantly associated with the number of cancers (22). Data from 1995-2008 revealed higher rates of MPCs among females compared to males, with 17.2% according to SEER rules and 14.5% according to IARC rules for females versus 15.8% (SEER) and 14.4% (IARC) for males (14). Furthermore, individuals undergoing prolonged immunosuppression, such as organ transplant recipients, face a higher risk of developing skin, lung, and head and neck cancer (31).

## Diagnosis

The development of new imaging methods allowed the early detection of tumors that were previously ignored. The techniques that allow a better view of soft tissue, such as positron emission tomography-computed tomography (PET-CT) have enhanced the detection of additional malignant tumors. After a PET-CT scan in 1912 patients, 4.1% of suspicious lesions were identified, 1.2% of which were histologically confirmed second primaries (32). These tumors were in the thyroid, colon, breast, esophagus, bile duct, and head and neck. In 200 patients who underwent a PET-CT scan for esophageal cancer staging, a synchronous MPC was identified in 17% of them (33).

An atypical metastatic spread is one of multiple clinical characteristics that can indicate a high possibility of MPCs. Tumor metastases can be atypical in terms of their characteristics or site (prostate cancer with radiologically lytic bone metastases), number (head and neck cancer with a single pulmonary nodule), and chronology (new metastatic spread years after initial diagnosis). Other features that may suggest a high likelihood of MPCs are the recurrence in patients exposed to environmental carcinogens, atypical values in tumor markers (prostate cancer with low prostate-specific antigen and extensive liver metastases), and imaging (discovery of suspicious lesions during staging or follow-up). Previous treatment of the individuals is also important. Hematological malignancy in patients with previous chemotherapy or secondary malignancies (especially if in the same site) after radiotherapy can be a sign of high MPC risk (28).

When MPCs are suspected, histological confirmation is important. When the primary cancer tissue is available for diagnosis, it makes the diagnosis easier, especially in cases of undifferentiated histology (28).

## Screening and genetic counseling

Cancer genetic risk assessment and counseling involve a comprehensive process to identify and advise high-risk cancer patients and their families. Advancements in cancer genetics, including the expanded adoption of multigene panel testing, have transformed the clinical approach to screening high-risk patients and their families. Strategies that streamline the screening process are the search for a known pathogenic variant (PV) identified in a relative or testing of individuals with early-onset disease, multiple relatives with cancer, or MPCs (See Table 3). Generally, genetic testing is recommended for those with personal or family history suggestive of genetic cancer susceptibility when the results will aid in risk management and treatment (34).

**Table 3.** Criteria for considering genetic testing

- A relative presenting a confirmed pathogenic germline variant associated with increased susceptibility to cancer.
- Diagnosis of cancer at an early age, such as colon cancer before age 50.
- Occurrence of multiple distinct cancer types in the same individual.
- Cancer diagnoses across multiple relatives, particularly with patterns such as:
    - Several first-degree relatives affected by the same type of cancer.
    - Family history, including breast or ovarian cancer.
    - Family history, including both colon and endometrial cancer.
- Cancer affecting both paired organs, such as the two kidneys or breasts.
- Diagnosis of male breast cancer, adrenocortical carcinoma, carcinoid tumors, diffuse gastric cancer, fallopian tube/primary peritoneal cancer, leiomyosarcoma, medullary thyroid cancer, paraganglioma/pheochromocytoma, renal cell carcinoma of chromophobe, hybrid oncocytotic, or oncocytoma histology, sebaceous carcinoma, or sex cord tumors with annual tubules.
- Presence of birth defects linked to recognized inherited cancer syndromes, such as specific non-cancerous conditions or physical anomalies.
- Being a member of a racial or ethnic group with documented increased susceptibility to specific inherited cancer syndromes, and having one or more of the above features as well

*Note:* Adapted from (35,36)

Predictive genetic testing identifies inherited gene PVs, enabling the prediction of an individual's susceptibility to specific types of cancer and future disease risks (37). Unlike traditional diagnostic tests, these assessments are designed to detect potential health issues before the onset of symptoms, allowing for the early identification of individuals at risk (38)

Multigene testing can detect pathogenic or likely pathogenic (P/LP) variants that remain undetected by single-gene testing. This strategy is particularly beneficial when multiple genes could explain an inherited cancer syndrome, making phenotype-directed testing through a multigene panel more efficient and/or cost-effective based on personal and family history. It may also be considered for individuals with suggestive family history despite negative testing for a specific syndrome (34,39–41).

Genetic counseling for patients with MPCs should adhere to several key principles. Firstly, if a PV is identified, relatives should be offered testing for that specific PV, and subsequent counseling should be tailored accordingly. In instances where no PV is found, counseling should be based on the individual tumor types observed in the proband, rather than the combination. For instance, if a woman has experienced multiple primary colon and breast cancers, her daughters should be provided with appropriate screening for both colon and breast cancer, tailored to the age at which their mother was diagnosed (42). Table 4 includes common tumor sites and the criteria that warrant assessment for cancer predisposition with the most frequent genes.

**Table 4.** Assessment of cancer predisposition by tumor type

| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| Basal cell carcinoma (BCC) | >5 cumulative BCCs or BCC dx at age <30 and one additional nevoid basal cell carcinoma syndrome criterion (See Table 7) in the same person | Nevoid basal cell carcinoma syndrome | AD | *PTCH1*<br>*SUFU* | 109400<br>607035 |
| Brain | Brain tumor dx at age <18 if any of the following criteria are met:<br>–Café-au-lait macules and/or other signs of NF1, or hypopigmented skin lesions<br>–Consanguineous parents<br>–Family history of Lynch syndrome-associated cancer<br>–Second primary cancer<br>–Sibling with a childhood cancer | Constitutional mismatch repair deficiency syndrome | AR | *MLH1, PMS2, MSH6, MSH2* | 276300, 600259, 600678, 609309 |
| | Brain tumor and two additional cases of any Lynch syndrome-associated cancer (See Table 8) in the same person or relatives | Lynch syndrome | AD | *MSH2, MLH1, PMS2, MSH6* | 120435, 120436, 600259, 600678 |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| | Brain tumor and one additional Li-Fraumeni syndrome tumor (See Table 9) in the same person or two relatives, one dx at age ≤45 | Li-Fraumeni syndrome | AD | *TP53* | 151623 |
| | Astrocytoma and melanoma in the same person or two first-degree relatives | Melanoma-astrocytoma syndrome | AD | *CDKN2A* | 155755 |
| | Subependymal giant cell astrocytoma and one additional Tuberous sclerosis complex criterion (See Table 10) in the same person | Tuberous sclerosis complex | AD | *TSC1, TSC2* | 191100, 191092 |
| | Medulloblastoma and ≥10 cumulative adenomatous colon polyps in the same person | Familial adenomatous polyposis | AD | *APC* | 175100 |
| | Medulloblastoma (Primitive Neuro Ectodermal Tumors) dx at age <18 and one additional nevoid basal cell carcinoma syndrome criterion (See Table 7) in the same person | Basal cell nevus syndrome | AD | *PTCH1* | 109400 |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| Breast cancer, female | Breast cancer dx at age ≤50<br>Triple-negative breast cancer dx at age ≤60<br>≥2 primary breast cancers in the same person | Hereditary breast and ovarian cancer syndrome | AD | *BRCA1, BRCA2, PALB2* | 604370, 612555, 610355 |
| | Ashkenazi Jewish ancestry and breast cancer at any age<br>≥3 cases of breast, ovarian, pancreatic, and/or aggressive prostate cancer in close relatives, including the patient<br>Breast cancer and one additional Li-Fraumeni syndrome tumor (See Table 9) in the same person or in two relatives, one dx at age ≤45 | Li-Fraumeni syndrome | AD | *TP53* | 151623 |
| | Breast cancer and ≥1 Peutz-Jeghers polyp in the same person | Peutz-Jeghers Syndrome | AD | *STK11* | 175200 |
| | Lobular breast cancer and diffuse gastric cancer in the same person<br>Lobular breast cancer in one relative and diffuse gastric cancer in another, one dx at age <50 | Diffuse gastric and lobular breast cancer syndrome | AD | *CDH1* | 137215 |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| | Breast cancer and two additional Cowden syndrome criteria (See Table 11) in the same person | Cowden syndrome | AD | *PTEN* | 158350 |
| Breast cancer, male | Single case present | Hereditary breast and ovarian cancer syndrome | AD | *BRCA1, BRCA2, PALB2* | 604370, 612555, 610355 |
| Colorectal cancer | Colorectal cancer dx at age <50<br>Colorectal cancer dx at age ≥50 if there is a first-degree relative with colorectal or endometrial cancer at any age<br>Synchronous or metachronous colorectal or endometrial cancers in the same person | Lynch syndrome | AD | *MSH2, MLH1, PMS2, MSH6* | 120435, 120436, 600259, 600678 |
| | Colorectal cancer showing mismatch repair deficiency on tumor screening | Mismatch repair cancer syndrome | AR | *MLH1* | 276300 |
| | Colorectal cancer and two additional cases of any Lynch syndrome-associated cancer (See Table 8) in the same person or close relatives | *MUTYH*-associated polyposis | AR | *MUTYH* | 608456 |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| | Colorectal cancer and two additional Cowden syndrome criteria (See Table 11) in the same person | Cowden syndrome | AD | *PTEN* | 158350 |
| | Colorectal cancer and one additional Li-Fraumeni syndrome tumor (See Table 9) in the same person or two relatives, one dx at age ≤45 | Li-Fraumeni syndrome | AD | *TP53* | 151623 |
| | Colorectal cancer with ≥10 cumulative adenomatous colon polyps in the same person | *MUTYH*-associated polyposis | AR | *MUTYH* | 608456 |
| | | Familial adenomatous polyposis | AD | *APC* | 175100 |
| Endometrial cancer | Endometrial cancer dx at age <50<br>Endometrial cancer dx at age ≥50 if there is a first-degree relative with colorectal or endometrial cancer at any age<br>Synchronous or metachronous colorectal or endometrial cancer in the same person | Lynch syndrome | AD | *MSH2, MLH1, PMS2, MSH6* | 120435, 120436, 600259, 600678 |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| | Endometrial cancer showing mismatch repair deficiency on tumor screening<br>Endometrial cancer and 2 additional cases of any Lynch syndrome-associated cancer (See Table 8) in the same person or in close relatives | | | | |
| | Epithelial endometrial cancer and two additional Cowden syndrome criteria (See Table 11) in the same person | Cowden syndrome | AD | *PTEN* | 158350 |
| Gastric cancer | ≥2 cases of gastric cancer, one dx at age <50 in close relatives<br>≥3 cases of gastric cancer in close relatives<br>Diffuse gastric cancer dx at age <40<br>Diffuse gastric cancer and lobular breast cancer in the same person<br>Diffuse gastric cancer in one relative and lobular breast cancer in another, one dx at age <50 | Diffuse gastric and lobular breast cancer syndrome | AD | *CDH1* | 137215 |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| | Gastric cancer and 2 additional cases of any Lynch syndrome -associated cancer (See Table 8) in the same person or in close relatives | Lynch syndrome | AD | *MSH2, MLH1, PMS2, MSH6* | 120435, 120436, 600259, 600678 |
| Leukemia | Leukemia dx at age <18, if any of the following criteria are met:<br>-Café-au-lait macules and/or other signs of NF1, or hypopigmented skin lesions<br>-Consanguineous parents<br>-Family history of Lynch syndrome-associated cancers<br>-Second primary cancer<br>-Sibling with childhood cancer | Constitutional mismatch repair deficiency syndrome | AR | *MLH1, PMS2, MSH6, MSH2* | 276300, 600259, 600678, 609309 |
| | Leukemia and one additional Li-Fraumeni syndrome (See Table 9) in the same person or 2 close relatives, one dx at age ≤45 | Li-Fraumeni syndrome | AD | *TP53* | 151623 |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| Melanoma | ≥3 cases of melanoma and/or pancreatic cancer in close relatives<br>≥3 primary melanomas in the same person<br>Melanoma and pancreatic cancer in the same person<br>Melanoma and astrocytoma in the same person or 2 first-degree relatives | Familial atypical mole and malignant melanoma | AD | *CMM1* | 155600 |
| | | Melanoma-astrocytoma syndrome | AD | *CDKN2A* | 155755 |
| Ovarian/Fallopian tube/primary peritoneal cancer | Single case present in the patient or a first-degree relative | Hereditary breast and ovarian cancer syndrome | AD | *BRCA1, BRCA2, PALB2* | 604370, 612555, 610355 |
| | | Lynch syndrome | AD | *MSH2, MLH1, PMS2, MSH6* | 120435, 120436, 600259, 600678 |
| Pancreatic cancer | Pancreatic cancer dx at any age, if any of the following criteria are met:<br>-≥2 cases of pancreatic cancer in close relatives | Hereditary breast and ovarian cancer syndrome | AD | *BRCA1, BRCA2, PALB2* | 604370, 612555, 610355 |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| | -≥2 cases of breast, ovarian, and/or aggressive prostate cancer in close relatives<br>-Ashkenazi Jewish ancestry | | | | |
| | Pancreatic cancer and ≥1 Peutz–Jeghers polyp in the same person | Peutz-Jeghers Syndrome | AD | *STK11* | 175200 |
| | Pancreatic cancer and two additional cases of any Lynch syndrome -associated cancer (See Table 8) in the same person or close relatives | Lynch syndrome | AD | *MSH2, MLH1, PMS2, MSH6* | 120435, 120436, 600259, 600678 |
| | ≥3 cases of pancreatic cancer and/or melanoma in close relatives<br>Pancreatic cancer and melanoma in the same person | Familial atypical mole and malignant melanoma | AD | *CMM1* | 155600 |
| Prostate cancer | ≥2 cases of prostate cancer dx at age ≤55 in close relatives<br>≥3 first-degree relatives with prostate cancer | Hereditary prostate cancer | AD<br><br>XL | *RNASEL*<br>*HOXB13*<br>*AR*<br>*Xq27-q28* | 601518<br>610997<br>176807<br>300147 |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| | Aggressive (Gleason score >7) prostate cancer and ≥2 cases of breast, ovarian, and/or pancreatic cancer in close relatives | Hereditary breast and ovarian cancer syndrome | AD | *BRCA1, BRCA2, PALB2* | 604370, 612555, 610355 |
| Renal cancer | RCC with clear cell histology, if any of the following criteria are met:<br>-dx at age <50<br>-Bilateral or multifocal tumors<br>-≥1 close relative with clear cell RCC | von Hippel-Lindau syndrome | AD | *VHL* | 193300 |
| | | Birt-Hogg-Dube syndrome | AD | *FLCN* | 135150 |
| | RCC with papillary type 1 histology | Hereditary papillary renal cancer | | *PRCC* | 605074 |
| | | | | *MET* | 605074 |
| | RCC with papillary type 2 histology<br>RCC with collecting duct histology<br>RCC with tubulopapillary histology | Hereditary leiomyomatosis and renal cell cancer | AD | *FH* | 150800 |
| | RCC with Birt-Hogg-Dube-related histology (chromophobe, oncocytoma, oncocytic hybrid) | Birt-Hogg-Dube syndrome | AD | *FLCN* | 135150 |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| | Urothelial carcinoma (or transitional cell carcinoma) and 2 additional cases of any Lynch syndrome-associated cancer (See Table 8) in the same person or in relatives | Lynch syndrome | AD | *MSH2, MLH1, PMS2, MSH6* | 120435, 120436, 600259, 600678 |
| | RCC and 2 additional Cowden syndrome criteria (See Table 11) in the same person | Cowden syndrome | AD | *PTEN* | 158350 |
| | Angiomyolipomas of the kidney and one additional Tuberous sclerosis complex criterion (See Table 10) in the same person | Tuberous sclerosis complex | AD | *TSC1, TSC2* | 191100, 191092 |
| Thyroid cancer | Medullary thyroid cancer, OMIM | Multiple endocrine neoplasia type 2 | AD | *RET* | 171400, 155240, 162300 |
| | Nonmedullary thyroid cancer and one additional Carney complex criterion in the same person:<br>-Spotty skin pigmentation on lips, conjunctiva and inner | Carney complex | AD | *PRKAR1A* | 160980 |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| | or outer canthi, and/or vaginal or penile mucosa<br>-Myxoma (cutaneous and mucosal)<br>-Cardiac myxoma<br>-Breast myxomatosis or fat-suppressed magnetic resonance imaging findings suggestive of this diagnosis<br>-Acromegaly due to growth hormone–producing adenoma<br>-Large cell calcifying Sertoli cell tumor or characteristic calcification on testicular ultrasonography<br>-Primary pigmented nodular adrenocortical dysplasia<br>-Thyroid carcinoma (nonmedullary) or multiple hypoechoic nodules on thyroid ultrasonography in a young patient<br>-Psammomatous melanotic schwannoma<br>-Blue nevus, epithelioid blue nevus (multiple)<br>-Breast ductal adenoma (multiple)<br>-Osteochondromyxoma | | | | |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| | Nonmedullary thyroid cancer and 2 additional Cowden syndrome criteria (See Table 11) in the same person | Cowden syndrome | AD | *PTEN* | 158350 |
| | Papillary thyroid cancer (cribriform-morular variant) | Familial adenomatous polyposis | AD | *APC* | 175100 |
| Sarcoma (non-Ewing sarcoma) | Sarcoma and one additional Li-Fraumeni syndrome tumor (See Table 9) in the same person or in 2 close relatives, one dx at age ≤45<br>-Sarcoma dx at age <18 | Li-Fraumeni syndrome | AD | *TP53* | 151623 |
| Pheochromocytoma/ paraganglioma | Single case present in the patient or a first-degree relative | Hereditary pheochromocytoma/ paraganglioma syndrome | AD<br>AD<br>AD<br>AD<br>AD<br>AD | *SDHB*<br>*SDHD*<br>*SDHC*<br>*SDHAF2*<br>*MAX*<br>*TMEM127* | 115310<br>168000<br>605373<br>601650<br>154950<br>613403 |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

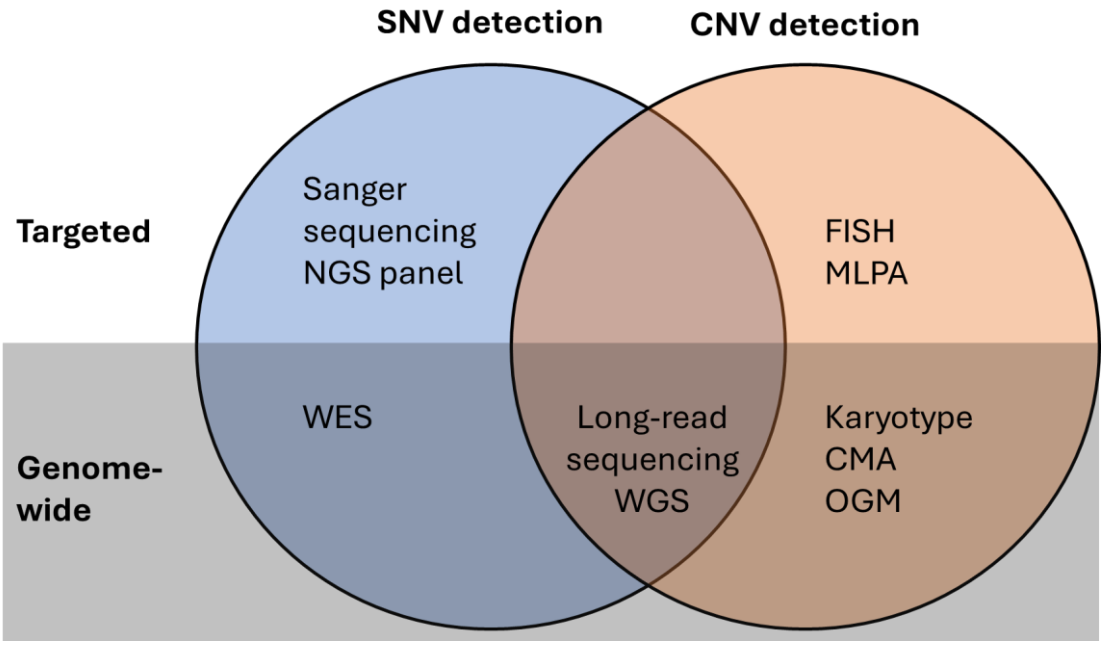
| Cancer in the patient or first-degree relative | Genetic counseling reference criteria | Syndrome(s) to consider | Inheritance | Gene/locus | OMIM |
|---|---|---|---|---|---|
| | | von Hippel-Lindau syndrome | AD | *VHL* | 193300 |
| | | Multiple endocrine neoplasia type 2 | AD | *RET* | 171400 |
| Retinoblastoma | Single case present in the patient or a first-degree relative | Hereditary retinoblastoma | AD | *RB1* | 180200 |

AD – autosomal dominant, AR – autosomal recessive, BCC – Basal cell carcinoma, dx – diagnosis, NF1 – neurofibromatosis type 1, RCC – renal cell carcinoma, XL – X-linked. *Note:* adapted from: (57)

## Genetic testing techniques

Genetic testing techniques for cancer predisposition include cytogenetics, microarrays, DNA and RNA sequencing (See Figure 3). Classical cytogenetic studies rely on two main methods: karyotyping and fluorescence in situ hybridization (FISH). These two methods involve microscopic examination of chromosomes in individual cells or tissues fixed on a microscope slide (43). A karyotype is a visual representation of the number and structure of chromosomes, offering a low-resolution, genome-wide screening method for detecting chromosomal variants. This method is limited to living, actively dividing cells analyzed during metaphase, when chromosomes are at their most condensed state. A karyotype can identify numerical and structural chromosomal abnormalities visible under a microscope, such as gains or losses of chromosomes, as well as deletions, insertions, duplications, and translocations spanning approximately 5–10 megabases. Notably, this method enables direct evaluation of genomic complexity, clonal evolution, and unrelated clones within individual cells, facilitating the classification of abnormalities within a clone and distinguishing between related or unrelated clones. A key advantage of a karyotype lies in its ability to detect clones present at very low frequencies in a sample, particularly in myeloid neoplasms (43,44).

**Figure 3.** Overview of genetic testing techniques




CMA – Chromosomal Microarray, CNV – Copy Number Variant, FISH – fluorescence in situ hybridization, MLPA – Multiplex Ligation-Dependent Probe Amplification, NGS – Next-Generation Sequencing, OGM – Optical Genome Mapping, SNV – Single Nucleotide Variant, WES – Whole Exome Sequencing, WGS – Whole Genome Sequencing.

FISH utilizes fluorescently labeled DNA probes specifically designed to target recurrent, clinically significant chromosomal rearrangements or copy number variations. Unlike karyotyping, FISH does not provide an overview of the entire chromosomal composition of a cell but focuses solely on genomic regions complementary to the chosen probes. This technique complements karyotyping by enabling analysis in non-dividing cells, including paraffin-embedded tissues, and delivering rapid detection of specific chromosomal or genetic abnormalities. Such findings can assist in diagnosing conditions like gene fusions in leukemias and gene rearrangements in lymphoma. With higher resolution than karyotyping, FISH can detect submicroscopic abnormalities and permits the rapid examination of numerous cells. This capability is particularly advantageous for identifying low-level clones, such as in cases of minimal residual disease or early relapse (43,45).

DNA microarrays are used to identify copy number alterations throughout the genome. This testing can be performed using two distinct approaches: chromosomal microarray (CMA, previously known as array-CGH) and single nucleotide polymorphism (SNP) arrays.

CMA provides valuable complementary information to karyotype and FISH analyses. In certain tumors where copy number alterations, rather than gene rearrangements, play a pivotal role in disease management (such as neuroblastoma, Wilms tumor, and most central nervous system tumors) CMA may serve as the primary diagnostic tool. By hybridizing tumoral DNA to whole-genome copy number or SNP microarrays, DNA gains, losses, and amplifications that might go unnoticed with conventional cytogenetic techniques are detected. Furthermore, SNP probes allow the identification of regions with copy-neutral loss of heterozygosity, which could contain critical tumor-related genes. CMA offers a resolution of several hundreds to thousands of base pairs, depending on the number of probes used (46,47).

In CMA, patient and reference DNA are labeled with distinct fluorescent dyes, typically green for patient DNA and red for reference DNA. An array slide is prepared with oligonucleotide DNA probes—short DNA sequences designed to hybridize to specific genomic regions. The probes are unevenly distributed, with some providing general "backbone" coverage and others focusing on gene-rich areas, syndrome-associated regions, or specific genes of interest. The labeled DNA samples are applied to the array slide, where they competitively bind to the probes. Following hybridization, the slide is washed to remove unbound DNA and scanned to measure fluorescence at each probe location. The intensity and color of the fluorescence are analyzed by computer software: yellow indicates equal amounts of patient and reference DNA, red signifies more reference DNA than patient DNA (suggesting a deletion), and green represents more patient DNA than reference DNA (suggesting a duplication) (48).

A SNP array slide is prepared using allele-specific DNA probes designed to target regions of SNP variation among individuals. Patient DNA is hybridized to the slide, where it binds to the probes.

Following hybridization, the slide is scanned to measure the fluorescence at each probe location. The intensity of the fluorescence indicates the alleles present at the targeted SNP sites in the patient's genome. Specialized software analyzes this data to identify regions of copy number variation by evaluating the nucleotides present at each SNP site. At each SNP, an individual may be heterozygous (AB or BA) or homozygous (AA or BB). Regions with deletions are characterized by a loss of signal intensity and the appearance of homozygous SNPs across the affected area. In contrast, regions with duplications exhibit altered allele ratios compared to normal regions, leading to an increase in signal intensity (48).

Hybrid arrays integrate SNP and oligonucleotide probes to overcome the uneven genomic distribution of SNPs, which can create coverage gaps in standard SNP arrays. By incorporating oligonucleotide probes alongside SNP-specific probes, these arrays achieve more uniform genomic coverage. Additionally, this design allows for targeted analysis of regions or genes associated with specific syndromes, thereby increasing their value for both diagnostic and research purposes (48).

Sequencing technologies enable the determination of nucleic acid sequences. To date, three generations of sequencing technologies can be distinguished. The first generation includes methods developed in the mid-1970s, such as the Maxam-Gilbert chemical degradation method (49) and the Sanger method (50), which relies on polymerase arrest using dideoxynucleotides. The second generation encompasses high-performance sequencing technologies commercialized in the mid-1990s, which, despite varying principles, require signals derived from multiple identical DNA molecules (short-read sequencing). These include sequencing by hybridization (Affymetrix, now largely displaced) (51), sequencing by ligation (Polonator, by Dover/Harvard), pyrosequencing (currently discontinued), sequencing by synthesis (Illumina), and ion semiconductor sequencing (Ion Torrent, Thermo Fisher Scientific) (52). More recently, third-generation sequencing (long-read sequencing) technologies have emerged such as RS II system from Pacific Biosciences (Menlo Park, CA, USA) and Oxford Nanopore Technologies (ONT, Oxford, UK), capable of detecting signals from single nucleic acid molecules (53).

Sanger sequencing, short-read sequencing, and long-read sequencing, are highly effective for detecting various genetic alterations, including single-nucleotide variants (SNVs), copy number variants (CNVs), and rearrangements. They have a range of potential clinical applications, including whole genome sequencing (WGS), whole exome sequencing (WES), gene panel testing, and single-gene testing. RNA sequencing is particularly efficient for identifying gene fusions, while DNA sequencing can detect SNVs, CNVs, and fusions depending on the assay design. Long-read sequencing, which requires fresh or frozen tissue samples, can be clinically applied to identify large or complex structural rearrangements and to assess methylation status (46).

Optical genome mapping (OGM) is a cytogenomic tool that facilitates genome-wide analysis of copy number alterations, balanced rearrangements (such as translocations, inversions, and insertions), and complex structural rearrangements with high resolution. This method relies on the use of linearized strands of high molecular weight DNA, which are much longer than the DNA sequences analyzed by current second- and third-generation sequencing technologies, achieving average read lengths greater than 200 kilobases. In comparison, the RS II system from Pacific Biosciences has an average read length of 10–16 kilobases, while Oxford Nanopore Technologies devices are practically limited to about 20 kilobases when significant human genome coverage is required. However, with substantial effort, an N50 read length of 100 kilobases can be achieved for low-coverage human genome analysis. The use of long DNA molecules in OGM facilitates the mapping of repetitive and complex regions more effectively than with shorter molecules. This capability allows for the creation of genome maps that can span entire chromosomal arms while still detecting insertions and deletions as small as 500 bp. Other CNVs require a minimum size of 30 kilobases to be detected (46,47).

Finally, there are polymerase chain reaction (PCR)-derived methods also used in the evaluation of cancer predisposition. These include testing for MSI using PCR (54) and multiplex ligation-dependent probe amplification (MLPA) evaluation of cancer predisposition genes such as *BRCA1* and *BRCA2* in breast cancer patients (55).

MLPA employs pairs of oligonucleotide probes that can be multiplexed in a single reaction, enabling the screening of all exons in a gene, for example. Each probe pair binds exclusively to its target sequence, and if the probes are adjacent, their ends ligate. Each probe in the pair contains a primer binding site at its end, so PCR amplification occurs only if the probes are ligated. The resulting amplification products are separated by size using capillary electrophoresis. The amount of amplification product correlates with the quantity of the target sequence in the sample. By comparing the peak heights of the amplification products from the sample with those from a reference sample with a known copy number, dosage abnormalities such as exon deletions or duplications can be identified (56).

## Genomic instability and DNA damage response

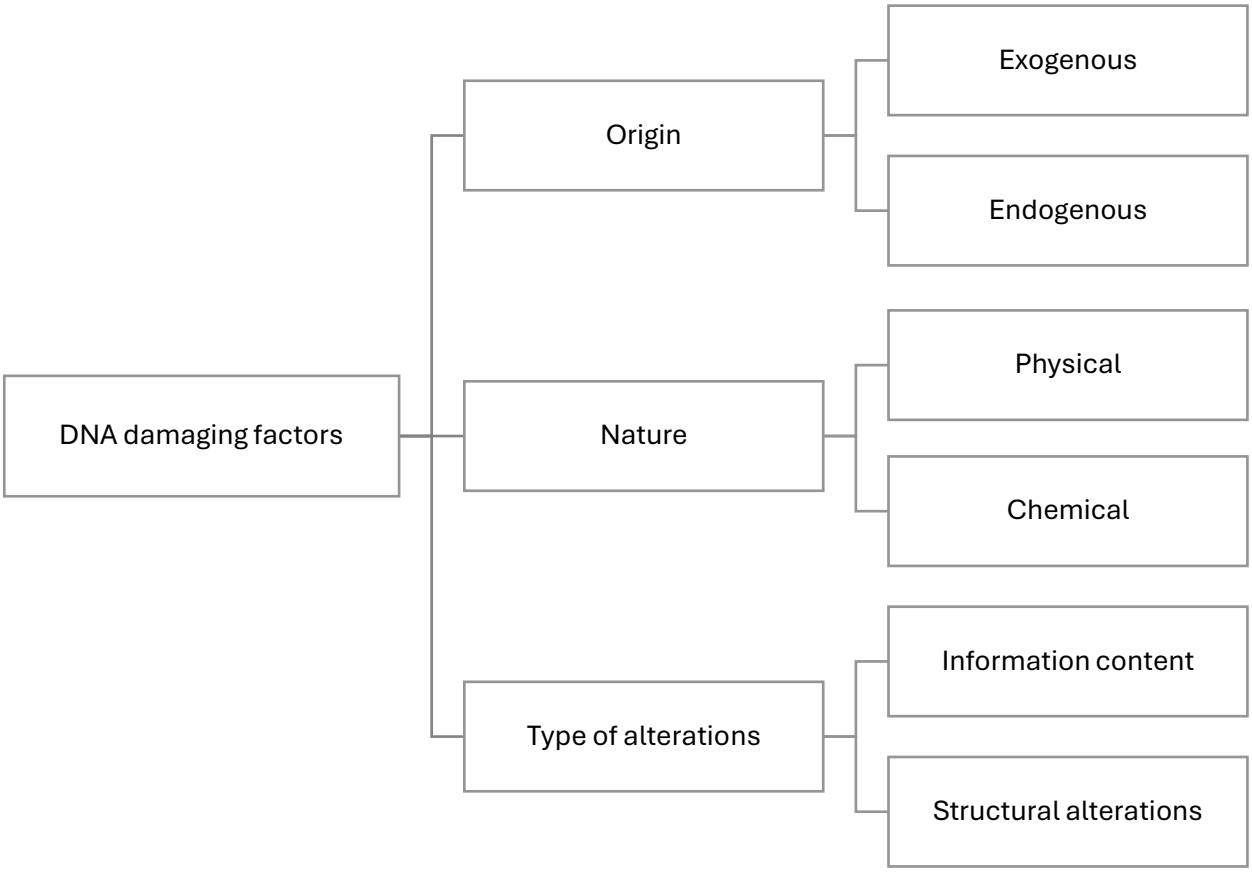
Preserving genomic stability is critical for cell survival and reproduction. Damage to the DNA molecule can compromise genomic stability, leading to a higher-than-normal rate of mutation in the cells (genomic instability), and inducing carcinogenesis. This process comprises a cascade response of triggering proto-oncogenes (e.g. *EGFR*, *MYC,* and *RAS* families) while simultaneously suppressing anti-oncogenes (e.g. *TP53*) (58–60).

## DNA damage

DNA damage can occur through either a direct or indirect pathway, both culminating in the disruption of the molecule's chemical bonds and the alteration of DNA's structure and properties. Direct damage occurs when either endogenous or exogenous materials directly contact with the molecule. At the same time, in the indirect pathway, these substances activate other products (e.g. free radicals) that subsequently damage DNA. Nonetheless, certain DNA-damaging agents, like UV light, can act as both direct and indirect agents (via reactive oxygen species) (59–61).

DNA damage factors vary in their origins, nature, and the types of alterations they generate (See Figure 4).

**Figure 4.** Types of DNA damage factors



Endogenous factors, such as oxidative phosphorylation, tend to be more severe and/or extensive compared to exogenous factors (e.g. environmental influences). Moreover, endogenous factors are more common than exogenous ones (See Table 5). Eukaryotic cells typically lose several thousand bases daily due to base hydrolysis alone. Based on their nature, DNA damage factors can be divided into physical (such as UV radiation, ionizing radiation, and, under certain circumstances, infrared, microwaves, and radio waves) and chemicals (including alkylating agents, oxidizing agents, and chemicals that create DNA-DNA or DNA-protein crosslinks). Content-altering agents typically act at a nucleotide level (e.g. mismatch), while structure-altering agents affect higher-order structures (such as Z-conformation or triple-strand DNA). Finally, cytotoxic agents damage the entire cell, whereas genotoxic ones specifically target DNA (62).

**Table 5.** Endogenous and exogenous DNA damaging agents

| Endogenous | Exogenous |
|---|---|
| Replication errors | Ionizing radiation |
| DNA base mismatches | UV radiation |
| Topoisomerase-DNA complexes | Chemical agents |
| Spontaneous base deamination | ▪ Alkylating agents |
| Abasic sites | - Dietary components |
| Oxidative DNA damage | - Tobacco smoke |
| DNA methylation | - Biomass burning |
| | - Industrial processing |
| | - Chemotherapeutic agents |
| | ▪ Crosslinking agents |
| | - Cyclophosphamide |
| | - Cisplatin |
| | - Psoralen |
| | ▪ Aromatic amines |
| | - 2-aminofluorene |
| | - N-acetyl-2-Aminofluorene |
| | ▪ Polycyclic aromatic hydrocarbons |
| | - Benzo[α]pyrene |
| | ▪ Reactive electrophiles |
| | - 4-Nitroquinoline 1-oxide |
| | ▪ Toxins |
| | - Aflatoxin B1 |

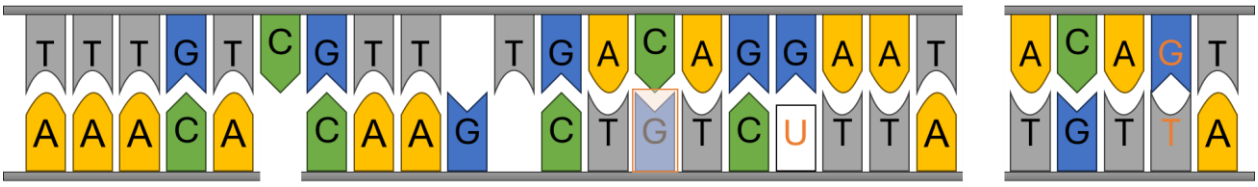
*Note:* (63)

Solar UV radiation reaching the Earth's surface includes UVA (315–400 nm) and long UVB (290–315 nm) wavelengths, while shorter wavelengths (short UVB and UVC) are blocked by the ozone layer. UVB is carcinogenic, inducing mutagenic DNA damage through photon absorption that generates covalent bonds between adjacent pyrimidines. These bonds can occur at all potential dipyrimidine sites (CC, CT, TC, and TT), resulting in two major UVR-induced DNA lesions: cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP). CPDs are particularly mutagenic and play a central role in UVR-induced skin cancers. They account for 70% of UVB-induced DNA lesions and are responsible for the formation of the UV signature mutations (C → T transitions) at dipyrimidine sites, particularly at sites containing cytosine (CT, TC, and CC) (64).

It is well established that, unlike UVB radiation, the less energetic UVA photons primarily exert their biological effects on cells and skin through oxygen-dependent mechanisms. These effects target a broad range of cellular components, including membranes, proteins, and DNA. Exposure to UVA radiation induces oxidative DNA damage in cells and human skin, primarily generating 8-oxo-7,8-dihydroguanine (8-oxoGua), along with smaller amounts of oxidized pyrimidine bases and oligonucleotide strand breaks (65).

On average, each human cell is subject to approximately 70 000 DNA lesions daily. Most of these lesions are single-strand DNA breaks (SSBs), followed by a formation of an abasic site (depurination/depyrimidination). Double-strand breaks (DSBs) are the least frequent yet most severe lesions for the cells (61,66) (See Figure 5).

**Figure 5.** Types of DNA lesions and mutations with estimated frequencies in human cells



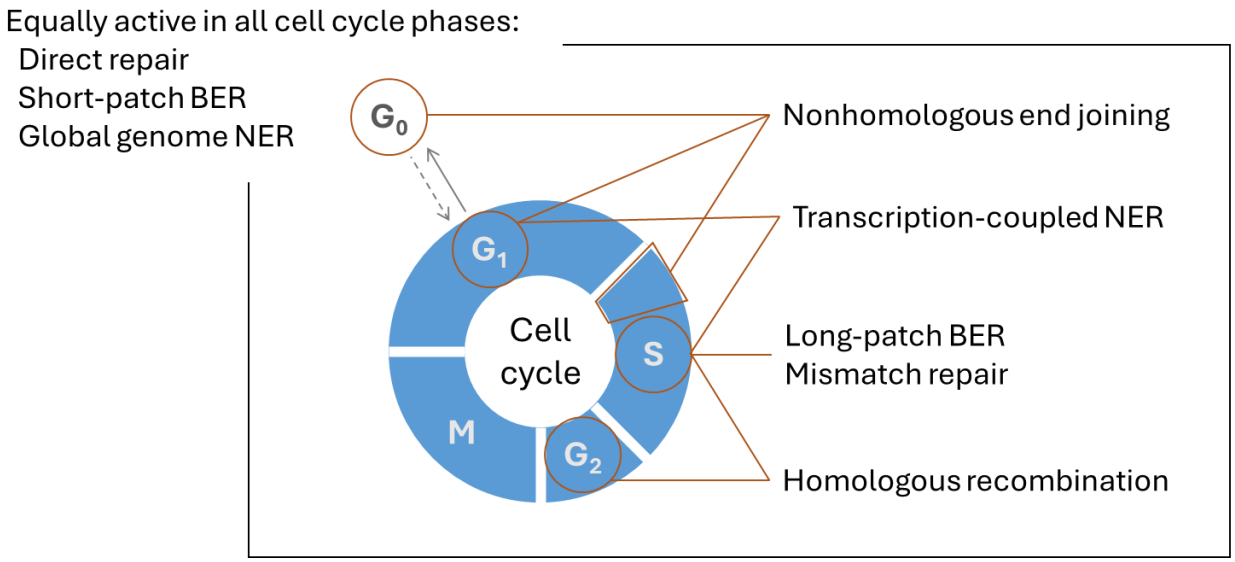

| **Damage** | Single-strand break | Depurination/ depyrimidination | 8-oxoG | Deamination | Double-strand break | Mismatch |
|---|---|---|---|---|---|---|
| **Estimated frequency** (per cell per day) | 55000 | 12000/600 | 2800 | 192 | 25 | N/d |

8-oxoG – 8-hydroxyguanine, N/d – not determined. *Note*: Adapted from (61).

## DNA repair

Cells are equipped with multiple systems to reduce the deleterious effects of DNA damage, namely DNA repair, damage tolerance, cell cycle checkpoints, and cell death pathways. DNA damage response (DDR) pathways sense DNA damage, signal its presence, and physically remove the damage. Five main DNA repair pathways act through different stages of the cell cycle (See Figure 6), including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and nonhomologous end joining (NHEJ). Additionally, a few specific lesions can be removed by direct repair (DR) and interstrand crosslinking (ICL) repair(63).

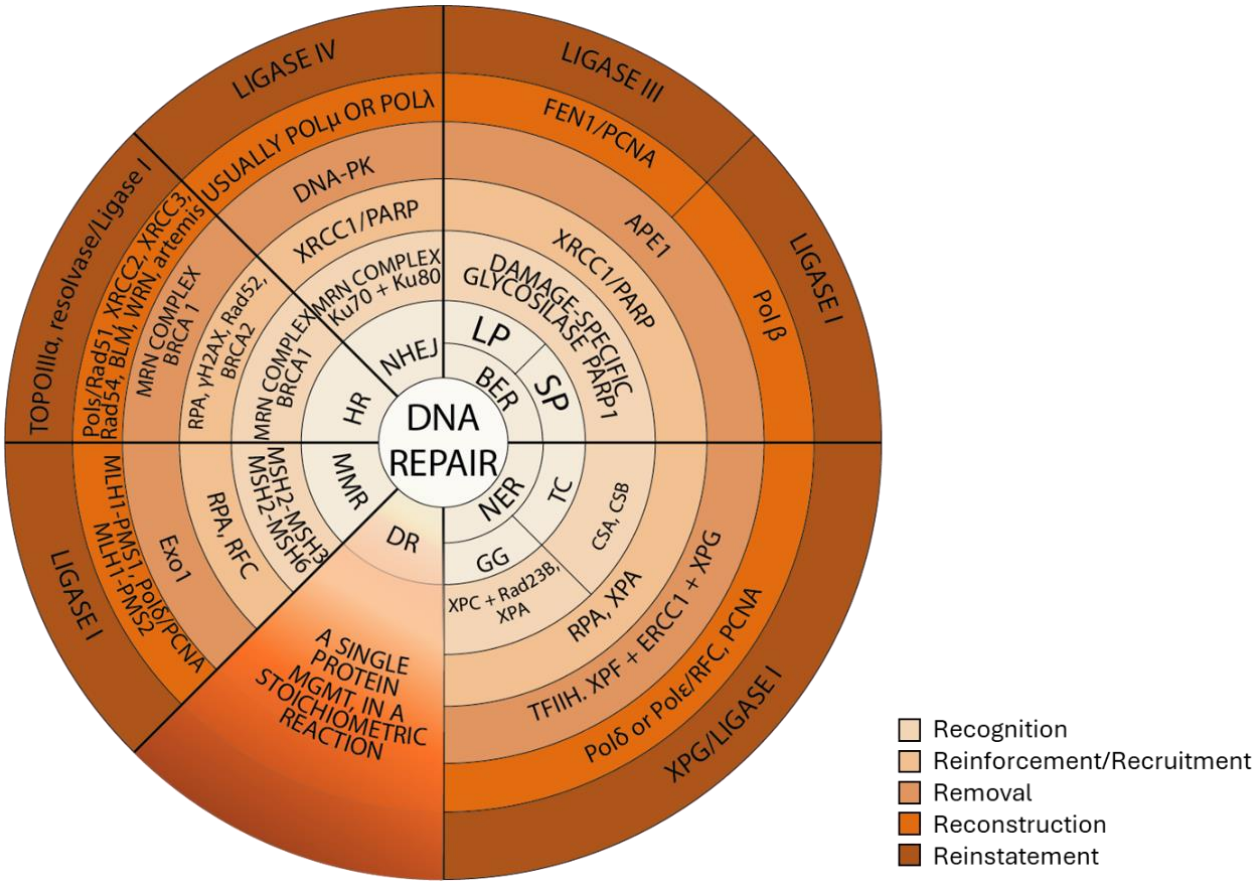
**Figure 6.** Activation of DNA pathways during different cell cycle phases




Direct repair, short-patch BER, and global genome NER are active through all the cell cycle phases. Nonhomologous end joining acts during $G_0$, $G_1$, and early S phases, while long-patch BER, mismatch repair, and transcription-coupled NER act during the S phase. Cell cycle phases: $G_0$ – resting phase, $G_1$ – growth, S – DNA synthesis, $G_2$ – growth and preparation for mitosis, M – mitosis. BER – base excision repair, NER – nucleotide excision repair. *Note:* (67).

Except for direct repair, all DNA repair pathways involve five main steps: recognition, reinforcement/recruitment, removal, reconstruction, and reinstatement (See Figure 7). In the initial recognition step, glycosylases and helicases detect and locate the damage. Subsequently, during the reinforcement/recruitment phase, the damage site is stabilized, and additional proteins are attracted to aid in repair by scaffolding and stabilizing proteins. During removal, endonucleases and lyases eliminate damaged bases from the DNA strand, preparing the site for new bases. Polymerases and progressivity factors then insert new nucleotides during the reconstruction step, while exonucleases and ligases restore the correct DNA configuration in the reinstatement phase (67).

**Figure 7.** DNA repair pathways




DR – direct repair, BER – base excision repair, MMR – mismatch repair, NER – nucleotide excision repair, NHEJ – nonhomologous end joining, HR – homologous recombination, GG – global genome NER, TC – transcription-coupled NER, LP – long-patched BER, SP – short-patched BER. *Note:* (67)

The direct repair mechanism relies on a single protein to eliminate DNA damage and lesions, making it less error-prone and efficient in maintaining genetic information without involving incisions in the sugar-phosphate backbone or base excision (68). Direct repair primarily refers to the repair of pyrimidine dimers caused by UV exposure or other factors and the repair of alkylated bases (69). In humans, direct repair is carried out by two different pathways: the O6-methylguanine-DNA methyltransferase (MGMT) and the alkylated DNA repair protein B (AlkB) homologs. The MGMT pathway is present in all cells. Its mechanism involves the direct transfer of the alkyl group from the oxygen in the DNA to a cysteine residue in its active site. This reaction reverses the base damage, but the alkylation of MGMT results in its inactivation, followed by ubiquitination and proteasomal degradation. Alternatively, the AlkB homolog pathway reversibly removes numerous modified bases but principally 1-methyladenine and 3-methylcytosine residues (70,71).

BER is activated in response to oxidative, alkylating, and deamination DNA damage. This mechanism operates through two common pathways: short-patch (repairing a single nucleotide) and long-patch (repairing at least two nucleotides) (72,73). Four key proteins are involved in BER – DNA glycosylase,

apurinic/apyrimidinic (AP) endonuclease, DNA polymerase, and DNA ligase (74). Currently, at least 11 distinct mammalian DNA glycosylases have been identified, excluding mitochondrial isoforms, which typically arise from alternative splicing. DNA glycosylases are classified as monofunctional or bifunctional based on their activities. Monofunctional glycosylases, such as uracil-DNA glycosylase, have only glycosylase activity, creating apurinic/apyrimidinic sites, while bifunctional glycosylases, like NTHL1 and NEIL1, also have β-lyase and β,δ-lyase activity, respectively. Cleavage of the DNA strand via β-elimination or β,δ-elimination produces ends that must be processed before base excision BER can continue. (75,76).

NER is a versatile repair mechanism that removes various types of lesions, including UV-induced damage, bulky chemical adducts, and intrastrand cross-links. In eukaryotes, NER can be divided into two distinct pathways, the global genome (GG-NER) and the transcription-coupled NER (TC-NER). GG-NER can take place anywhere in the genome, whereas TC-NER specifically targets and accelerates the repair of lesions on the transcribed strand of active genes. GG-NER is initiated by the GG-NER specific factor XPC-RAD23B, sometimes assisted by UV-DDB (UV-damaged DNA-binding protein). In contrast, TC-NER initiates when RNA polymerase stalls at a lesion, with assistance from TC-NER-specific factors CSA, CSB, and XAB2. GG-NER and TC-NER rely on the core NER factors to complete the excision process (77–79).

Two main mechanisms repair DNA double-strand breaks: HR and NHEJ. NHEJ uses enzymes such as nucleases, DNA polymerases, and ligases, which have multifunctional roles in DNA repair (80). This pathway employs various proteins to identify, remove, polymerize, and ligate DNA ends (81). Endogenous damage from reactive oxygen species (ROS) causes 10-50 DSBs per cell daily in tumor cells (82). NHEJ is the primary pathway in mammalian cells for repairing DSBs from ionizing radiation, which can lead to chromosomal translocations and genomic instability if not repaired correctly (83). Major NHEJ proteins include KU70 (XRCC6), KU80 (XRCC5), dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4-XLF, and ligase 4. DNA-PKcs inhibitors prevent DSB re-joining and stimulate cytotoxicity, and defects in NHEJ cause sensitivity to ionizing radiation and lymphocyte excision (84,85).

HR involves various proteins and is highly accurate, repairing collapsed replication forks, single-ended DSBs, and ICLs, thus re-establishing genomic stability (86). Pathogenic variants in one or more genes encoding HR proteins can impair the entire pathway. Tumor-suppressor genes like *BRCA1*, *BRCA2*, and *ATM* are involved in HR, and DSBs caused by ionizing radiation, and topoisomerase I poisons (Camptothecin, Irinotecan and Topotecan) occur more frequently in tumors with defective HR, enhancing the efficacy of cytotoxic drugs. Additionally, HR deficient tumors are more vulnerable to platinum-based therapies (87,88).

Crucial steps in HR include RAD51 phosphorylation and accumulation, dependent on the proto-oncogene *ABL1*, with ABL1 inhibitors increasing cell responsiveness to crosslinking agents and ionizing radiation (89,90). ATM is needed for the initiation of the DSB repair by HR. Upon DNA damage, ATM is recruited to the damaged site and leads to subsequent check-point activation during the G1/S checkpoint. ATM and ATR phosphorylate multiple downstream targets such as p53, H2AX, and BRCA1 either directly or via *CHEK2* gene coding checkpoint kinase 2 protein activation (91,92). The central reaction of HR is the homology search and DNA strand invasion mediated by the Rad51-ssDNA presynaptic filament, which aligns the invading 3'-end with a homologous template duplex to initiate repair synthesis. Rad51 catalyzes both the homology search and DNA strand exchange. Among the mediator proteins, the tumor suppressor BRCA2 is particularly significant. BRCA2 is essential for radiation-induced Rad51 focus formation in vivo and serves as a critical regulatory hub within the HR pathway (93).

The association and colocalization of BRCA1 with RAD51 in nuclear foci of mitotic cells provided early evidence of BRCA1's role in HR repair. These foci are observed both before and after DNA damage, highlighting BRCA1's involvement in repairing both intrinsic and induced DNA damage. The interactions of BRCA1 and BRCA2 with RAD51 suggest a functional link among these proteins in RAD51-mediated DNA damage repair. While BRCA2 directly facilitates RAD51-mediated repair, BRCA1 appears to function through a more complex mechanism, involving interactions with additional proteins (94).

MMR recognizes and repairs erroneous insertions, deletions, and base mis-incorporations that occur during DNA replication and recombination, as well as some forms of DNA damage. Defects in MMR increase the spontaneous mutation rate and induce multistage carcinogenesis (95). The MMR pathway corrects DNA mismatches generated during replication, preventing permanent mutations in cell divisions. Many human cancers, hereditary or non-hereditary, are linked to MMR inactivation, with some DNA damage requiring MMR for cell cycle arrest or programmed apoptosis. MMR plays a crucial role in the DNA damage response, eradicating severely damaged cells and suppressing short-term mutagenesis and long-term tumorigenesis (96). The MMR system was first identified in *Escherichia coli*, where mismatches in DNA triggered a repair response upon transformation into the bacterial cell. Key *E. coli* genes implicated in this process include *MutS*, *MutL*, *MutH*, and *uvrD* (ultraviolet repair protein D). Comparative studies in model organisms, such as bacteria and *Saccharomyces cerevisiae*, have demonstrated that MMR mechanisms and proteins are highly conserved across species, from prokaryotes to humans. In *E. coli*, MMR begins with the MutS protein, which detects mismatches in double-stranded DNA. Upon binding the lesion, MutS undergoes a conformational change, forming a homodimer and stabilizing its interaction with the mismatch. It then recruits MutL, which mediates interactions with downstream proteins such as MutH and uvrD. MutH, an endonuclease, cleaves the

newly synthesized strand at hemimethylated GATC sites, ensuring strand-specific repair. uvrD, a helicase, unwinds the DNA for mismatch removal. While prokaryotes rely on DNA methylation for strand discrimination, this mechanism remains unclear in eukaryotes (97). Microsatellite instability (MSI), a hypermutator phenotype resulting from MMR deficiencies, is associated with various tumors, including those associated with Lynch syndrome (98).

In response to specific DNA damage, one or multiple repair pathways are activated. Mismatched bases resulting from replication are addressed by the MMR. The Fanconi anemia (FA) pathway restores ICLs generated by natural or synthetic compounds. UV radiation, environmental mutagens, endogenous DNA damaging agents, and ROS cause SSBs that are repaired with BER and NER. DSBs triggered by ionizing radiation are handled either by NHEJ if the DNA ends are protected by the Ku70/Ku80 complex, or by homology-directed repair (HDR)/microhomology-mediated end joining (MMEJ)/single-strand annealing (SSA), depending on the length of the homology DNA fragments available (77,99).

## Genetics of multiple primary cancers

The emergence of genomic analysis using genetic technologies and next-generation sequencing (NGS) has significantly broadened our understanding of cancer susceptibility and genomic changes within cancer cells. It is now recognized that malignant tumor development requires the emergence of a new cell population harboring a diverse array of genetic and epigenetic changes, some of which will be driver mutations. These driver mutations can initiate tumorigenesis by conferring selective advantages over the neighboring cells, as they occur in specific cancer driver genes. Since the establishment of genetics, one of the main goals has been the identification of these cancer-driver genes (1,100).

Mutations targeting oncogenes, tumor suppressor genes, including “caretaker” genes (= DNA repair genes) directly involved in MPCs are the main genetic factors contributing to the development of MPCs. Additionally, the increasing number of genome-wide studies has shed light on the potential influence of common genetic markers shared among diverse tumors (pleiotropic loci) and low penetrance variants that elevate the risk of MPCs. A pleiotropic locus can increase the probability of developing MPCs in an individual while also influencing the risk of various cancer types. Furthermore, cancer treatment can alter the risk of developing MPCs if DNA-repair genes are altered, particularly in cases of treatment-exposure-related MPCs (101,102).

## MPC development mechanisms

The mechanisms involved in MPC development include MSI, p53 inactivation, alterations in the receptor tyrosine kinases (RTK)-RAS pathway, and in the DSB repair systems, and others (See Figure 8).

Microsatellites are intrinsically hypermutable short and simple repeating DNA sequences that are repaired with the MMR mechanism. In cases of impaired MMR, the excess of replication errors alters the microsatellite length and is called MSI. MMR abnormalities have been linked to MPCs, especially digestive tumors. Five percent of cancer patients are diagnosed with multiple colorectal cancers (CRCs). Metachronous CRCs have a higher rate of MSI compared to synchronous CRCs and have mainly a hereditary pattern with inactivation of MMR genes (101,103).

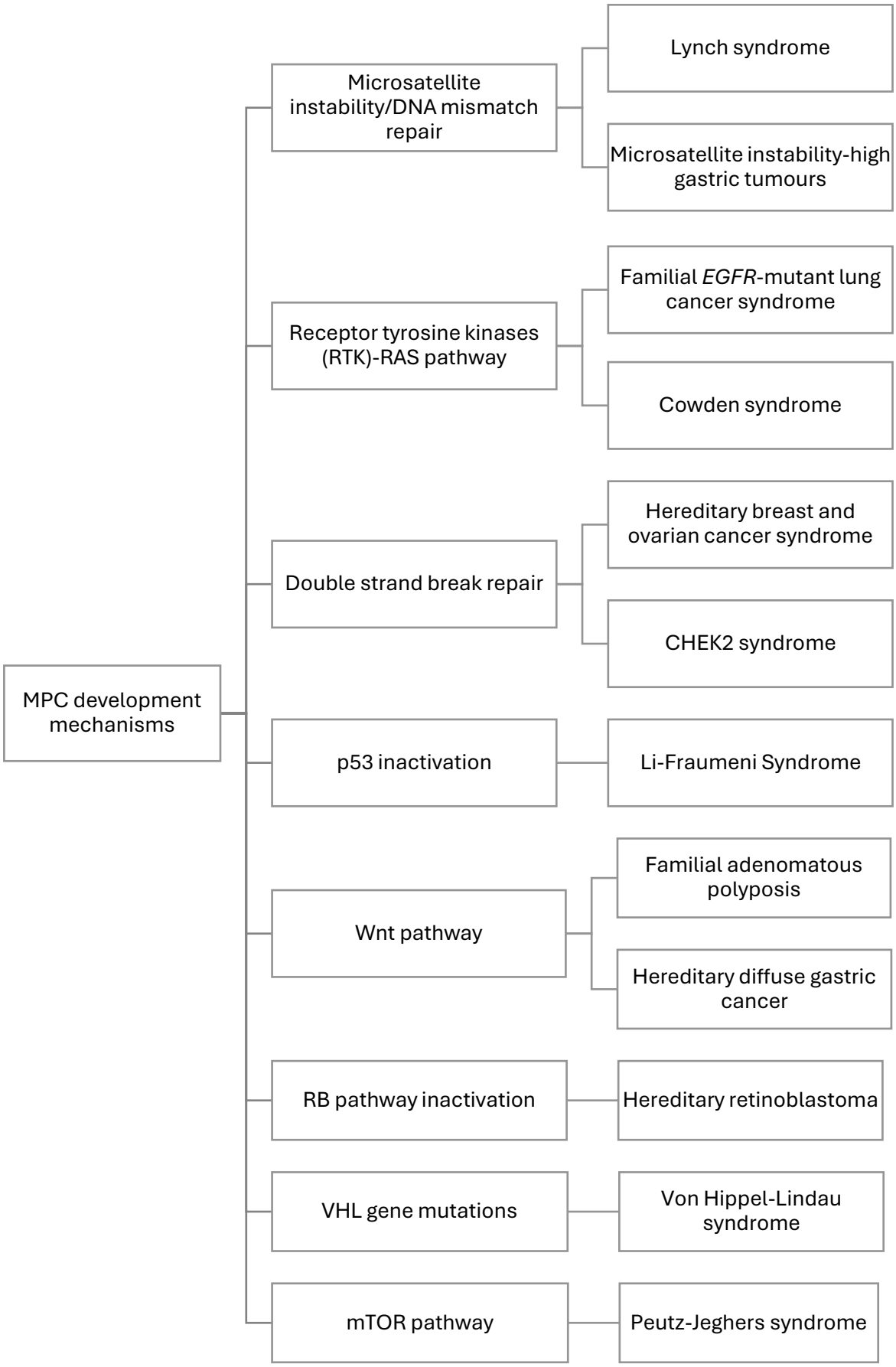
DSB repair abnormalities can also significantly increase the risk of MPCs. HR is one of the main DSB repair mechanisms, which are crucial for preserving genomic integrity and normal cellular function. Key driver genes, such as Breast cancer type 1 (*BRCA1*), and Breast cancer type 2 (*BRCA2*) genes, play significant roles in the HR repair process (101).

Germline PVs in *BRCA1/2* occurred in 42.9% of breast cancer patients with a second primary tumor. This percentage dropped to 22.7% when the subsequent cancer was not ovarian but soared to 84.4% if the second primary cancer originated in the ovary. Additionally, genes like Cadherin 1 (*CDH1*), Phosphatase and tensin homolog (*PTEN)*, Serine/threonine kinase 11 (*STK11*) and *TP53* can be mutated in cases of hereditary breast and ovarian cancer (104).

*CHEK2* is an important protein in the DDR network in an ATM-dependent manner. This gene plays a crucial role in regulating cell cycle checkpoints, halting cell division to facilitate repair processes. When phosphorylated, *CHEK2* can trigger apoptosis through TP53-dependent or independent pathways. Recognized for its high prevalence of germline PVs, *CHEK2* is commonly incorporated into diagnostic NGS panels (101).

The p53 protein is pivotal in tumor suppression, serving as a central hub for various signaling pathways. Somatic PVs in *TP53* are present in over 50% of cancers, leading to the survival and proliferation of damaged cells, and ultimately promoting cancer invasion and metastasis. Although there is notable variability, malignancies induced by p53 inactivation consistently follow a predictable pattern of genome evolution. This leads to deficient repair mechanisms and genetic instability, which are thought to be significant contributors to MPCs (105,106).

**Figure 8.** Multiple primary cancers development mechanisms and hereditary syndromes




*Note*: (107–112)

## Monogenic inheritance. Cancer predisposition genes and syndromes

Hereditary cancer predisposition syndromes encompass a diverse range of genetic diseases that increase the risk of developing tumors. Among these, some severe inherited disorders involve widespread organ failures, with cancer susceptibility being just one aspect of their clinical presentation (examples include Bloom syndrome, Fanconi anemia, Nijmegen breakage syndrome, and ataxia-telangiectasia). Most of these syndromes are caused by the inactivation of genes crucial for DNA repair and often manifest with severe immune deficiency (113,114). In contrast, individuals affected by "genuine" hereditary cancer syndromes typically do not exhibit noticeable physical abnormalities; their distinction from healthy individuals lies primarily in their markedly heightened predisposition to develop cancer in specific organs (See Table 6) (115).

Hereditary cancers appear to be the most prevalent category of disorders with vertical transmission. Unlike well-known genetic diseases such as cystic fibrosis or phenylketonuria, which are typically rare, conditions like *BRCA1/2*-related hereditary breast-ovarian cancer or *MLH1/MSH2*-associated Lynch syndrome are much more common, with a population frequency about 8-30 times higher, approaching approximately 1 in 300 to 1 in 400 people (116,117). It is estimated that at least 2% of apparently healthy individuals carry inherited variants associated with significantly increased and often life-threatening risks of specific types of cancer, with these figures potentially higher in populations where there is a strong founder effect (118,119).

Most hereditary cancer predisposition syndromes mainly involve cancers specific to certain organs or tissues. However, with the advent of hereditary cancer registries and large datasets, it has become evident that many hereditary cancer syndromes are linked to a broader range of cancers than initially recognized, although most of the newly discovered associations show only a slight increase in lifetime cancer risk. For instance, *BRCA1* and *BRCA2* were originally identified as genes associated with breast and ovarian cancers. Recent findings suggest that individuals with PVs in *BRCA1/2* may also face a borderline elevated likelihood of developing nearly all major types of cancer (120–126).

**Table 6.** List of hereditary cancer predisposition syndromes

| Syndrome | Prevalence | Inheritance | Involved Genes |
|---|---|---|---|
| Hereditary paraganglioma-pheochromocytoma syndrome | 1–9:1,000,000 | AD (*SDHA, SDHB, SDHC, TMEM127*) Probable paternal inheritance (*SDHD, SDHAF2, MAX*) | *SDHA, SDHAF2, SDHB, SDHC, SDHD, MAX, TMEM127* |
| Carney Complex | U | AD | *PRKAR1A* |
| Neurofibromatosis type 1 | 1:2600 | AD | *NF1* |
| Neurofibromatosis type 2 | 1:60,000 | AD | *NF2* |
| Schwannomatosis | 1:70,000 | AD | *SMARCB1, LZTR1* |
| Multiple endocrine neoplasia type 1 | 1:10,000 | AD | *MEN1* |
| Multiple endocrine neoplasia type 2A | 1:44,000 | AD | *RET* |
| Multiple endocrine neoplasia type 2B | 1:700,000 | AD | *RET* |
| Familial medullary thyroid carcinoma | 1:233,000 | AD | *RET* |
| Multiple endocrine neoplasia type 4 | <1:1,000,000 | AD | *CDKN1B* |
| Hyperparathyroidism-jaw tumor syndrome | U | AD | *CDC73* |
| Parathyroid carcinoma syndrome | U | AD | *CDC73* |
| Nijmegen Breakage syndrome | 1:100,000 | AR | *NBN* |
| Von Hippel–Lindau syndrome | 1:36,000 | AD | *VHL* |

AD – autosomal dominant, AR – autosomal recessive, M – mixed, U – unknown, X – X-linked. *Note:* (127)

| Syndrome | Prevalence | Inheritance | Involved Genes |
|---|---|---|---|
| Hereditary papillary renal carcinoma syndrome | U | AD | *HPRC* (*MET* protooncogene) |
| Hereditary leiomyomatosis and renal cancer cell syndrome | <1:500 | AD | *FH* |
| Tuberous Sclerosis Complex | 1:5800 | AD | *TSC1, TSC2* |
| Birt–Hogg–Dubé syndrome | 1:500,000 | AD | *FLCN* |
| Li–Fraumeni syndrome | 1:3500 | AD | *TP53* |
| Bloom syndrome | U | AR | *BLM* |
| Familial GIST | U | AD | *KIT, PDGFRA* |
| BRCA1- and BRCA2-associated hereditary cancer syndrome | 1:500 (*BRCA1*)<br>1:225 (*BRCA2*) | AD | *BRCA1, BRCA2* |
| CHEK2-associated hereditary cancer syndrome | 1:937 (*CHEK2* R95) | AD | *CHEK2* |
| PALB2-associated hereditary cancer syndrome | 1:1250 | AD | *PALB2* |
| RAD51C-associated hereditary cancer syndrome | 1:1600 | AD | *RAD51C* |
| RAD51D-associated hereditary cancer syndrome | U | AD | *RAD51D* |
| ATM-associated hereditary cancer syndrome | 1:100 | AR | *ATM* |
| Ataxia telangiectasia | 1:40,000–300,000 | AR | *ATM* |

AD – autosomal dominant, AR – autosomal recessive, M – mixed, U – unknown, X – X-linked. *Note:* (127)

| Syndrome | Prevalence | Inheritance | Involved Genes |
|---|---|---|---|
| Peutz–Jeghers syndrome | 1:25,000–280,000 | AD | *STK11* |
| *BARD1*-associated hereditary cancer syndrome | U | AD | *BARD1* |
| *BRIP1*-associated hereditary cancer syndrome | 1:500 | AD | *BRIP1* |
| Fanconi Anemia | 1–9:1,000,000<br>The carrier frequency of FA is 1/181 in the general population in North America and 1:93 in Israel. Specific populations have a founder effect with increased carrier frequencies (1 per 100 or less) | M (AR, X) | AR: *FANCA, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG (XRCC9), FANCI, FANCJ/BRIP1, FANCL, FANCM, FANCN/PALB2, FANCP/SLX4, FANCQ/ERCC4, FANCR/RAD51, FANCS/BRCA1, FANCT/UBE2T, FANCU/XRCC2, FANCV/REV7, FANCW/RFWD3, and FANCY/FAP100*<br>*X:* a hemizygous pathogenic variant in *FANCB* |
| Familial atypical mole-malignant melanoma syndrome | U | AD | *CDKN2A, CDK4* |

AD – autosomal dominant, AR – autosomal recessive, M – mixed, U – unknown, X – X-linked. *Note:* (127)

| Syndrome | Prevalence | Inheritance | Involved Genes |
|---|---|---|---|
| Nevoid basal cell carcinoma | 1:31,000–164,000 | AD | *PTCH1, SUFU* |
| Xeroderma pigmentosum | 1:1,000,000 (EU, USA), 1:22,000 (JAP) | AR | *DDB2 (XPE), ERCC1, ERCC2 (XPD), ERCC3 (XPB), ERCC4 (XPF), ERCC5 (XPG), POLH (XPV), XPA, XPC* |
| *BAP1* tumor predisposition syndrome | U | AD | *BAP1* |
| Shelterin complex genes hereditary cancer syndrome | U | AD | *POT1, ACD, TERF2IP* |
| *TERT* hereditary cancer syndrome | U | AD/AR | *TERT* |
| *DICER1* tumor predisposition syndrome | U | AD | *DICER1* |
| Lynch syndrome | 1:279 | AD | *MLH1, MSH2, MSH6, PMS2, EPCAM* |
| *RPS20*-associated hereditary nonpolyposis colorectal cancer syndrome | U | AD | *RPS20* |
| Familial adenomatous polyposis | 1:8000 | AD | *APC* |
| Attenuated familial adenomatous polyposis | U | AD | *APC* |
| Gastric adenocarcinoma and proximal polyposis of the stomach | U | AD | *APC* |

AD – autosomal dominant, AR – autosomal recessive, M – mixed, U – unknown, X – X-linked. *Note:* (127)

| Syndrome | Prevalence | Inheritance | Involved Genes |
|---|---|---|---|
| Polymerase proofreading-associated polyposis | U | AD | *POLE, POLD1* |
| *MUTYH*-associated polyposis | 1:20,000 (carrier 1:100) | AR | *MUTYH* |
| *NTHL1* tumor syndrome | U | AR | *NTHL1* |
| *MSH3*-associated polyposis | <1:1,000,000 | AR | *MSH3* |
| *MLH3*-associated polyposis | U | AR | *MLH3* |
| Juvenile polyposis syndrome | U | AD | *BMPR1A, SMAD4* |
| Hereditary mixed polyposis syndrome | U | AD | *GREM1* |
| Sessile serrated polyposis cancer syndrome | U | AD | *RNF43* |
| Cowden syndrome | 1:200,000 | AD | *PTEN* |
| Hereditary diffuse gastric cancer syndrome | U | AD | *CDH1* |
| Hereditary pancreatitis | 1–9:1,000,000 | AD (*PRSS1, CFTR, SPINK1, CPA1, CTRC, CASR, CEL*)<br>X (*CLDN2*)<br>AR (*CFTR, SPINK1, TRPV6*) | *PRSS1, SPINK1, CFTR, CTRC, CLDN2, CPA1 (Putative genes: CEL, CELP, CASR, GGT1, TRPV6)* |
| Howel–Evans syndrome | <1:1,000,000 | AD | *RHBDF2* |
| *EGFR*-associated genetic susceptibility | U | AD | *EGFR* |
| NBN hereditary cancer syndrome | U | AD | *NBN* |

AD – autosomal dominant, AR – autosomal recessive, M – mixed, U – unknown, X – X-linked. *Note:* (127)

The main syndromes that increase the risk of developing multiple primary cancers are described below.

**Nevoid basal cell carcinoma**

Nevoid basal cell carcinoma, also known as Gorlin-Goltz syndrome, is an autosomal dominant multisystemic disease marked by the appearance of multiple jaw keratocysts, typically starting in the second decade of life, and/or basal cell carcinomas (BCCs), which usually begin in the third decade (See Table 7). Many affected individuals have a distinctive appearance, characterized by macrocephaly, frontal bossing, coarse facial features, and facial milia. Most also exhibit skeletal anomalies, such as bifid ribs and wedge-shaped vertebrae. Ectopic calcification, particularly in the falx cerebri, occurs in 90% of affected individuals by age 30. Cardiac fibromas occur in about 2% of individuals, while ovarian fibromas are seen in approximately 20%. About 5% of children with this disease develop medulloblastoma, usually the desmoplastic subtype. The risk of developing medulloblastoma is significantly higher in individuals with a pathogenic variant in the *SUFU* gene (33%) compared to those with a *PTCH1* pathogenic variant (less than 2%), with peak incidence occurring at one to two years of age. Life expectancy for individuals with nevoid basal cell carcinoma is generally not significantly different from the average. Approximately 20%-30% of individuals with the disease harbor *de novo* PVs, while 70%-80% of patients inherit the disease from an affected parent. The estimated prevalence varies from 1/57,000 to 1/256,000, with a male to female ratio of 1:1 (128,129).

**Table 7.** Nevoid basal cell carcinoma syndrome criteria

| **Major criteria** |
|---|
| Lamellar calcification of the falx in an individual younger than age 20 |
| Jaw keratocyst |
| Palmar or plantar pits |
| Multiple basal cell carcinomas (>5 in a lifetime) or a basal cell carcinoma diagnosed before age 30 (excluding basal cell carcinomas that develop after radiotherapy) |
| First-degree relative with nevoid basal cell carcinoma syndrome |
| **Minor criteria** |
| Childhood medulloblastoma (primitive neuroectodermal tumor) |
| Lymphomesenteric or pleural cysts |
| Macrocephaly (occipital frontal circumference >97th percentile) |
| Cleft lip or cleft palate |
| Vertebral or rib anomalies observed on x-ray |

| |
|---|
| Preaxial or postaxial polydactyly |
| Ovarian or cardiac fibromas |
| Ocular anomalies (cataracts, developmental defects, and pigmentary changes of the retinal epithelium) |

*Note:* adapted from (57)

**Lynch syndrome**

Lynch syndrome is the most common hereditary form of CRCs, resulting from autosomal dominant PVs in the mismatch repair genes *MLH1*, *MSH2* (including methylation due to a deletion of the 3' end of *EPCAM* including the polyadenylation signal), *MSH6*, or *PMS2* (130). Lynch syndrome is associated with increased lifetime risks for colorectal (40–80%), endometrial (25–60%), ovarian (4–24%), and gastric (1–13%) cancers (131,132). Lynch syndrome accounts for 2% to 4% of all CRCs and approximately 2.5% of endometrial cancer cases (130). Additionally, individuals with Lynch syndrome have elevated risks for urothelial carcinoma, glioblastoma, and sebaceous, biliary, small bowel, and pancreatic adenocarcinomas (133–135) (See Table 8). The lifetime cancer risks are generally lower in those with *MSH6* and *PMS2* PVs. Most tumors (77–89%) in individuals with Lynch syndrome exhibit MSI due to defective mismatch repair (136).

**Table 8.** Tumors associated with Lynch syndrome

| |
|---|
| Colorectal adenocarcinoma |
| Endometrial adenocarcinoma |
| Urothelial carcinoma (ureter and renal collecting ducts) |
| Gastric cancer |
| Ovarian cancer |
| Small bowel cancer |
| Glioblastoma |
| Sebaceous adenocarcinoma |
| Biliary tract cancer |
| Pancreatic cancer |

*Note:* adapted from (57)

**Li-Fraumeni syndrome**

Li-Fraumeni syndrome is an autosomal dominant cancer predisposition syndrome caused by PVs in the *TP53* gene. It is characterized by a higher risk of developing five primary types of tumors: adrenocortical carcinomas, breast cancer, central nervous system tumors, osteosarcomas, and soft-tissue sarcomas (See Table 9), often before the age of 50, with many patients developing MPCs (137,138). The lifetime risk of cancer for these individuals is over 70% for men and over 90% for women (139).

**Table 9.** Tumors associated with Li–Fraumeni syndrome

| |
|---|
| Soft-tissue sarcoma |
| Osteosarcoma |
| Brain tumor |
| Breast cancer (often early onset) |
| Adrenocortical tumor |
| Leukemia |
| Bronchoalveolar cancer |
| Colorectal cancer |

*Note:* adapted from (57)

Early diagnosis, particularly before age 30, and specific types of malignancies, such as adrenocortical tumors or choroid plexus tumors diagnosed before age 18, are strong indicators of a *TP53* PV, with the likelihood of identifying a PV approaching 80% and 100%, respectively (138,140,141). Children diagnosed with sarcoma also have a higher likelihood of having Li-Fraumeni syndrome (142).

*De novo* germline *TP53* pathogenic variants are found in approximately 7% to 20% of cases (139). Genetic evaluation is recommended for individuals with a personal or family history (first-degree relative) that includes: two or more close relatives with a Li-Fraumeni syndrome spectrum tumor, one diagnosed at or before age 45, breast cancer diagnosed before age 30, two or more Li-Fraumeni syndrome tumors in the same person, one diagnosed at or before age 45, adrenocortical tumors, choroid plexus tumors, childhood sarcoma (57).

**Tuberous sclerosis complex**

Tuberous sclerosis complex (TSC) is an AD disorder caused by PVs in the *TSC1* and *TSC2* genes. It is characterized by the development of tumors in the brain, kidneys, and heart, along with various skin and neurological abnormalities (143,144). Brain lesions in TSC are diverse and include subependymal nodules, cortical hamartomas, focal cortical hypoplasia, and heterotopic gray matter. Renal lesions,

such as angiomyolipomas and cysts, typically appear during childhood and become more common with age (144,145). Approximately two-thirds of newborns with TSC have one or more cardiac rhabdomyomas, which are largest at birth and tend to regress over time (146). Skin lesions are observed in nearly all individuals with TSC, though none are unique to the disease (144).

Interestingly, about two-thirds to three-fourths of TSC cases result from *de novo* PVs (147). Clinical diagnosis is based on a combination of major and minor criteria (see Table 10). Referral for genetic evaluation should be considered for any individual with a personal or family history (first-degree relative) of any two major or minor diagnostic criteria (57).

**Table 10.** Tuberous sclerosis complex criteria

| **Major criteria** |
|---|
| Facial angiofibromas or forehead plaque |
| Nontraumatic ungual or periungual fibroma |
| Hypomelanotic macules (≥3) |
| Shagreen patch (connective tissue nevus) |
| Cortical tuber in the brain |
| Subependymal glial nodule |
| Subependymal giant cell astrocytoma |
| Multiple retinal nodular hamartomas |
| Cardiac rhabdomyomas, single or multiple |
| Lymphangiomyomatosis |
| Renal angiomyolipoma |
| **Minor criteria** |
| Multiple, randomly distributed pits in dental enamel |
| Hamartomatous rectal polyps |
| Bone cysts |
| “Confetti” skin lesions |
| Multiple renal cysts |
| Nonrenal hamartoma |
| Cerebral white matter radial migration lines |
| Retinal achromic patch |
| Gingival fibromas |

*Note:* adapted from (57)

**Cowden syndrome**

Cowden syndrome, also known as PTEN hamartoma tumor syndrome, is an AD cancer predisposition syndrome characterized by multiple hamartomas and an increased risk of malignant transformation. These lesions can arise in tissues derived from any of the three embryonic germ layers (148). The estimated prevalence of the disease is 1 in 250,000, though this may be underestimated due to the syndrome's variable expression and the subtlety of its features, which can occur in the general population and be easily overlooked (149). There is a higher prevalence in females and most reported cases are in Caucasians (150). Diagnosis of Cowden syndrome typically occurs between the ages of 13 and 65 (151).

Cowden syndrome is caused by PVs in the *PTEN* gene and is associated with benign skin findings and significantly increased lifetime risks of several cancers: breast cancer (30–85%, often early-onset), follicular thyroid cancer (10–38%), renal cell cancer (34%), endometrial cancer (5–28%), colorectal cancer (9%), and possibly melanoma (6%) (152–155). The mucocutaneous manifestations of Cowden disease are so common that some of them are considered pathognomonic, such as facial trichilemmoma, acral keratosis, and papillomatous lesions (156). Clinical diagnosis is based on a combination of major and minor criteria (See Table 11). It is recommended to refer individuals who meet any three criteria from the major or minor diagnostic lists for genetic evaluation (57).

**Table 11.** Cowden syndrome diagnostic criteria

| **Major criteria** |
|---|
| Breast cancer |
| Endometrial cancer (epithelial) |
| Thyroid cancer (follicular) |
| Gastrointestinal hamartomas (including ganglioneuromas but excluding hyperplastic polyps; ≥3) |
| Lhermitte–Duclos disease (adult) |
| Macrocephaly (≥97th percentile: 58cm for adult women, 60cm for adult men) |
| Macular pigmentation of the glans penis |
| Multiple mucocutaneous lesions (any of the following): |
| Multiple trichilemmomas (≥3, at least 1 proven by biopsy) |
| Acral keratoses (≥3 palmoplantar keratotic pits and/or acral hyperkeratotic papules) |
| Mucocutaneous neuromas (≥3) |
| Oral papillomas (particularly on tongue and gingival), multiple (≥3) OR biopsy proven OR diagnosed by a dermatologist |

| Minor criteria |
|---|
| Autism spectrum disorder |
| Colon cancer |
| Esophageal glycogenic acanthosis (≥3) |
| Lipomas (≥3) |
| Intellectual disability (i.e., intelligence quotient ≤75) |
| Renal cell carcinoma |
| Testicular lipomatosis |
| Thyroid cancer (papillary or follicular variant of papillary) |
| Thyroid structural lesions (e.g., adenoma, multinodular goiter) |
| Vascular anomalies (including multiple intracranial developmental venous anomalies) |

*Note:* adapted from (57)

**Birt–Hogg–Dubé syndrome**

Birt–Hogg–Dubé (BHD) syndrome is an AD disorder caused by PVs in the *FLCN* gene, characterized by a combination of skin lesions, renal tumors, and lung cysts (See Table 12) (157). The skin lesions, which include fibrofolliculomas, perifollicular fibromas, trichodiscomas, angiofibromas, and acrochordons, typically appear in the 30s and 40s and increase with age. The median age at diagnosis for renal tumors is 48 years, with a range from 31 to 71 years (57). Renal tumors occur in 12-34% of individuals with BHD, with the mean age of onset between 46-52 years, though cases have been reported in patients as young as 20 years (158–160). The risk of developing renal tumors is seven times higher in individuals with BHD compared to their unaffected siblings (161). In BHD individuals, the most common renal cell carcinoma (RCC) type is the hybrid oncocytic tumor (50%), featuring characteristics of chromophobe RCC and renal oncocytoma. Additionally, BHD patients may develop chromophobe RCC (35%), clear cell RCC (9%), and, less frequently, renal oncocytoma (5%) (162,163). Multiple bilateral lung cysts, often associated with spontaneous pneumothorax, are also a characteristic feature of BHD syndrome.

**Table 12.** Birt–Hogg–Dubé syndrome criteria

- ≥5 Birt–Hogg–Dubé–associated facial or truncal papules
- Early-onset (<50 years old), bilateral or multifocal clear cell renal carcinoma
- Renal cancers with Birt–Hogg–Dubé histology (chromophobe, oncocytoma, or oncocytic hybrid)
- Lung cysts associated with multiple spontaneous pneumothoraxes.

*Note:* (57)

**Constitutional mismatch repair deficiency**

Constitutional mismatch repair deficiency is a recessive condition caused by biallelic PVs in MMR genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*), including *MSH2* methylation due to a deletion of the 3' end of *EPCAM* including the polyadenylation signal. The tumor spectrum of this syndrome can be categorized into four main groups: hematological malignancies, brain tumors, Lynch syndrome-associated tumors, and other malignancies (164,165). Individuals with this deficiency often exhibit neurofibromatosis type 1–like features, such as café-au-lait macules, which are observed in most cases, and skinfold freckling, Lisch nodules, neurofibromas, and tibial pseudoarthrosis, which are reported in fewer cases. Notably, a family history of cancer is not always present in individuals with constitutional mismatch repair deficiency (57,165).

Referral for evaluation should be considered for anyone with a personal history of or a first-degree relative with Lynch syndrome-associated cancer in childhood or another type of childhood cancer accompanied by one or more of the following features:

- Café-au-lait macules, skinfold freckling, Lisch nodules, neurofibromas, tibial pseudoarthrosis, or hypopigmented skin lesions
- Family history of LS-associated cancer
- A second primary cancer
- A sibling with a childhood cancer
- Consanguineous parents (57).

**Familial adenomatous polyposis and attenuated familial adenomatous polyposis**

Familial adenomatous polyposis (FAP) and attenuated FAP are AD disorders caused by PVs in the *APC* gene. Both are characterized by adenomatous colon polyps and a significantly increased lifetime risk of CRC: nearly 100% for individuals with FAP and 70% for those with attenuated FAP (166,167). Classic FAP is clinically diagnosed when an individual has more than 100 adenomatous polyps in the colon, while attenuated FAP is characterized by having 30 to 100 polyps (168).

In addition to CRC, individuals with FAP have increased risks for other cancers, including duodenal cancer (4–12%), pancreatic cancer (~2%), cribriform morular variant of papillary thyroid cancer (1–2%), hepatoblastoma by age 5 (1–2%), and medulloblastoma (<1%) (169–172). They may also exhibit extracolonic manifestations such as congenital hypertrophy of the retinal pigmented epithelium, osteomas, dental abnormalities, benign cutaneous lesions like epidermoid cysts and fibromas, and desmoid tumors. *APC* PVs were identified in 80% of patients with 1,000 or more adenomas, 56% of those with 100–999 adenomas, 10% with 20–99 adenomas, and 5% with 10–19 adenomas (173).

Referral for genetic evaluation should be considered for individuals with a personal history of or a first-degree relative with any of the following:

- 10 or more adenomatous colon polyps, with or without CRC or other FAP-associated cancers
- Cribriform morular variant of papillary thyroid cancer
- Desmoid tumor
- Hepatoblastoma diagnosed before age 5.

**Familial gastrointestinal stromal tumor**

Familial gastrointestinal stromal tumor (GIST) is an AD disease linked to PVs in the *KIT*, *PDGFRA*, *SDHB*, and *SDHC* genes. People with germline *KIT* PVs may exhibit hyperpigmentation, mast cell tumors, or dysphagia, while large hands are associated with *PDGFRA* PVs (174,175). NF1 patients are also prone to developing GISTs (176). GIST tumors can occur throughout the gastrointestinal tract but are most found in the stomach and small intestine, and less frequently in the colorectum and esophagus (177). Rarely, tumors may arise in the mesentery and omentum, known as extra-GISTs (178). Sporadic GISTs can also occur outside of familial and syndromic contexts, and these multiple tumors may be mistakenly identified as recurrent or metastatic disease (179–181).

**Hereditary breast–ovarian cancer syndrome (HBOC)**

The most common breast cancer susceptibility genes, *BRCA1* and *BRCA2*, are well known for their association with the risk of developing ovarian and pancreatic cancer. However, with advances in NGS analysis technology, researchers have identified several non-BRCA genes that also contribute to the risk of breast and ovarian cancers (See Table 13) (182).

**Table 13.** HBOC genes and candidate HBOC predisposing genes

| Gene | Cancer type | Relative cancer risk (95%CI) (p value) | Absolute cancer risk (95%CI) |
|---|---|---|---|
| *BRCA1* | Female Breast cancer | OR 9.25 (8.20-10.30) (<0.0001) | >60%<br>Primary: 72% (65-79%) by age 80y, Contralateral: By 20y after diagnosis of primary: 40% (35-45%)<br>~55% by age 80y |

OR – Odds Ratio, RR – Relative Risk, SE – Standard Error, SIR – Standardized Incidence Ratio, SRR – Standardized Rate Ratio, y – years. *Note:* adapted from (183)

| Gene | Cancer type | Relative cancer risk (95%CI) (p value) | Absolute cancer risk (95%CI) |
|---|---|---|---|
| | Male Breast cancer | OR: 1.80 (0.30-6.80) (0.30)<br>RR: 4.30 (1.09-16.96) (0.04) | 0.2-1.2%<br>1.2% (0.22-2.8%) to age 70y<br>0.40% (0.10-1.50%) by age 80y |
| | Ovarian cancer | OR 35.26 (29.60-42.00) (<0.0001)<br>SRR 11.78 (10.42-13.28) (<0.0001) | >39-58%<br>44% (36-55%) by age 80y |
| | Pancreatic cancer | OR 2.58 (1.54-4.05) (0.002)<br>RR 2.36 (1.51-3.68) (<0.001) | ≤5%<br>Males: 2.90% (1.90-4.50%) by age 80y<br>Females: 2.30% (1.50-3.60%) by age 80y |
| | Prostate cancer | SIR 2.35 (1.43-3.88) relative to the population incidence. At most, moderate risk: RR 2-4 <65y, 1-2 >65y | 7-26%<br>21% (3-34%) by age 75y<br>29% (17-45%) by age 85y |
| | Melanoma | OR 2.86 (significance not reached)<br>RR 0.64 (0.14-2.95) (0.56) | No definitive association |
| | Stomach cancer | RR 2.17 (1.25-3.77) (0.01) | Males: 1.6% (0.7-4.0%) by age 80y<br>Females: 0.7% (0.3-1.7%) by age 80y |
| *BRCA2* | Female Breast cancer | OR 5.67 (5.14-6.30) (<0.0001) | >60%<br>Primary: 69% (61-77%) by age 80y<br>Contralateral: 26% (20-33%) 20y after diagnosis of primary<br>~45% by age 80 |
| | Ovarian cancer | OR 11.91 (9.87-14.40) (<0.0001)<br>SRR 7.97 (7.00-9.01) (<0.0001) | 13-29%<br>17% (11-25%) by age 80y |

OR – Odds Ratio, RR – Relative Risk, SE – Standard Error, SIR – Standardized Incidence Ratio, SRR – Standardized Rate Ratio, y – years. *Note:* adapted from (183)

| Gene | Cancer type | Relative cancer risk (95%CI) (p value) | Absolute cancer risk (95%CI) |
|---|---|---|---|
| | Male Breast cancer | OR 13.9 (8.50-22.50) (<0.0001)<br>RR 44.03 (21.32-90.93) (<0.001) | 1.80-7.10%<br>7.1% (SE 5.2-8.6%) to age 70y;<br>8.4% (SE 6.2-10.6%) to age 80y<br>6.8% (3.2-12%) by age 70y<br>3.80% (1.9-7.7%) by age 80y |
| | Pancreatic cancer | OR 6.20 (4.62-8.17) (<0.001)<br>RR 3.34 (2.21-5.06) (<0.001) | 5-10%<br>Males: 3% (1.7-5.4%) by age 80y<br>Females: 2.30% (1.3-4.2%) by age 80y |
| | Prostate cancer | SIR 4.45 (2.99-6.61) relative to the population incidence<br>RR 2.22 (1.63-3.03) (<0.001) | 19-61%<br>27% (17-41%) by age 75y; 60% (43-78%) by age 85y |
| | Melanoma | Evidence for a statistically significant association is conflicting | No definitive association |
| | Stomach cancer | RR 3.69 (2.40-5.67) (<0.001) | Males: 3.5% (2.1-6.1%) by age 80y<br>Females: 3.5% (1.9-6.4%) by age 80y |
| *PALB2* | Female Breast cancer | OR 4.87 (4·20-5·65) (<00001)<br>RR 7.18 (5.82-8.85) (<00001) | 41-60%<br>53% (44-63%) by age 80y<br>~40% by age 80y |
| | Ovarian cancer | OR 2.13 (1·420-3·207) (0.0003)<br>RR 2.91 (1.40-6.04) (4.1x10-3)<br>SRR 3.08 (1.93-4.67) (1.2x10-5) | 3-5%<br>5% (2-10%) by age 80y |
| | Male Breast cancer | OR 6.60 (1.70-21.10) (0.013)<br>RR 7.34 (1.28-42.18) ($2.6x12^{-5}$) | 0.90% by age 70y<br>1% (0.20-5%) by age 80y |
| | Pancreatic cancer | RR 2.37 (1.24-4.50) ($8.7x10^{-3}$)<br>OR 7.69 (3.88-14.44) ($6.01 x10^{-7}$) | 5-10%<br>2-3% (1-5%) by age 80y |

OR – Odds Ratio, RR – Relative Risk, SE – Standard Error, SIR – Standardized Incidence Ratio, SRR – Standardized Rate Ratio, y – years. *Note:* adapted from (183)

| Gene | Cancer type | Relative cancer risk (95%CI) (p value) | Absolute cancer risk (95%CI) |
|---|---|---|---|
| *ATM* | Female Breast cancer | OR 2.42 (2.16-2.71) (<0.0001) | 20-40%<br>~22% by age 80y |
| | Ovarian cancer | OR 2.0 (1.33-2.94) (0.001)<br>SRR 2.25 (1.69-2.94) ($1.8x10^{-7}$) | 2-3% |
| *BRIP1* | Ovarian cancer | OR 4.94 (4.07-6.00) (<0.0001)<br>SRR 4.99 (3.79-6.45) (<0.0001) | 5-15% |
| | Female Breast cancer | OR 1.11 (0.80-1.53) (0.54) | Insufficient data to define |
| *BARD1* | Female Breast cancer | OR 2.33 (1.83-2.97) (<0.0001) | 20-40%<br>~20% by age 80y |
| | Ovarian cancer | OR 1.40 (0.69-2.90) (0.47) | No established association |
| *CHEK2* | Female Breast cancer | OR 2.47 (2.02-3.05) (0.001) | 20-40%<br>~ 24% by age 80y |
| | Ovarian cancer | OR 0.43 (0.29-0.63) (0.84)<br>SRR 0.98 (0.75-1.27) (0.87) | No established association |
| *CDH1* | Female Breast cancer | OR 2.66 (1.68-4.20) (<0.0001) | 41-60% |
| *MLH1* | Ovarian cancer (LS-Associated) | OR 1.44 (0.53-3.90) (0.68)<br>SRR 2.20 (0.81-4.78) (0.12) | 4-20% |
| | Female Breast cancer | OR 0.68 (0.466-0.97) (0.05) | <15% |
| *MSH2* | Ovarian cancer (LS-Associated) | OR 3.98 (1.82-8.70) (0.0007)<br>SRR 13.91 (8.82-20.87) (<0.0001) | 8-38% |
| | Female Breast cancer | OR 1.67 (1.17-2.34) (0.0054) | <15% |
| *MSH6* | Ovarian cancer (LS-Associated) | OR 4.08 (2.43-6.85) (<0.0001)<br>SRR 5.04 (3.70-6.70) (<0.0001) | ≤1-13% |
| | Female Breast cancer | OR 1.73 (1.37-2.2) (<0.0001) | <15% |

OR – Odds Ratio, RR – Relative Risk, SE – Standard Error, SIR – Standardized Incidence Ratio, SRR – Standardized Rate Ratio, y – years. *Note:* adapted from (183)

| Gene | Cancer type | Relative cancer risk (95%CI) (p value) | Absolute cancer risk (95%CI) |
|---|---|---|---|
| *MUTYH* | Breast/Ovarian cancer | Insufficient data<br>OR 1.00 (0.83-1.21) (0.99) | Insufficient evidence |
| *NBN* | Breast cancer | OR 1.22 (0.98-1.53) (0.083) | Insufficient evidence |
| | Ovarian cancer | OR 2.12 (1.35-3.50) (0.002)<br>SRR 2.03 (1.27-3.08) (0.004) | Insufficient Data |
| *PMS2* | Female Breast cancer | OR 0.97 (0.787-1.20) (0.81) | <15% |
| | Ovarian cancer (LS-Associated) | 0.71 (0.29-1.70) (0.56) | 1.3-3% by age 80y |
| *PTEN* | Female Breast cancer | OR 5.40 (3.15-9.23) (<0.0001) | 40-60% (historical cohort data);<br>>60% (projected estimates) |
| | Ovarian Cancer | OR 5.47 (1.26-23.8) (0.08) | No established association |
| *RAD51C* | Ovarian cancer | RR 7.55 (5.60-10.19) (<0.0001)<br>OR 5.59 (4.42-7.07) (<0.0001)<br>SRR 5.12 (3.72-6.88) (<0.0001) | 10-15%<br>11% (6-21%) by age 80y |
| | Female Breast cancer | RR 1.99 (1.39-2.85) (<0.0001)<br>OR 1.93 (1.20-3.11) (0.07) | 20-40%<br>21% (15-29%) by age 80y |
| *RAD51D* | Ovarian cancer | RR 7.60 (5.61-10.30) (<0.0001)<br>OR 6.94 (4·028-13·140) (<0.0001)<br>SRR 6.34 (5.10-9.44) (<0.0001) | 10-20%<br>13% (7-23%) by age 80y |
| | Female Breast cancer | RR 1.83 (1.24-2.72) (0.002)<br>OR 1.72 (0.88-3.51) (0.12) | 20-40%<br>20% (14-28%) by age 80y |
| *STK11* | Female Breast cancer | OR 1.10 (0.32-3.80) (0.88) | 32-54% |
| *TP53* | Female Breast cancer | OR 4.36 (3.27-5.81) (<0.0001) | >60% |
| | Ovarian cancer | OR 5.05 (2.41-10.58) (<0.0001) | No established association |

OR – Odds Ratio, RR – Relative Risk, SE – Standard Error, SIR – Standardized Incidence Ratio, SRR – Standardized Rate Ratio, y – years. *Note:* adapted from (183)

### *BRCA1*- and *BRCA2*-Associated Hereditary Breast and Ovarian Cancer

*BRCA1*- and *BRCA2*-Associated Hereditary breast-ovarian cancer (HBOC) syndrome is an AD disorder caused by PVs in the *BRCA1* and *BRCA2* genes. This syndrome significantly increases the risk of developing various cancers, including early-onset and/or multiple primary breast cancers, male breast cancer, and epithelial ovarian, fallopian tube, or primary peritoneal cancers. Additionally, individuals with HBOC syndrome have higher risks of pancreatic, prostate, and melanoma cancers. *BRCA1* PVs, in particular, are strongly linked to triple-negative breast cancer, while *BRCA2* is associated with an increased risk of prostate cancer (184–187).

Women with ovarian cancer have a 13-18% likelihood of carrying a *BRCA1* or *BRCA2* PV, and 15-20% of men with breast cancer have these PVs (188–190). The general population prevalence is about 1 in 300 for *BRCA1* PVs and 1 in 800 for *BRCA2* PVs, but certain populations, such as Ashkenazi Jewish, Icelandic, and Mexican Hispanic, have higher rates due to founder PVs (191).

Referral for genetic evaluation should be considered for individuals with a personal or family history (first-degree relative) of the following:

- Breast cancer diagnosed at or before age 50
- Triple-negative breast cancer diagnosed at or before age 60
- Two or more primary breast cancers in the same person
- Ovarian, fallopian tube, or primary peritoneal cancer
- Ashkenazi Jewish ancestry with breast or pancreatic cancer at any age
- Male breast cancer.

Additionally, referral is recommended for those with a family history of three or more cases of breast, ovarian, pancreatic, or aggressive prostate cancer (Gleason score ≥7), excluding families where all three cases are aggressive prostate cancer (192,193).

### Non-BRCA genes related to HBOC

A review of the genetic causes of familial breast cancer revealed that only 25% of cases involved *BRCA1* or *BRCA2* variants. In 5% of patients, variants in four other high-risk genes—*CDH1*, *PTEN*, *STK11*, and *TP53*—were identified. Additionally, variants in medium-penetrance genes were found in another 5% of cases, while 14% were linked to low-penetrance genes. Notably, the causative gene remained unknown in 51% of cases (194). Most of the known susceptibility genes for HBOC encode tumor suppressors involved in genome stability pathways, particularly HR repair. Some of these genes also play a role in MMR and interstrand DNA cross-link repair through the Fanconi anemia pathway (195).

**Hereditary diffuse gastric cancer**

Hereditary diffuse gastric cancer (HDGC) is an AD cancer syndrome characterized by a high prevalence of diffuse gastric cancer and lobular breast cancer. It is largely caused by inactivating germline PVs in the tumor suppressor gene *CDH1*, though PVs in *CTNNA1* are found in a minority of HDGC families (196–198). HDGC is now estimated to have a worldwide population incidence of 5–10 per 100,000 births (199). *CDH1* PVs occur in 25–50% of those meeting the HDGC criteria (196).

The International Gastric Cancer Linkage Consortium has established guidelines for the clinical management of HDGC, including recommendations for *CDH1* genetic testing (199). Referral for genetic testing should be considered for individuals when one of the following criteria has been met and cancer diagnosis has been confirmed (See Table 14). Individuals who meet the criteria for genetic testing and test negative for a *CDH1* variant should then be considered for *CTNNA1* analysis.

**Table 14.** Hereditary diffuse gastric cancer criteria

| **Family criteria (First- or second-degree blood relative)** |
|---|
| ≥2 cases of gastric cancer in family regardless of age, with at least one diffuse gastric cancer |
| ≥1 case of diffuse gastric cancer at any age, and ≥1 case of lobular breast cancer at age <70 years, in different relatives |
| ≥2 cases of lobular breast cancer in relatives <50 years of age |
| **Individual criteria** |
| Diffuse gastric cancer at age <50 years |
| Diffuse gastric cancer at any age in individuals of Māori ethnicity |
| Diffuse gastric cancer at any age in individuals with a personal or family history (first-degree relative) of cleft lip or cleft palate |
| History of diffuse gastric cancer and lobular breast cancer, both diagnosed at age <70 years |
| Bilateral lobular breast cancer, diagnosed at age <70 years |
| Gastric in situ signet ring cells or pagetoid spread of signet ring cells in individuals <50 years of age |

*Note:* (199)

**Hereditary leiomyomatosis and renal cell cancer**

Hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome, also known as Reed syndrome, is a rare genetic disorder caused by PVs in the *FH* gene. This syndrome predisposes individuals to develop multiple cutaneous leiomyomas, RCC, and in women, uterine leiomyomas (200). Those affected by HLRCC typically present with various types of RCC, including papillary type 2, collecting duct, and

tubulopapillary carcinomas (201,202). Genetic counseling referral is recommended for individuals with cutaneous leiomyomas and RCC, as 85% of those with cutaneous leiomyomas (some isolated cases and some with a family history of uterine leiomyoma or RCC) have been found to carry an *FH* PV in studies (203–206). Additionally, a *FH* PV has been identified in 17% of patients with papillary type 2 RCC. *FH* gene PVs have been identified in 76% to 93% of families with clinical features consistent with HLRCC (207,208). This disorder has been reported in approximately 200 families worldwide, but it may be underdiagnosed (209).

**Hereditary melanoma**

Hereditary melanoma, or familial atypical mole and malignant melanoma, is an AD disease primarily caused by PVs in the *CDKN2A/ARF* gene and the *CDK4* gene (210). Germline susceptibility to the disease has also been associated with PVs in other high-penetrance melanoma predisposition genes, including *BAP1* (breast cancer associated protein-1), *TERT* (telomerase reverse transcriptase), and *POT1* (protection of telomeres 1), or with variants in intermediate-risk genes, such as *MC1R* (melanocortin 1 receptor) and *MITF* (microphthalmia-associated transcription factor) (211–213). This condition is characterized by the presence of numerous melanocytic nevi (typically more than 50) and a family history of melanoma (214). A positive family history is defined as a family in which either two first-degree relatives or three or more melanoma patients on the same side of the family (irrespective of the degree of relationship) are diagnosed with melanoma (215). Approximately 5–10% of cutaneous melanomas occur in families with hereditary melanoma predisposition (216). Individuals with hereditary melanoma also have a 17% risk of developing pancreatic cancer by age 75 (214).

In general, familial melanoma cases have an earlier age at diagnosis, around 34 years, compared to 54 years for other melanoma cases. The cancer risk for individuals with familial melanoma is between 50-90% (217). A study of 466 families with at least three cases of melanoma found that 38% had *CDKN2A* PVs, although the penetrance and detection rates can vary by region (218). *CDKN2A* PVs are relatively rare in families with pancreatic cancer alone but can occur in up to 11% of families that have both pancreatic cancer and melanoma (219).

**Hereditary papillary renal cell carcinoma**

Hereditary papillary RCC is a rare familial disorder caused by activating PVs in the *MET* gene. It is characterized by an increased risk of developing papillary type 1 RCC, with patients typically having multiple kidney tumors and a heightened risk of tumors in both kidneys. In a study of 129 patients with papillary RCC, 6% (8 out of 129) were found to have a germline *MET* PVs. Currently, no other types of cancer or noncancerous health issues are associated with hereditary papillary RCC (220,221).

**Hereditary paraganglioma-pheochromocytoma syndrome**

Hereditary paraganglioma**-**pheochromocytoma (PGL/PCC) syndrome is an AD disease caused by PVs in the *SDHB*, *SDHD*, *SDHC*, *SDHAF2*, *MAX*, and *TMEM127* genes. This syndrome significantly increases the risk of developing paragangliomas and pheochromocytomas, as well as certain other cancers, including, GIST, pulmonary chondromas, and clear cell RCC (222,223). Studies indicate that 8–25% of individuals with these tumors have hereditary PGL/PCC syndrome due to a germline PV in the *SDHB*, *SDHC*, or *SDHD* genes. The prevalence of hereditary PGL/PCC syndrome is higher in individuals with a family history of these tumors or other clinical factors, such as multiple tumors, recurrent, early-onset disease, or head and neck location (224–228).

PVs in *SDHD*, *SDHAF2*, and possibly *MAX* demonstrate parent-of-origin effects, causing disease almost exclusively when paternally inherited. An individual who inherits an *SDHD* or *SDHAF2* pathogenic variant from their father is at high risk of developing PGLs and PCCs, whereas those who inherit these variants from their mother are usually not at risk, though exceptions can occur (222).

**Hereditary retinoblastoma**

Hereditary retinoblastoma is an AD disease caused by PVs in the *RB1* gene, characterized by a malignant tumor of the retina, typically occurring before age 5. Approximately 40% of all retinoblastoma cases are hereditary, and 80-90% of individuals with *RB1* gene PVs develop ocular tumors (229,230). Retinoblastoma was the first disease for which a genetic etiology of cancer was described, and *RB1* was the first identified tumor suppressor gene. In 1971, Knudson proposed the hypothesis that retinoblastoma is caused by two mutational events, laying the foundation for understanding the genetic basis of cancer (231). Individuals with a family history of retinoblastoma, bilateral tumors, or multifocal tumors are at the highest risk for hereditary retinoblastoma (229).

Survival rates for retinoblastoma patients are high, but they face a significantly increased risk of developing secondary cancers, primarily pinealoblastoma, osteosarcoma, sarcoma (particularly radiogenic sarcoma), and melanoma (232–234). This predisposition to secondary cancers is attributed to genetic susceptibility and past radiation treatment for retinoblastoma (235–237). Chemotherapy, particularly with alkylating agents, has also been associated with an increased risk of bone cancer in retinoblastoma survivors (238–240), although it is less commonly linked to soft tissue sarcomas (241). Long-term survivors have a 20-fold increased risk of developing and dying from these non-ocular cancers (238,242).

**Juvenile polyposis syndrome**

Juvenile polyposis syndrome is a rare AD disorder characterized by the presence of multiple juvenile-type hamartomatous polyps throughout the gastrointestinal tract and a significantly increased risk of colorectal cancer (243). This syndrome is caused by PVs in the *SMAD4* (20%) and *BMPR1A* (20%) genes, with about 50%-60% of juvenile polyposis syndrome patients having a germline PV in one of these genes (244). The term "juvenile polyp" refers to a specific histologic type of polyp rather than the age at diagnosis (245).

Juvenile polyposis syndrome is defined by the presence of five or more juvenile polyps in the colorectum, juvenile polyps throughout the gastrointestinal tract, or any number of juvenile polyps combined with a positive family history of juvenile polyposis syndrome (243). Individuals with juvenile polyposis syndrome have a cumulative colorectal cancer risk of up to 68% by the age of 60 years (244). The risk for other gastrointestinal cancers, including those of the stomach, upper gastrointestinal tract, and pancreas, ranges from 9% to 50% in families with juvenile polyposis syndrome (245).

Extraintestinal features of juvenile polyposis syndrome can include valvular heart disease (11%), telangiectasia or vascular anomalies (9%, particularly in *SMAD4* carriers), and macrocephaly (11%) (246). Additionally, some individuals with juvenile polyposis syndrome due to *SMAD4* PVs may exhibit symptoms of hereditary hemorrhagic telangiectasia (247,248).

**Melanoma–astrocytoma syndrome**

Melanoma–astrocytoma syndrome is a rare AD condition caused by PVs in the *CDKN2A* and *p14ARF* genes, and possibly the *ANRIL* antisense noncoding RNA. This syndrome is characterized by an increased risk for melanoma and astrocytomas (57). The genetic basis of this tumor predisposition syndrome was elucidated in 1998 by Bahuau et al., who identified deletions in the INK4 locus at chromosome 9p21.3 in families previously reported in 1993 and 1997 (249). The INK4 locus contains the *CDKN2A* and *CDKN2B* tumor suppressor genes. *CDKN2A* encodes the p16INK4a cyclin-dependent kinase inhibitor and, in an alternative reading frame, p14ARF, an inhibitor of p53 signaling. *CDKN2B* encodes p15INK4b, a cyclin-dependent kinase inhibitor closely related to p16INK4a (250,251).

*CDKN2A* is a major susceptibility gene for familial cutaneous melanoma, typically through inactivating PVs or gene deletions (250,251). Familial melanoma-astrocytoma syndrome is considered an AD variant of familial melanoma syndrome, involving heterozygous germline *CDKN2A* inactivation that also predisposes individuals to astrocytomas and occasionally other neural tumors, including peripheral nerve sheath tumors and meningiomas (252). The tumor spectrum in this syndrome is not well understood, with fewer than 15 families described in the medical literature (253).

**Multiple endocrine neoplasia type 1**

Multiple endocrine neoplasia type 1 (MEN1), also known as Wermer syndrome, is a rare and highly penetrant AD disorder caused by germline PVs in the *MEN1* gene, which encodes the protein menin. MEN1 is characterized by an increased risk of developing both endocrine and nonendocrine tumors (254). The prevalence of MEN1 is estimated to be between 3-20 per 100,000 people (255,256).

Individuals with two MEN1 manifestations had a 26% chance of having a *MEN1* PV (257). Due to the relatively low PV detection rates in sporadic cases, no single MEN1-associated tumor is sufficient to warrant genetic counseling referral (57), except for gastrinoma, of which 20% are due to *MEN1* PVs (258).

The age-related penetrance of MEN1 exceeds 50% by age 20 and 95% by age 40 (258). MEN1 syndrome is marked by the development of various combinations of over 20 different types of endocrine and nonendocrine tumors. These tumors often show loss of heterozygosity (LOH) at chromosome 11q13, where the *MEN1* gene is located, leading to biallelic loss of *MEN1* (258–262). Additionally, instances of geographical clustering of MEN1 due to founder effects have been reported (263).

**Multiple endocrine neoplasia type 2**

Multiple endocrine neoplasia type 2 (MEN2) is an AD neuroendocrine neoplasia predisposition syndrome caused by gain-of-function mutations in the *RET* gene (264). MEN2 is characterized by increased risks for medullary thyroid cancer (up to 100%), pheochromocytomas (up to 50%), and parathyroid disease (up to 30%) (265–267). The syndrome occurs in approximately 1 in 200,000 live births and presents with variable penetrance depending on the specific subtype and associated genotype (265).

MEN2 has several subtypes, each with distinct age-related penetrance, frequencies, and clinical presentations of component neoplasia. As many as 25% of unselected individuals with medullary thyroid cancer have a *RET* PV (268). Additionally, genetic testing for *RET* PVs in individuals with nonsyndromic pheochromocytomas has found a PV in about 5% of cases in some studies (269), though other studies report lower rates (227).

***MUTYH*-associated polyposis**

*MUTYH*-associated polyposis (MAP) is a recessive condition resulting from biallelic PVs in the *MUTYH* gene, leading to a significantly increased risk of developing adenomatous colon polyps and CRC, with a lifetime risk as high as 80–90% (270). MAP was first identified in 2002 by a Welsh research group (271). Individuals with MAP may develop a varying number of adenomatous polyps, ranging from just a few to over 100, which can cause overlap with FAP, attenuated FAP, and Lynch syndrome (272,273).

Although MAP accounts for less than 1% of all CRCs, it is associated with a 28-fold increased risk of CRC (274,275). Testing for MAP is typically conducted alongside testing for the APC gene, especially in patients with 10 or more adenomatous polyps. MAP testing is also considered for patients diagnosed with colorectal cancer before age 50, particularly if their tumors show mismatch repair proficiency and Lynch syndrome has been excluded (57).

The prevalence of biallelic *MUTYH* PVs varies with the number of adenomas: 2% in patients with 1000 or more adenomas, 7% in patients with 100–999 adenomas, 7% in patients with 20–99 adenomas, and 4% in patients with 10–19 adenomas (173).

**Peutz–Jeghers syndrome**

Peutz-Jeghers syndrome (PJS) is an AD disorder caused by PVs in the *STK11* gene, characterized by distinct mucocutaneous hyperpigmentation seen in areas such as the mouth, lips, nose, eyes, genitalia, or fingers. Individuals with PJS also develop multiple hamartomatous polyps throughout the gastrointestinal tract, which significantly increases the risk for various cancers. Specifically, there is a heightened risk for CRC (39% between ages 15 and 64), pancreatic cancer (36%), gastric cancer (29%), and small intestinal cancer (13%) (276,277). The incidence of PJS is estimated to be between 1 in 50,000 to 1 in 200,000 live births, with a prevalence ranging from 1 in 8,300 to 1 in 280,000 individuals (277–283).

In addition to gastrointestinal cancers, individuals with PJS face increased risks for other malignancies, including breast cancer (54%), ovarian sex cord tumors with annular tubules (21%), adenoma malignum of the cervix (10%), and Sertoli cell tumors of the testes (9%) (276). The hamartomatous polyps characteristic of PJS are composed of glandular epithelium supported by smooth muscle cells that are contiguous with the muscularis mucosa (57).

**Von Hippel–Lindau syndrome**

Von Hippel-Lindau (VHL) syndrome is an AD familial neoplastic syndrome caused by PVs in the *VHL* gene (284). It encompasses a spectrum of tumors affecting multiple organs, with the most common manifestations being hemangioblastomas in the brain, spinal cord, and retina; clear cell RCC; pheochromocytomas and paragangliomas; and pancreatic neuroendocrine tumors (285). Additional findings include endolymphatic sac tumors and papillary cystadenomas of the epididymis and broad ligament. VHL affects approximately 1 in 36,000 live births, with a penetrance exceeding 90% by age 65. Symptoms typically appear in the second decade of life, and about half of patients are symptomatic at diagnosis, often presenting with cerebellar hemangioblastomas (284,286).

*VHL* PVs are detected in 10–40% of individuals with isolated central nervous system hemangioblastoma, 46% with isolated retinal capillary hemangioma, 3–11% with isolated pheochromocytoma, and approximately 20% with an endolymphatic sac tumor (226,227,287–290). Referral for genetic counseling should be considered for individuals with personal or first-degree family history of clear cell RCC (especially bilateral or multifocal, diagnosed before age 50, or with close relatives affected), central nervous system hemangioblastoma, pheochromocytoma, endolymphatic sac tumor, or retinal capillary hemangioma. Unilateral, solitary RCC diagnosed at or after age 50 alone does not typically warrant genetic counseling referral (57,289,291).

**_DICER1_ syndrome**

*DICER1* syndrome, also known as pleuropulmonary blastoma familial tumor susceptibility syndrome, is an AD genetic disorder caused by PVs in the *DICER1* gene (292). The penetrance of germline *DICER1* PVs for clinical phenotypes has been calculated to be ~ 5% by age 10 years, increasing to ~ 20% by age 50 (293). This syndrome has been linked to endocrine tumors, multinodular goiter, pleuropulmonary blastoma, cystic nephroma, and ovarian Sertoli-Leydig Cell Tumors (294).

*DICER1* syndrome-related tumors usually harbor an additional somatically acquired missense PV in the RNase IIIb cleavage domain of the gene. This second PV fulfills Knudson's two-hit hypothesis, but in contrast to the classic model, with a few exceptions, the event on the second allele does not fully suppress *DICER1* function (295). Instead, hotspot PVs in the RNase IIIb domain interfere with *DICER1*'s ability to process miRNAs (296–298).

Approximately 87% of *DICER1* PVs are inherited, while about 13% are *de novo* (299). Somatic mosaicism is also observed in around 10% of *DICER1* syndrome cases (300). Those patients with mosaic PVs in the RNase IIIb hotspot develop a greater number of disease foci at significantly younger ages compared with non-RNase IIIb-mosaic individuals. In contrast, those with mosaic loss-of-function PVs tend to have one or two foci of disease. Regardless of the type of mosaic variant, a second PV is present (including LOH) (299,300).

A subset of *DICER1* syndrome tumors presents biallelic *DICER1* alterations limited to the tumor (i.e. biallelic somatic PV). These patients have a single-organ disease and are not considered syndromic or at risk of developing other *DICER1* syndrome symptoms, although an unrecognized mosaicism of the identified loss-of-function PV should be considered (301,302).

## Digenic inheritance and oligogenic inheritance

The first documented case of digenic inheritance in human disease was for retinitis pigmentosa in 1994. This was a compelling report because it included data from multiple pedigrees and showed that the protein products of the two implicated genes interacted with each other (303).

When a germline PV is identified, for a few CPGs it enables the use of targeted therapies for patients and the inclusion of their relatives in cancer surveillance programs. The advent of NGS technologies, which allow for the simultaneous analysis of multiple cancer predisposition genes (CPGs) with reduced time and cost, has significantly increased the use of multigene panel testing over the past decade. This has led to the identification of a growing number of individuals carrying two or more PVs in CPGs. As more genes are included in these panels, the occurrence of this phenomenon is expected to rise (304). Whitworth and colleagues have termed this condition Multilocus Inherited Neoplasia Alleles Syndrome (MINAS) (305).

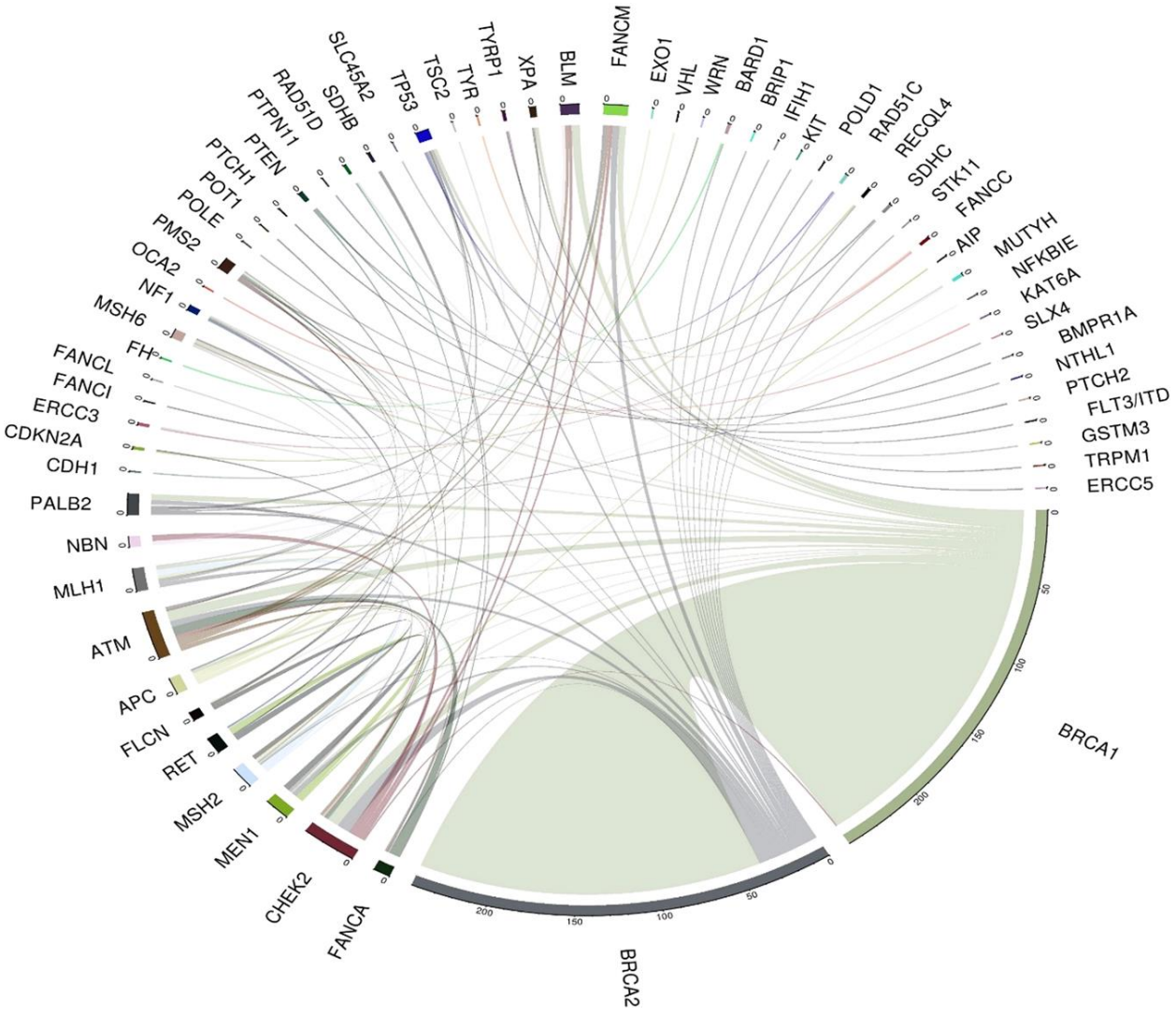
In addition to well-known cancer predisposition syndromes such as the ones described in the previous section, the presence of at least two independent, monoallelic germline PVs in different genes within the same signaling pathway has been described. These combined monoallelic double hits likely contribute to the clinical cancer phenotype by disrupting the affected signaling pathway. Importantly, these phenomena, which result from inherited combined digenic low-penetrance PVs, can occur even when parents show no clinical signs and the family history appears unremarkable (306).

Similarly, observations in breast cancer patients have suggested that low-penetrance cancer susceptibility SNVs act as modifier genes in both *BRCA1/BRCA2* PVs carriers and non-carriers, thereby increasing cancer risk. This phenomenon might involve genes acting as modifiers within the same cancer pathway or low-penetrance SNVs in individuals without *BRCA1/BRCA2* PVs (307,308). Additionally, combined monoallelic PVs in Fanconi anemia/breast cancer (*FA/BRCA*) pathway genes have been identified in patients with more severe disease phenotypes. The *FA/BRCA* pathway is crucial for maintaining genome integrity and plays essential roles in the DDR (306).

NGS can significantly accelerate the discovery of human digenic inheritance. By enabling the simultaneous sequencing of numerous genes, NGS allows the identification of disease-relevant PVs in two genes within a single experiment. However, it does not solve the challenge of determining which PVs are relevant to the phenotype, a task that is more complex in digenic inheritance than in monogenic inheritance (303).

A big review that compiled the published literature about digenic inheritance in CPGs was published in 2022. It included 385 individuals with MINAS, who presented 430 unique P/LP variants across 63 CPGs. The analysis revealed that 78.5% (287 out of 385) of these cases carried a *BRCA1* and/or *BRCA2* variant (See Figure 9). Among these individuals, 108 (28%) presented with MPCs. Of these 108 cases, 2 (1.9%) had an unknown number of MPCs, 75 (69%) had two primary tumors, 18 (17%) had three, and 13 (12%) had four or more. The most common combinations of MPCs were Breast-Ovarian (33 cases), Breast-Breast (24 cases), and Colon-Colon (6 cases) (304).

**Figure 9.** Circos plots illustrating combinations of cancer predisposition genes involved in individual cases of MINAS (n = 385)



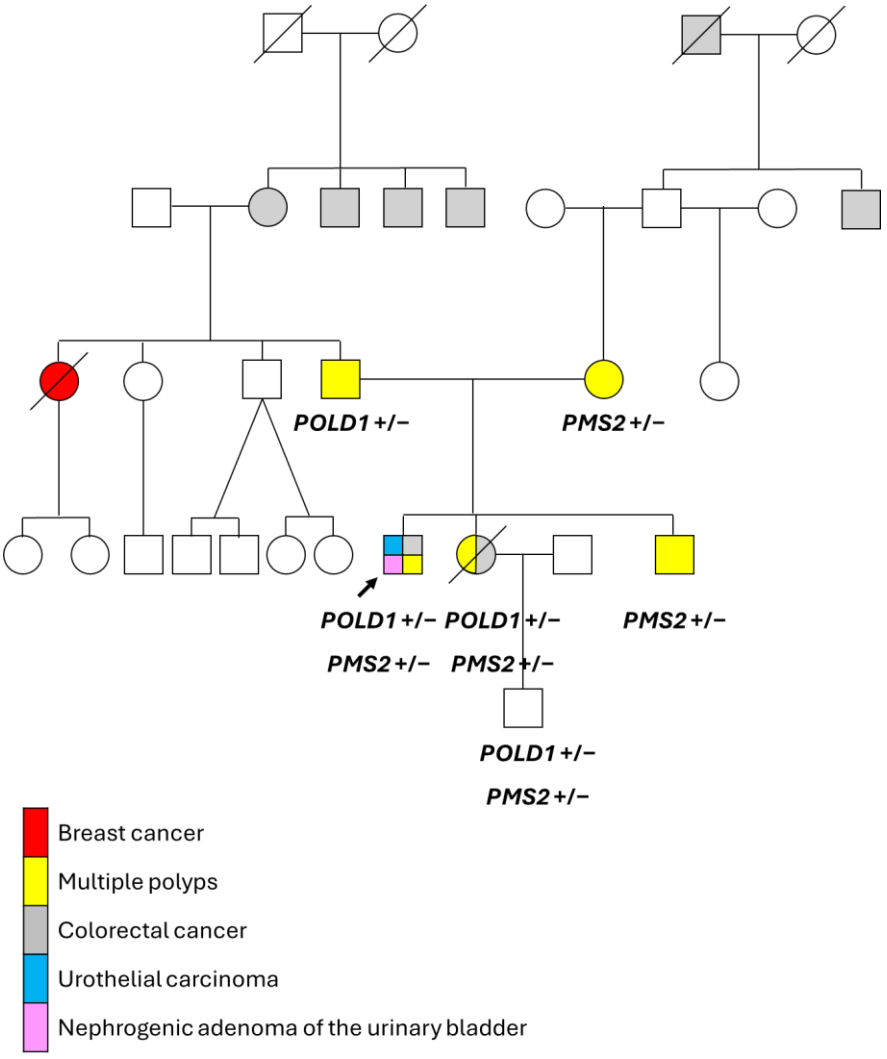

Note: (304)

In a big German study, out of 1485 total germline sequenced patients, 138 patients had one AD PV, 59 patients presented one AR PV (heterozygous), 6 patients had two AD PVs, 5 patients – a combination of one AD PV and one AR PV, two patients had homozygous germline PVs in an AR gene (*FANCA* and

*MUTYH*), one patient had two AD and one heterozygous AR PVs, and one patient presented two AR germline PVS in two different genes (309).

Three families with cases of childhood/adolescent and young adult cancer caused by digenic inheritance of heterozygous PVs in *PMS2* + *POLE* and *PMS2* + *POLD1* were reported. Two siblings with multiple adenomas and CRC had a maternally inherited heterozygous *PMS2* PV, that was not suspected in the family, and a paternally inherited *POLD1* PV (See Figure 10) (310).

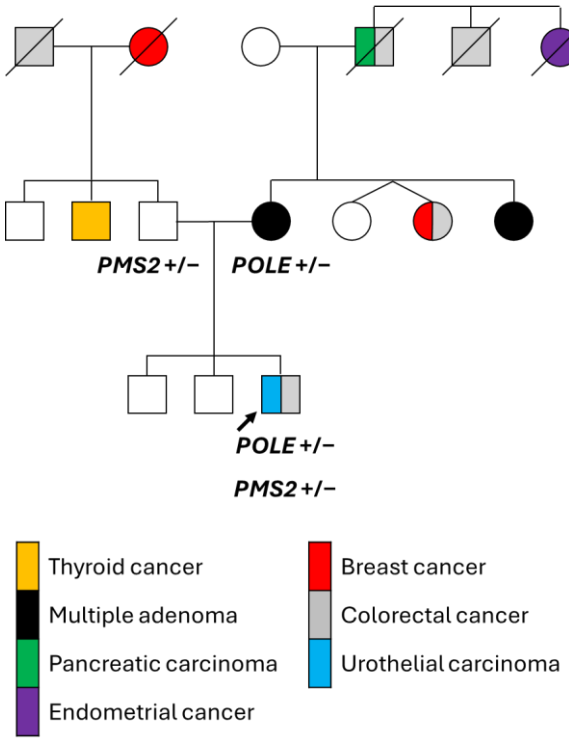
**Figure 10.** Segregation of pathogenic variants in a colorectal cancer family




*POLD1*+/- heterozygous PV in *POLD1*, *PMS2*+/- heterozygous PV in *PMS2*. *Note*: (310)

In one of the *PMS2* + *POLE* families, several maternal relatives of the proband, who developed colorectal cancer at 16 and high-grade urothelial carcinoma at 19, also had early-onset CRC. However, polymerase proofreading-associated polyposis (PPAP) was not initially suspected in the family (See Figure 11).

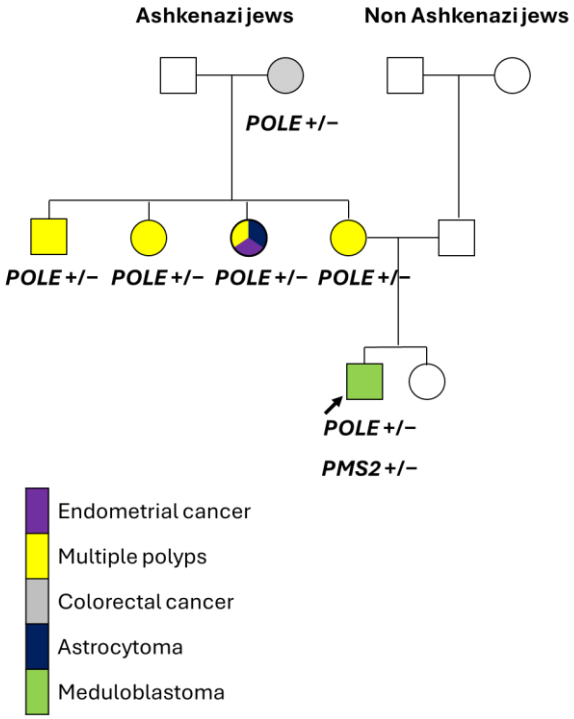
**Figure 11.** Segregation of pathogenic variants in a colorectal cancer family




*POLE*+/- heterozygous PV in *POLE*, *PMS2*+/- heterozygous PV in *PMS2*. Note: (311)

The second case described a patient from a family with a known *POLE* PV, who developed medulloblastoma at 4.5 years old. Analysis revealed the patient inherited the *POLE* PV from the mother and had a de novo *PMS2* (See Figure 12).

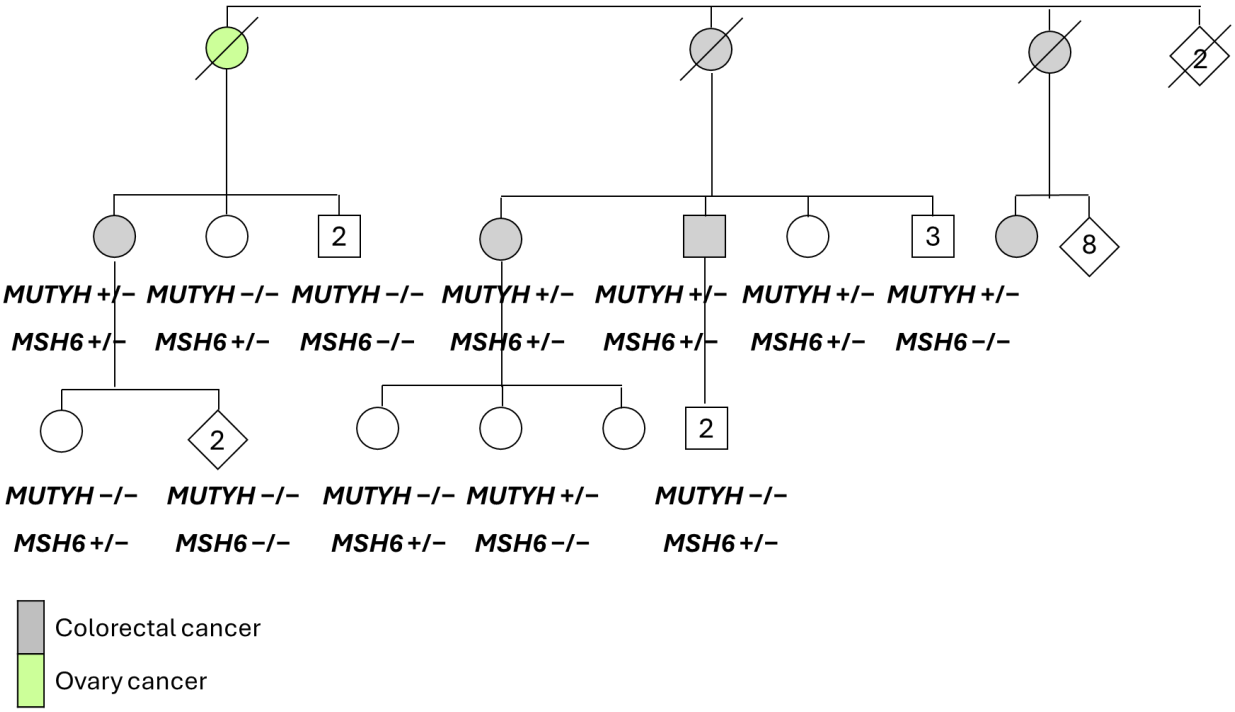
**Figure 12.** Segregation of pathogenic variants in a family with a proband with multiple cancers




*POLE*+/- heterozygous PV in *POLE*, *PMS2*+/- heterozygous PV in *PMS2*. *Note*: (312)

Another CRC family presented digenic inheritance of germline PVs in MSH6 and MUTYH genes. The family comprised 7 cancer patients divided over two generations (See Figure 13). Fourteen relatives, all unaffected by cancer or polyposis, were genotyped for these variants, identifying one additional carrier of both variants, five MSH6-only carriers, and four MUTYH-only carriers (313). The involvement of MSH6 and MUTYH in oxidative DNA damage repair, along with their physical interaction that enhances MUTYH's repair activity, supports the association of variants in these genes (314).

**Figure 13.** Segregation of pathogenic variants in a family with a proband with multiple colorectal cancers




*MUTYH*+/- heterozygous PV in *MUTYH*, *MSH6*+/- heterozygous PV in *MSH6*. *Note*: (313)

The coinheritance of monoallelic splice site variants in OGG1 (involved in the repair of 7,8-dihydro-8-oxoguanine) and missense MUTYH has also been described in a female with advanced synchronous colon cancer and adenomas at 36 years old. Segregation analysis within the family revealed that the patient's mother and two aunts, who were diagnosed with adenomas at ages 66 and 67 CRC at age 56, respectively, were heterozygous carriers of the MUTYH variant. Additionally, the OGG1 variant was inherited from the father, who had only a single polyp at age 65 (315).

A report of a coinheritance of BRCA2 and CYLD germline PVs in a family with malignant cylindroma and breast cancer emphasizes the importance of the recognition of double heterozygotes for the patient's treatment. A 29-year-old man presented with cylindromas on his scalp since he was 8 years old, inherited from his mother who had CYLD cutaneous syndrome. The proband's father and paternal grandmother had early-onset breast cancer, while his 33-year-old sister was diagnosed with breast cancer and CYLD cutaneous syndrome. Aged 28 years, the proband presented a malignant metastatic

cylindroma of the scalp. Somatic sequencing demonstrated LOH including the BRCA2 and CYLD loci. The germline and somatic PVs in this patient supported the use of unlicensed therapeutics that target BRCA deficiency, such as poly (ADP-ribose) polymerase inhibitor (PARPi), which have not previously been used to treat malignant cylindroma (316).

The occurrence of a single patient carrying PVs in both BRCA1 and BRCA2 genes is rare, except in specific subpopulations with founder effects. Studies have reported that the prevalence of double heterozygotes ranges from 0.2% to 0.8% across various ethnic groups, but it reaches 1.8% among Ashkenazi Jewish individuals (317). Approximately thirty studies have identified families with breast and ovarian cancer that harbor PVs in both BRCA1 and BRCA2 (318).

Similar to monogenic cancer predisposition syndromes (CPS), digenic inheritance cases can exhibit unusual phenotype-genotype correlations. In an 11-year-old girl diagnosed with metastatic osteosarcoma, researchers identified two simultaneous monoallelic germline PVs in BRIP1 and HIPK2 using trio WES. BRIP1 PVs were previously linked to breast cancer but not to osteosarcoma. Notably, the girl's mother, who passed on the BRIP1 variant, was diagnosed with breast cancer at age 46. The HIPK2 PV, inherited from the father, affects a critical regulator of the DDR pathway and plays a key role in DNA double-strand break repair (306).

In a comprehensive review, it was estimated that 14.6% of patients with 2 PVs with one outside the BRCA1/BRCA2 genes (13 out of 89) had at least one tumor type not typically associated with the relevant CPGs, such as clear cell RCC in a patient with variants in both BRCA1 and MLH1 PVs. In a second cohort, an atypical tumor phenotype was observed in 15.8% (12 out of 76) of non-BRCA1/BRCA2 tumor cases. However, not all studies provided individual patient-level data (304).

Four examples of atypical MINAS phenotypes included a woman diagnosed with breast cancer and Waldenstrom’s disease at 58 years old, carrying PVs in BRCA1 and BLM (319), and another woman diagnosed with breast cancer, melanoma, and CRC, with a PV in FANCC and a P/LP variant in TYR (320). Additionally, there was a woman diagnosed with lobular breast cancer at 51 years, followed by follicular adenoma and thyroid micropapillary carcinoma at 52 years, carrying PVs in PMS2 and CDH1 (321), and a woman diagnosed with cutaneous leiomyomas at 40 years, followed by colorectal polyposis at 52 years, found to be heterozygous for P/LP variants in FH and BARD1 (322). In these cases, Waldenstrom disease, colorectal cancer, thyroid carcinoma, and colorectal polyposis occurred despite not being associated with any of the relevant MINAS CPGs. Each case involved MPCs and rare CPGs combinations, making it unclear whether these were manifestations of synergy between the CPGs or coincidental. The presence of MPCs of unusual types might have prompted genetic testing in these patients (304). Additional examples of oligogenic conditions and their associated causative variants are available in the Oligogenic Diseases Database (OLIDA) (323).

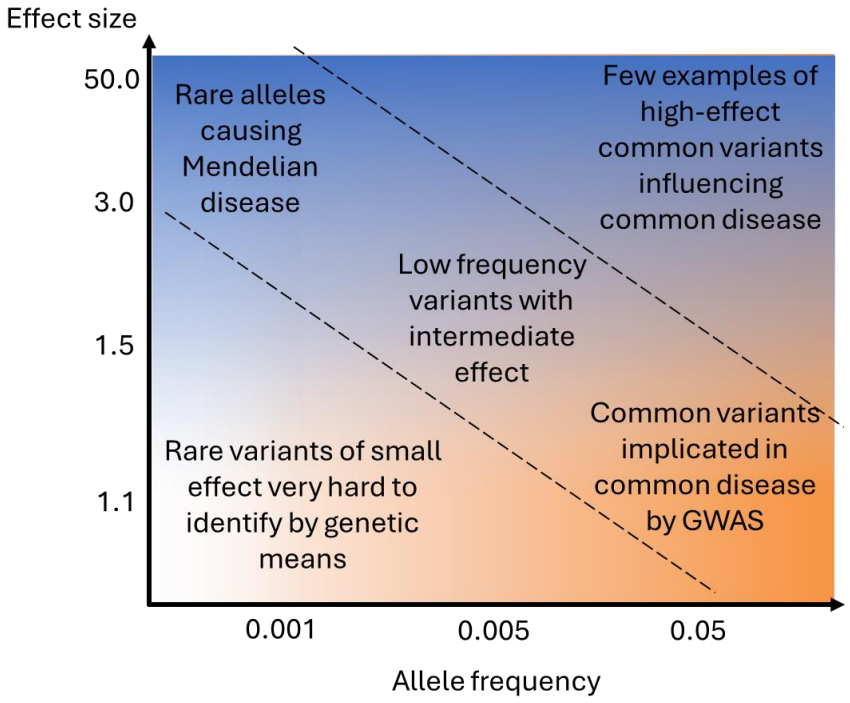
## Polygenic risk scores and mutational signatures

In the context of complex diseases such as cancer, both polygenic risk scores (PRS) and mutational signatures are tools used to understand and predict disease risk with potential clinical applications. It has been demonstrated that strong connections exist between PRS derived from germline genetic data and mutational signatures from the somatic mutational patterns in tumors. These correlations likely signify the roles of hormone regulation and immune responses in cancer development and progression, shedding light on the underlying mechanisms driving these processes (324).

### Polygenic risk scores

The germline genome holds the code for susceptibility or resistance to cancer. Studying it unveils variants in genes associated with cancer predisposition and susceptibility. Each cancer type and subtype exhibit a distinct genetic architecture comprised of very rare variants with substantial effects, more common moderately impactful variants, and common variants with minor effects (see Figure 14). These common variants can be combined in polygenic scores for cancer susceptibility (325–327). Sometimes not PVs, but the combination of many common variants contributes to cancer development risk.

**Figure 14.** Spectrum of distribution of susceptibility alleles according to their frequency and strength of genetic effect




High penetrant mutations (very low-frequency alleles with a high effect size) have been the easiest to identify as causing Mendelian disease using standard genetic techniques, while common susceptibility alleles (high frequency with a low effect size) were discovered in genome-wide association studies (GWAS). *Note*: adapted from (1)

Numerous fields within genetics now employ combinations of variants that collectively account for a significant portion of disease risk variation. Combining variants into PRS can explain a larger proportion of disease risk compared to single high penetrance variants. For instance, PRS derived from genome-wide association studies (GWAS) have identified loci used for cancer screening, risk prediction, and risk stratification purposes (328,329). PRS combine all the SNVs identified in a GWAS, giving greater weight to the SNVs more strongly associated with cancer, creating a single score. Individuals with higher PRS are anticipated to have higher disease risk than those with lower PRS (330,331). PRS have been described in breast cancer (327,332), prostate cancer (333), CRC (334), lung cancer (335) and ovarian cancer (336).

PRS effectively stratify breast cancer risk in the general population (327). Studies have shown that for some breast cancer predisposition genes, the risk associated with a P/LP variant combines multiplicatively with the risk derived from the PRS (337,338). In the case of single genes with a more moderate effect, the adjustment by the PRS alone can alter an individual's final risk classification and guide their corresponding clinical management. As data on polygenic risk has been primarily derived from studies restricted to populations of European ancestry, its applicability to people from other ethnic backgrounds remains uncertain (339). However, recent research suggests that the established PRS may offer some value, at least within Asian populations (340).

In population health, PRS application can identify groups at risk for cancer, enabling early interventions to decrease disease burden. These interventions include earlier or more frequent screening for disease biomarkers, lifestyle modifications, dietary adjustments, or preventive medical interventions. Additionally, PRS testing can identify individuals who could benefit from participation in new treatment trials (341).

**Mutational signatures**

Cancer arises from the accumulation of somatic mutations, distinct from the germline variants that are present in every cell. These somatic mutations, occurring in all human cells and persisting throughout life, result from various mutational processes, including inherent inaccuracies in DNA replication, exposure to external or internal mutagens, enzymatic DNA modifications, and impaired DNA repair mechanisms, giving rise to distinctive combinations of mutation types known as mutational signatures (324,342).

Mutational signatures are indicators of the mutational processes that were active throughout an individual's lifespan. Over recent years, various studies have unveiled more than 50 mutational signatures across different cancer types. However, the underlying causes of many of these signatures remain unknown. Mutational signatures encompass both processes commonly occurring across various cancer types and those specific to particular cancers. Signatures like SBS2 and SBS13 that are

linked to the enzymatic activity of the APOBEC family are present across multiple cancer types. On the contrary, signature SBS12 of an unknown etiology predominantly manifests in liver cancers. Another set of signatures reflect lifestyle choices and environmental exposures. Signatures like SBS4 correlate with tobacco smoking, while signatures SBS7a/b/c/d are influenced by exposure to ultraviolet light. Finally, some signatures arise from endogenous exposure. The clock-like signature SBS1 results from endogenous deamination of 5-methylcytosine (324,342–344).

## MPCs without familial predisposition

Outside of a familial context, MPCs may be linked to germline de novo PVs, low-penetrance PVs or mosaicism in predisposing genes. In a study of 100 patients with multiple primary melanoma (MPM) and no family history of melanoma, 9% were found to have germline PVs in the *CDKN2A* gene. The results indicated that six of the seven sporadic MPM cases shared a common ancestral PV. Therefore, de novo *CDKN2A* germline PVs associated with MPM are rare, but not non-existent (345).

PVs in the *TP53* gene have been described as both de novo and mosaic variants in Li-Fraumeni syndrome patients. Among 328 unrelated patients with *TP53* PVs identified by Sanger sequencing or Quantitative multiplex PCR, 40 cases were confirmed as de novo, with no correlation to parental age. Additionally, two mosaic PVs were identified: one in a child with adrenocortical carcinoma and the other in the unaffected father of a child with medulloblastoma. A re-analysis using NGS of 108 patients suspected of Li-Fraumeni syndrome but without detectable *TP53* PVs revealed six additional mosaic *TP53* variants in children and adults with adrenocortical carcinoma, choroid plexus tumors, breast cancer, osteosarcoma, and sarcoma. This study estimated that de novo PVs account for at least 14% of Li-Fraumeni syndrome cases, with around 20% of these variants occurring during embryonic development (346). In *BRCA1*- and *BRCA2*-Associated HBOC, de novo variants have been reported in ≤5% of cases (347,348), and inheritance of a PV from a mosaic parent has also been documented (349).

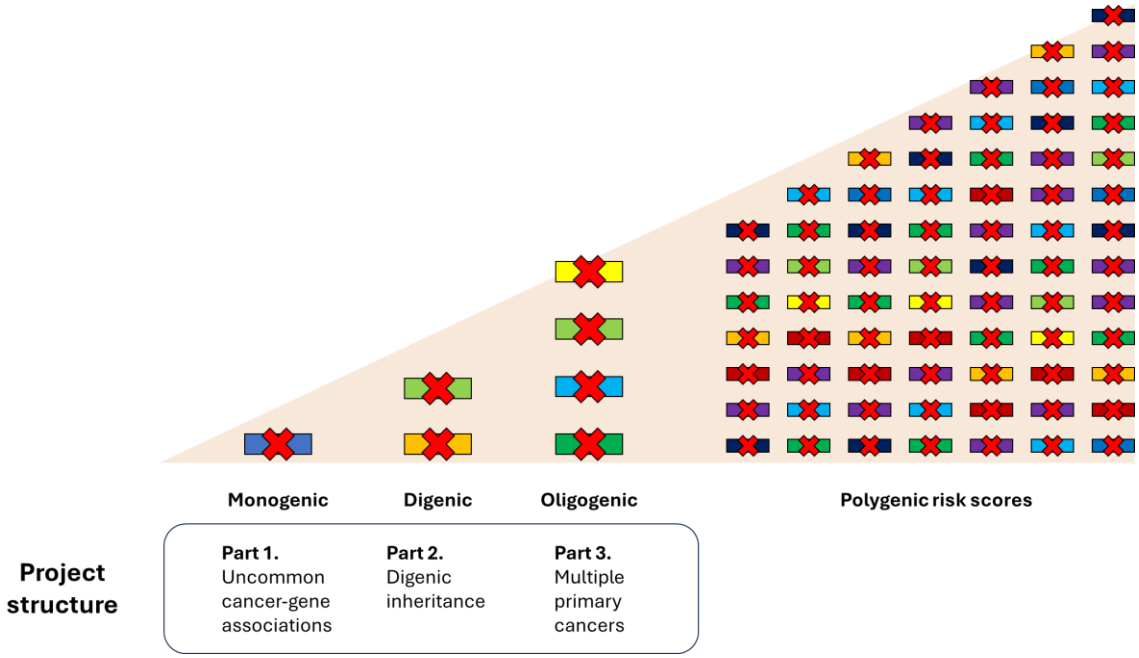
If a cancer risk variant is mosaic, it may evade detection by clinical tests due to their limited sensitivity. For instance, mosaicism has been identified in cases where activating *HIF2A* variants were present in multiple tumors but not in the blood of patients with multiple paragangliomas. This phenomenon could account for some individuals with MPCs who have no relevant family history. Genetic studies in such cases may still offer important insights, even without a family history suggestive of inherited predisposition (350,351).

# Overview of the project and aim of the thesis

## Project structure

Genetic cancer predisposition can be viewed as a continuous condition, starting with monogenic inheritance, and going through digenic, oligogenic and PRS (See Figure 15). In monogenic inheritance, very rare variants with very high effect in a single CPG predispose an individual to a tumor or a specific set of tumors. However, some patients may present with types of cancer that are unusual for a specific gene. When an individual has two low-frequency variants with high effects in two CPGs, this is known as digenic inheritance. Next, there are oligogenic combinations, typically involving 3-20 genetic loci (352) with variable allele frequencies and moderate effect sizes. These loci can interact in various ways, including as a dominant gene with its modifiers or as genes with similar effects in the disease. Finally, PRS involve hundreds or even thousands of genetic variants, each with a relatively high allele frequency but very low effect size, collectively increasing the risk of an individual developing cancer.

**Figure 15.** Cancer predisposition spectrum




Genetic counseling for cancer patients represents numerous challenges, particularly as genomic technologies evolve. The advent of NGS has shifted the landscape from single-gene testing to panel sequencing and even WES and WGS. These advancements have made testing more affordable and faster, leading to an increase in genetic testing outside the tumor phenotype. This has resulted in the discovery of previously unknown tumor-gene associations (Part 1. Uncommon cancer-gene associations). Additionally, enhanced surveillance and follow-up in families with known cancer

predisposition variants have uncovered further PVs, revealing complex inheritance patterns such as digenic inheritance (Part 2. Digenic inheritance). Moreover, testing patients with MPCs who lack known PVs in CPGs holds promise for expanding our understanding of the genetic basis of cancer (Part 3. Multiple primary cancers).

## Objectives

MPCs can be explained through monogenic, digenic, oligogenic or undetectable causes. This project focuses on three main MPCs mechanisms: monogenic (Part 1. Uncommon cancer-gene associations), digenic (Part 2. Digenic inheritance), and oligogenic (Part 3. Multiple primary cancers) aiming to refine genetic mechanisms behind high cancer susceptibility and enhance personalized care.

### Uncommon cancer-gene associations

This part investigates the controversial role of *BRCA1* in CRC. While *BRCA1*'s involvement in homologous recombination deficiency (HRD) and its importance in targeted cancer treatments are well-documented, its role in CRC remains unclear. This case series aims to clarify whether pathogenic *BRCA1* variants contribute to early-onset CRC development, besides its known role in breast and ovarian cancer risk.

### Digenic inheritance

This section explores the tumor risk associated with digenic inheritance involving known CPGs such as *BRCA1*, *BRCA2*, *ATM*, and *CHEK2*. The study describes six patients from five families who inherited combinations of pathogenic variants in these genes, including four patients with *BRCA2* and *ATM* variants, one with *BRCA2* and *BRCA1* variants, and one with *BRCA2* and *CHEK2* variants. These people had a wide range of tumors, including early cancers, multiple tumors or the absence of any malignant disease.

### Multiple primary cancers

This part explores the novel genetic mechanisms underlying tumor development in patients with MPCs diagnosed before the age of 45, particularly those who do not have a germline molecular genetic diagnosis after initial evaluation. This research aims to uncover additional CPGs and provide insights into the genetic factors contributing to high cancer susceptibility in these patients.

By investigating MPCs, rare cancer-gene associations, and digenic inheritance, the project aims to expand our understanding of genetic cancer predisposition. The findings will contribute to improved genetic counseling, early detection, and the development of personalized treatment strategies for patients with hereditary cancer risk.

# General methods

This section provides an overview of the general methodologies employed across the studies. For detailed methodologies specific to each study, please refer to the corresponding sections within the respective publications.

## Patient selection and data collection

The studies included cancer patients who underwent evaluation at the genetic department of the CHU of Liege. The inclusion criteria differed for each study (see Table 15). Patient clinical and genetic data was gathered from their medical charts.

**Table 15.** Inclusion criteria for the studies

| Inclusion criteria | Uncommon cancer-gene associations | Digenic inheritance | Multiple primary cancers |
|---|---|---|---|
| Young age | Young age at first cancer diagnosis | Yes | Yes |
| Cancer characteristics | Aggressive cancer | Breast cancer, multiple cancers | Multiple cancers |
| Cancer family history | Yes | Yes | No |

## Genetic evaluation

The studies involved the evaluation of both germline DNA, obtained from the patients' blood samples, and somatic DNA extracted from Formalin-Fixed Paraffin-Embedded (FFPE) tumor samples (See Table 16).

**Table 16.** Genetic evaluation in the studies

| | **Uncommon cancer-gene associations** | **Digenic inheritance** | **Multiple primary cancers** |
|---|---|---|---|
| Germline DNA | Targeted NGS<br>WES<br>Sanger sequencing | Targeted NGS<br>MLPA<br>Sanger sequencing | WES<br>CMA<br>Sanger sequencing |
| Somatic DNA | Targeted NGS<br>WES<br>CMA | - | WES |
| Bioinformatic evaluation | Mutational signatures | - | Mutational signatures<br>CNV detection in WES data |

CMA – Chromosomal Microarray Analysis, CNV – Copy Number Variation, MLPA – Multiplex Ligation-dependent Probe Amplification, NGS – Next Generation Sequencing, WES – Whole Exome Sequencing.

## Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and approved by the Comité d'Ethique Hospitalo-facultaire Universitaire de Liège (protocol code 2019/245, 28/10/2019 date of approval)

# Part 1. Uncommon cancer-gene associations

*BRCA1,* a gene encoding a multifunctional protein integral to many cellular pathways, has been linked to various neoplasms, including hereditary breast and ovarian cancers. However, its connection with CRCs remains debatable. The scarcity of data on young patients with *BRCA1* variants developing CRCs underscores the need for a deeper understanding of the role of these variants in early-onset CRCs.

This case series presents three patients diagnosed with aggressive early-onset CRC, each harboring heterozygous *BRCA1* PVs. Comprehensive molecular analyses, including WES, targeted NGS and CMA, unveiled the genetic landscape of these tumors. Notably, all tumors exhibited elevated HRD scores, with HRD-related mutational signatures contributing significantly to the somatic variant profiles.

Contradictory findings characterize existing data on the association between *BRCA1* variants and CRC risk. While some studies suggest an increased risk, others fail to establish a definitive link. However, emerging evidence hints at a potential correlation, as evidenced by the presence of *BRCA1* variants in CRC patients and high HRD scores in their tumors. These observations highlight the need for *BRCA1* testing in young patients with microsatellite-stable CRC and advocate for personalized treatment strategies, such as PARPi, in select cases.

The findings of this case series highlight the potential involvement of *BRCA1* germline PVs in CRC development through HRD. Incorporating *BRCA1* testing into the diagnostic workup of young CRC patients could facilitate tailored treatment approaches, potentially improving clinical outcomes in this subset of patients.

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# Case Report Series: Aggressive HR Deficient Colorectal Cancers Related to *BRCA1* Pathogenic Germline Variants

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**Objective:** The link between *BRCA1* and homologous recombination deficiency (HRD) in cancer has gained importance with the emergence of new targeted cancer treatments, while the available data on the role of the gene in colorectal cancer (CRC) remain contradictory. The aim of this case series was to elucidate the role of known pathogenic *BRCA1* variants in the development of early-onset CRC.

**Design:** Patients were evaluated using targeted next generation sequencing, exome sequencing and chromosomal microarray analysis of the paired germline and tumor samples. These results were used to calculate the HRD score and the frequency of mutational signatures in the tumors.

**Results:** Three patients with metastatic CRC were heterozygous for a previously known BRCA1 nonsense variant. All tumors showed remarkably high HRD scores, and the HRD-related signature 3 had the second highest contribution to the somatic pattern of variant accumulation in the samples (23% in 1 and 2, and 13% in sample 3).

**Conclusions:** A *BRCA1* germline pathogenic variant can be involved in CRC development through HRD. Thus, *BRCA1* testing should be considered in young patients with a personal history of microsatellite stable CRC as this could further allow a personalized treatment approach.

**Keywords: colorectal (colon) cancer, BRCA1, homologous recombination deficiency (HRD), exome sequencing (ES), case report**

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# INTRODUCTION

*BRCA1* is a tumor suppressor gene encoding a large protein that coordinates several cellular pathways including DNA repair, transcriptional regulation, cell-cycle control, centrosome duplication, and apoptosis (1). Pathogenic germline variants in *BRCA1* gene have been associated with familial risk of breast and ovarian cancers (OMIM: 604370) (2, 3). As early as in 1994, it was observed that women with a history of breast, endometrial, or ovarian cancer presented a statistically significant although small risk for subsequent colorectal cancer (CRC), suggesting the existence of common etiologic factors for the development of these tumors (4).

Data concerning young patients with *BRCA1* variants that develop CRC have been scarce. Germline pathogenic variants in *BRCA1* gene have not been causally linked to an increased risk of familial colorectal cancer, but the reports on the subject are contradictory (5–9). Indeed, patients carrying a germline *BRCA1* variant can develop a sporadic tumor, independently of *BRCA1* loss of function, highlighting the need to demonstrate the causal role of the variant in the cancer development (10).

The aim of this case series was to gain insight into the role of known pathogenic *BRCA1* variants in the development of early onset CRC

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# CASE DESCRIPTION

Three patients were diagnosed in 2020 and 2021 with aggressive early-onset CRC. The demographic, familial, clinical, histopathological, and molecular characteristics, as well as the treatment regimens of these patients are presented in Table 1.

The first case was referred to oncogenetic consultation due to the young age of presentation of an aggressive disease without evidence of Lynch syndrome (no mismatch repair deficiency or microsatellite instability) and history of a *BRCA1* pathogenic

**TABLE 1** | Patient characteristics.

| Parameter | Case 1 | Case 2 | Case 3 |
|---|---|---|---|
| Age (year) | 31 | 56 | 35 |
| Sex | Female | Female | Male |
| Medical history | None | Breast cancer at 36 y/o, contralateral breast cancer at 41 y/o | Ulcerative colitis |
| Family history | Maternal side: aunt breast cancer, grandmother CRC, great-grandmother uterine cancer | Paternal side: aunt CRC, grandmother ovary cancer, grandmother's sister breast cancer | Maternal side: five aunts breast cancer, grandmother ovary cancer. Paternal side: grandmother CRC |
| CRC localization and type | Right colon moderately differentiated adenocarcinoma | Well to moderately differentiated rectum adenocarcinoma | Mucinous appendix adenocarcinoma |
| TNM tumor staging | pT4aN2aM1a | cT3N1M1b | pT4bN0M0 at diagnosis, peritoneal relapse at month 5 |
| IHC and molecular tests on the tumor | | | |
| MSI-H | No | No | No |
| MLH1, MSH2, MSH6 and PMS2 protein expression | Normal | Normal | Normal |
| **Identified variants** | | | |
| Somatic pathogenic variants (heterozygous) | KRAS c.35G>A (p. Gly12Asp)<br>TP53 c.524G>A (p.Arg175His) | KRAS c.35G>A (p. Gly12Asp) | – |
| Germline pathogenic variants (heterozygous) | *BRCA1* c.1016dup (p.Val340Glyfs*6) | *BRCA1* c.3756_3759del (p.Ser1253Argfs*10) | *BRCA1* c.3841C>T (p.Gln1281*) |
| **Somatic CMA array results** | Partial gains and losses on Chr 1-3, 5- 9, 12, 13, 15-20 and X | Entire and partial gains and losses on Chr 1, 7, 8, 12, 13 and 18-20 | Normal |
| **HRD evaluation** | | | |
| HRD score | 59 | 61.15 | 66 |
| Proportions of mutational signatures with a proposed etiology | | | |
| SBS1 | 24% | 28% | 14% |
| SBS3 | 23% | 22% | 13% |
| SBS5 | 0% | 0% | 20% |
| Treatment | | | |
| Surgical | Right colectomy with lymph node dissection, ileocolonic anastomosis and metastasectomy of liver segments | Anterior rectum resection and hepatic surgery | Ileocolectomy with a lymph node dissection firstly and a posterior debulking surgery with IPCH after discovery of a peritoneal carcinomatosis |
| Chemotherapy | Pseudo-adjuvant chemotherapy with capecitabine-oxaliplatin followed by 7 cycles of chemotherapy with FOLFOX-bevacizumab | 6 cycles of FOLFOXIRI | Adjuvant chemotherapy with capecitabine-oxaliplatin regimen (Xelox) |

*CMA, chromosomal microarray analysis; FOLFOX, folinic acid; fluorouracil and oxaliplatin; FOLFOXIRI, fluorouracil; folinic acid; oxaliplatin; and irinotecan; HRD, homologous recombination deficiency; IHC, Immunohistochemistry; IPCH, Intraperitoneal chemohyperthermia; MMR, mismatch repair; MSI, microsatellite instability; SBS, Single Base Substitution; TNM, TNM Classification of Malignant Tumors; y/o, years old. Reference transcripts: BRCA1 NM_007294.3; KRAS NM_004985.5; TP53 NM_000546.6.*

variant in the family. Given the age at the diagnosis of CRC, genes associated with familial polyposis (*NTHL1*, *RNF43*, *SMAD4*, *BMPR1A*), CRC (*POLE*, *POLD1*) and Li-Fraumeni syndrome (*TP53*) were analyzed. However, the patient only carried the heterozygous *BRCA1* pathogenic variant NM_007294.3(BRCA1_v001):c.1016dup (p.Val340Glyfs*6) identified in her maternal aunt.

To further evaluate the disease, targeted next-generation sequencing (NGS) and a high-resolution (180K) chromosomal microarray analysis (CMA) were performed on the DNA extracted from the tumor (estimated proportion of tumor cells in the sample - 50%). After sequencing, the familial pathogenic variant *BRCA1* c.1016dup was identified at an allele frequency (AF) of 70%, suggesting a loss of heterozygosity at the *BRCA1* locus. Further analysis revealed a somatic variant of *TP53* NM_000546.6(TP53):c.524G>A (p.Arg175His) at an AF of 40%. The CMA showed multiple rearrangements indicating genomic instability (chromosomal partial gains and losses on chromosomes 1-3, 5- 9, 12, 13, 15-20 and X).

The personal and family history of cancer in case 2 already led in 2011 to the identification of the pathogenic *BRCA1* germline variant NM_007294.3(BRCA1_v001):c.3756_3759del (p.Ser1253Argfs*10). Taking this information into account, a CMA and NGS of the tumor DNA (estimated tumor infiltration – 30%) were performed, identifying the known germline *BRCA1* variant with an AF of 35% and an additional NM_004985.5 (KRAS_v001):c.35G>A variant with an AF of 23%. The CMA results were monosomies 18 and 19, trisomies 1q, 7, 8, 12, 13 and 20, partial chromosomal losses in the 1p region and partial chromosomal gains in the 1p region.

In case 3, CRC was diagnosed from a surgical specimen obtained after an appendectomy with the subsequent identification of a tumor-like lesion with low-grade dysplasia at the base of the cecum. Considering that the patient's mother carried a *BRCA1* germline variant, the patient DNA was tested, confirming the presence of the heterozygous *BRCA1* pathogenic variant NM_007294.3(BRCA1_v001):c.3841C>T (p.Gln1281*). Subsequently, *BRCA1* sequencing and CMA array on tumor DNA (sample estimated tumor infiltration – 20%) showed the *BRCA1* c.3841C>T family variant with an AF of 43%, while the CMA was normal.

The three variants are predicted to cause truncation of the translation in exon 10 (out of a total of 23) which will result in a severely shortened or absent protein due to nonsense-mediated decay of the mRNA. BRCA1 protein truncations downstream of this position have been described as pathogenic (11, 12). *BRCA1* c.1016dup and BRCA1 c.3841C>T variants were absent in 251174 control chromosomes in gnomAD, whereas *BRCA1* c.3756_3759del was present at an AF of 1.267e-05. BRCA1 c.1016dupA has been reported in the literature as a founder variant in Norway and Canada (13, 14) and also in multiple individuals affected with hereditary breast and ovarian cancer syndrome in other populations (15–18). Case 2 four-nucleotide deletion was widely reported in the literature in Polish and French-Canadian gynecological cancer patients (19, 20). The *BRCA1* variant present in case 3 has been reported as a France, Belgium, and Holland founder variant (21). ClinVar submitters including an expert panel (ENIGMA) cite the three variants as pathogenic. These data indicate that the three variants are highly likely to be associated with high breast and ovarian cancer risk.

Homologous recombination deficiency (HRD) evaluation can be performed using HRD score, an aggregate score of loss of heterozygosity (LOH), telomeric-allelic imbalance (TAI) and large-scale state transitions (LST). To confirm the HRD score in the CRC samples we used an alternative method of HRD detection by investigating single base substitution (SBS) signatures.

To assess homologous recombination deficiency (HRD) in CRC samples, a paired germline and tumoral DNA exome sequencing using Twist Comprehensive Exome Panel and Twist Human RefSeq Panel (according to the manufacturer's instructions) from all three patients was performed. We used Sequenza (22) to detect and quantify copy number variation and estimate tumor cellularity and ploidy. These results were used as an input to calculate the HRD score with a threshold of positivity ≥33 (23). Mutational signatures in the samples were analyzed Using MutationalPatterns R package (24) and COSMIC v2 signatures (25), taking only the somatic variants into account.

Through Sequenza, the estimated tumor cellularity was of 95% in the case 3 sample, while this value was lower for cases 1 and 2 – 22% and 27%, respectively. All three samples showed remarkably high HRD scores (59, 61.15 and 66, respectively), while no somatic copy number alteration was identified in *PALB2*, *BRCA1* and *BRCA2*.

The three most frequent SBS signatures with a proposed etiology in the samples were SBS1, 3 and 5 (see Figure 1). Signature 3 was the second most frequent signature with a contribution to 23% of the somatic pattern of variant accumulation in samples 1 and 2, and 13% in sample 3. While signature 1 and 5 reflect clock-like accumulation of somatic variants, signature 3 has been directly related to HRD (25).

# DISCUSSION

The existing data linking germline pathogenic variants in the *BRCA1* gene to an increased risk of CRC are scarce. Two large studies reported that *BRCA1* variants conferred approximatively a fivefold increased risk for CRC, especially in young patients from high-risk families (6, 26). Out of three recent metaanalyses, one of them found an increased risk of colorectal cancer associated with *BRCA1* variants (odds ratio = 1.49, 95% CI = 1.19 to 1.85, P < 0.001) (8), while the other two did not identify any increase in CRC risk among patients carrying a *BRCA1* variant (7, 9). A study evaluating a cohort of *BRCA1* or *BRCA2* pathogenic variant carriers mostly of Ashkenazi ancestry concluded that they may be prone to developing anal carcinoma and left-sided mucinous histology CRC (27). One single publication reported a young male patient with a *BRCA1* germinal variant who presented with rectal

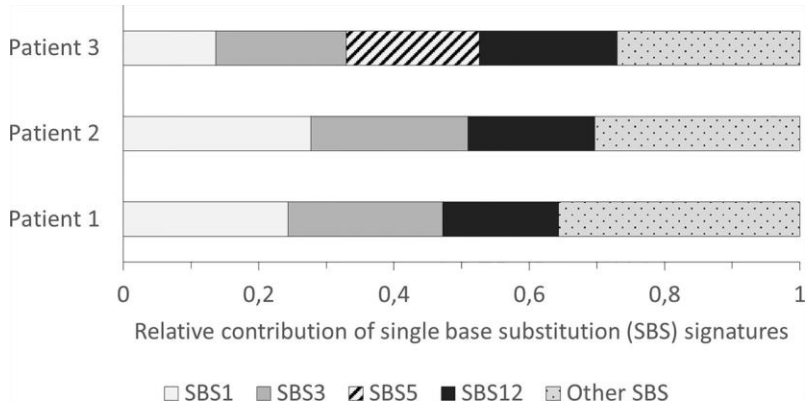


FIGURE 1 | Single base substitution (SBS) signatures identified in the patients with a relative contribution ≥ 15% of the global pattern.

adenocarcinoma and showed an excellent response to oxaliplatin-containing neoadjuvant therapy (28). These data thus remain contradictory and do not allow to recommend to screen for CRC in *BRCA1* variants heterozygotes, or to consider *BRCA1* pathogenic variants as a factor predisposing to familial CRC.

Given the frequency of CRC and of *BRCA1* variant heterozygotes in European populations (29), co-occurrence may be incidental rather than indicative of a causal relationship, as suggested previously (30). However, a few lines of evidence indicate that co-occurrence might be relevant.

Recently, a large report investigated the frequencies of various cancers, including CRCs, in 6902 men with BRCA variants (31). The probability for developing a CRC was, according to this report, two times lower in men with *BRCA2* variants than in *BRCA1* variant heterozygotes. As it seems unlikely that *BRCA2* variants had a protective role against CRCs, these data could indicate a slightly but significantly increased risk of these cancers in men with *BRCA1* variants.

In our samples, we did not evaluate *BRCA1* protein expression. Although we describe patients with aggressive metastatic cancer, the presence of low levels of *BRCA1* protein had a worse prognosis even in early-stage CRC (32).

In our study, we not only confirmed that the *BRCA1* germline variants were still present in the tumor (with evidence of positive selection in case 1), but we also demonstrate scars of HRD in the three tumors. Indeed, the presence of germline variants in HRD-associated genes alone is not sufficient to predict clinically relevant HRD. We highlighted the presence of specific mutational signatures (COSMIC signature 3) (33) and genomic instability characteristics (LOH, TAI and LST) (34–36), reflecting significant HRD, comparable with that observed in ovarian cancers with a *BRCA1* or *BRCA2* pathogenic variants. Interestingly, the initial somatic NGS analysis of cases 2 and 3 was not conclusive, possibly because of low tumor infiltration, but it could also be indicative of an epigenetic event leading to loss of *BRCA1* function and demonstrates the role of HRD testing even in cases where the mechanism driving HRD is not fully elucidated. Taken together, these observations indicate that germline *BRCA1* variants may, in a small proportion of variant carriers, play a driver role in CRC development or progression and that these patients might thus benefit from a treatment with poly (ADP-ribose) polymerase-inhibitors (PARPi). Indeed, clinical trials clearly demonstrated the efficacy of platinum-based chemotherapy and PARPi to treat BRCA mutated and/or HRD positive cancers inside the spectrum of BRCA-related cancers (37). Further evidence demonstrating that some CRC could be linked to BRCA deficiencies could open new perspectives for treatment with PARPi of these rare aggressive tumors.

The small number of patients and the bias in recruitment are the main limitations of our study, precluding to justify any specific surveillance or screening program in the absence of a personal or family history.

In conclusion, our data indicate that a *BRCA1* germline pathogenic variant can be involved in CRC development through HRD. Thus, *BRCA1* testing should be considered in young patients with a personal history of microsatellite stable CRC. This could further allow a personalized treatment approach with a PARPi.

# DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

# ETHICS STATEMENT

The "Comité d'Ethique Hospitalo-facultaire Universitaire de Liège" (CHU/University of Liège) approved the study. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

# AUTHOR CONTRIBUTIONS

Conceptualization: VB. Data curation: MF, MM, and VB. Formal analysis: MF. Funding acquisition: VB. Investigation: MF, MM, RT, CM, KS, ES, NL, CL, and CF. Methodology: MF, MM, RT, CM, CJ, and LP. Project administration: VB. Resources: VB. Supervision: CJ, LP, and VB. Validation: JR, YG, JC, and AS. Writing-original draft: MF and VB. Writing-review and editing: MF, MM, RT, CM, KS, ES, NL, CL, JR, YG, JC, AS, CJ, LP, VB, and CF. All authors contributed to the article and approved the submitted version.

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# Part 2. Digenic inheritance

Digenic inheritance, characterized by the simultaneous presence of PVs in two different CPGs, could have significant implications for cancer risk and management. Patients with digenic inheritance, specifically those harboring PVs in established cancer predisposition genes such as *BRCA1*, *BRCA2*, *ATM*, and *CHEK2*, exhibit a markedly elevated risk of developing multiple cancers at an earlier age compared to those with a single pathogenic variant. Despite extensive research on these individual genes, the cancer risk associated with simultaneous PVs in multiple CPGs remains largely unexplored. This study aims to elucidate the cancer risk in patients harboring combined PVs in HR CPGs examining six patients from five families, all of whom have multiple cancer diagnoses.

Family 1: This family presented with pathogenic variants in both *BRCA2* and *ATM* genes. The affected individuals included a patient with kidney cancer, prostate cancer and pancreatic adenocarcinoma, and his daughter with breast cancer.
Family 2: The proband in this family had a pathogenic variant in *BRCA2* and *ATM* and was diagnosed with breast cancer at a young age.
Family 3: This family also carried pathogenic variants in *BRCA2* and *ATM*. The male proband had a history of prostate cancer, gastric cancer, and pancreatic cancer.
Family 4: Pathogenic variants in both *BRCA2* and *BRCA1* were identified. The affected male had pancreatic cancer.
Family 5: This family exhibited pathogenic variants in *BRCA2* and *CHEK2*, with a female proband diagnosed with breast cancer.

The observation of MPCs at an early age of diagnosis in these patients shows the elevated cancer risk in individuals who are double heterozygous for PVs in HR-related CPGs. This study suggests that in families where patients show phenotypic diversity, early-onset, or unusual cancer types compared to other relatives, comprehensive genetic testing should include screening for additional CPGs beyond the initially identified variant. The findings advocate for a more inclusive cascade testing strategy to accurately assess and manage cancer risk in these high-risk families. By identifying multiple PVs, healthcare providers can implement more tailored surveillance and prevention strategies, thereby improving patient outcomes in those with a high genetic predisposition to cancer.

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# Digenic Inheritance of Mutations in Homologous Recombination Genes in Cancer Patients

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**Abstract:** Background/Objectives: *BRCA1*, *BRCA2*, *ATM*, and *CHEK2* are known cancer predisposition genes (CPGs), but tumor risk in patients with simultaneous pathogenic variants (PVs) in CPGs remains largely unknown. In this study, we describe six patients from five families with multiple cancers who coinherited a combination of PVs in these genes. Methods: PVs were identified using NGS DNA sequencing and were confirmed by Sanger. Results: Families 1, 2, and 3 presented PVs in *BRCA2* and *ATM*, family 4 in *BRCA2* and *BRCA1*, and family 5 in *BRCA2* and *CHEK2*. PVs were identified using NGS DNA sequencing and were confirmed by Sanger. The first family included patients with kidney, prostate, and breast cancer, in addition to pancreatic adenocarcinomas. In the second family, a female had breast cancer, while a male from the third family had prostate, gastric, and pancreatic cancer. The fourth family included a male with pancreatic cancer, and the fifth family a female with breast cancer. Conclusions: The early age of diagnosis and the development of multiple cancers in the reported patients indicate a very high risk of cancer in double-heterozygous patients associated with PVs in HR-related CPGs. Therefore, in families with patients who differ from other family members in terms of phenotype, age of diagnosis, or type of cancer, the cascade testing needs to include the study of other CPGs.

**Keywords:** digenic inheritance; double heterozygosity; familial cancer; BRCA1; BRCA2; ATM; CHEK2



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## 1. Introduction

Cancer predisposition syndromes (CPS) are now extensively studied, with an increasing proportion of cancer patients undergoing genetic testing [1]. This testing is based on the type of cancer, the number of cancer occurrences during the patient's life, the age at diagnosis, and the family history [2,3]. It is expected that 3 to 5% of cancers are linked to a causal variant in a cancer predisposition gene (CPG) [4]. As most CPS are transmitted in an autosomal dominant way, once a pathogenic variant (PV) is identified in a family, the geneticists propose a family cascade testing to search for the variant, and start with first-degree relatives [5].

Within the frame of inherited cancer predisposition, carriers of pathogenic variants (PVs) in a single gene have been extensively represented in the literature, and an ever-growing accumulation of data on the single gene-related cancer risk, based on multiple family histories, is available [6,7]. These data have led to gene-specific screening and follow-up recommendations for these carriers [3]. However, the coinheritance of heterozygous PV in two CPGs is a poorly studied event restricted to small case series and single case reports [8-10]. The exact frequency of double heterozygotes remains unknown, as is the case for their cancer risk and associated follow-up strategies [11]. Therefore, empirically, most genetic centers propose to apply the guidelines defined for the most dangerous gene to the follow-up of patients with two PVs in two different CPGs. However, the *BRCA1* and *BRCA2* PVs coinheritance, in the population-based Israeli national breast cancer cohort, was described in 2.2% of all carriers [12], and 17 double heterozygotes for CPGs were detected in a breast cancer cohort of people of Slavic ancestry which included 5391 patients [13].

Breast cancer gene 1 (*BRCA1*), breast cancer gene 2 (*BRCA2*), checkpoint kinase 2 (*CHEK2*), and ataxia-telangiectasia mutated (*ATM*) are CPGs, part of the homologous recombination (HR) pathway for double stand break (DSB) repair. This pathway preferentially uses the sister chromatid for error-free repair, and both the DNA damage response and the cell cycle checkpoints are crucial for initiating and regulating HR [14]. ATM participates in HR initiation and phosphorylation of CHEK2; BRCA1 facilitates DNA end resection [15], while BRCA2 aids in the formation of a DNA D-loop through the invasion of the nearby duplex DNA [16]. Finally, the BRCA2 protein is post-translationally modified by ATM [17].

HR is crucial for repairing severe replication lesions at replication forks, and can repair or bypass DNA lesions remaining due to inactivation of other pathways. Consequently, mutations in HR genes result in genomic instability, fueling further mutations that lead to cancer development [14,18]. This deficiency in the HR pathway makes tumor cells more sensible to poly-(ADP-ribose)-polymerase inhibitors, platinum derivatives, alkylating agents, mitomycin C, and other antitumor drugs that are used for the treatment of cancer patients [19-22].

PVs in *BRCA1*, *BRCA2*, *CHEK2*, and *ATM* have been linked to a wide variety of cancers [15]. *BRCA1* and *BRCA2's* PVs were associated with breast cancer, ovarian/fallopian cancer, pancreas cancer, prostate cancer, and melanoma, while breast, prostate, thyroid, kidney, colon and stomach cancers were related to PVs in *CHEK2* [23]. Germline heterozygous PVs in *ATM* increase the risks of breast, pancreatic, gastro-esophageal, colorectal, ovarian, prostate, thyroid, gastric, and head and neck cancers, as well as melanoma [24]. Given the frequencies of PVs in these genes, it is expected that cancer patients carrying two PVs should be rarely, but not exceptionally, observed. Moreover, as these genes act on the homologous recombination pathway, these double heterozygote patients might have a higher risk of HR dysfunction and thus a more severe cancer risk.

In this study we describe six patients from five families with multiple cancers who coinherited PVs in *BRCA2* and other HR genes—four patients with variants in *BRCA2* and *ATM,* one patient with *BRCA2* and *BRCA1,* and one patient with *BRCA2* and *CHEK2* PVs.

## 2. Materials and Methods

### *2.1. Ethical approval*

The study was conducted in accordance with the Declaration of Helsinki and approved by the Comité d'Ethique Hospitalo-facultaire Universitaire de Liège (protocol code 2019/245 and date of approval 28 October 2019).

### *2.2. Data collection*

Patient sex, age, age at diagnosis for each tumor, and personal and family history were extracted from the medical records. Data on cancer diagnosis and treatment were gathered from the institution's database. All of the patients read and signed an informed-consent document.

### 2.3. Genetic analysis

Genetic analysis was performed on DNA extracted from blood samples using QIAcube (QIAGEN Hilden, Germany) and STARlet (Seegene Inc. Seoul, South Korea) extraction instruments (See SupplementaryMaterials: DNA extraction methods). DNA purity and concentration were measured with NanoDrop (Thermo Fisher Scientific Waltham, Massachusetts, United States), and DNA underwent NGS panel sequencing (See Supplementary Data: Table S1). The bioinformatic analysis was performed using in-house demultiplexing pipelines and the in-house Humanomics pipeline (as described in [25]). Variant classification was performed according to the ACMG "Standards and guidelines for the interpretation of sequence variants" [26]. The in silico analysis of missense and splicing variants was performed using the aggregated score of the Franklin by Genoox tool (https://franklin.genoox.com, accessed on 17 May 2024), which includes the scores of SIFT, FATHMM, DANN, MetaLR, REVEL, MutationAssessor, PolyPhen-2, MutationTaster, PrimateAI, BayesDel, SpliceAI, dbscSNV, GERP, GenoCanyon, fitCons, MitoTip, and APOGEE. For the splicing variants, Human Splicing Finder [27] was used. Two databases, gnomAD (https://gnomad.broadinstitute.org/, accessed on 17 May 2024) and ALFA (https://www.ncbi.nlm.nih.gov/snp/docs/gsr/alfa, accessed on 17 May 2024), were used to retrieve the Minor Allele Frequency (MAF) data. The identified PVs were confirmed by Sanger sequencing (See Supplementary Data: Table S2).

## 3. Results

### 3.1. Frequency of double heterozygotes

Over the past 28 months, following the introduction of the new Hereditary Breast and Ovarian Cancer (HBOC) panel at our institution, a total of 2152 panels have been conducted in cases of cancer patients (1929 13-gene panels and 223 26-gene panels). In total, 121/1929 13-gene panels (6.27%) and 22/223 (9.8%) 26-gene panels were positive, containing a pathogenic or likely pathogenic result. Three patients (3/2152 patients, 0.14%) were double-heterozygous for CPG PVs. Two samples had two PVs in the 13-gene panel (2.2% of the 91 samples with PVs) and one in the 26-gene panel (5.6% of the 18 samples with PVs, see Table 1). Heterozygous variants in genes associated with a recessive instance of CPS, such as the *MUTYH* gene, were excluded from this analysis.

**Table 1.** Double heterozygote statistics in the institution.

| | 13-Gene Panel | | 26-Gene Panel | |
|---|---|---|---|---|
| | **Likely Pathogenic (n = 30)** | **Pathogenic (n = 91)** | **Likely Pathogenic (n = 4)** | **Pathogenic (n = 18)** |
| 1 variant per sample, n (%) | 30 (100.0) | 89 (97.8) | 4 (100.0) | 17 (94.4) |
| 2 variants per sample, n (%) | 0 (0.0) | 2 (2.2) | 0 (0.0) | 1 (5.6) |

In this study, we report two of the three double-heterozygous patients from whom we obtained informed consent, and one additional patient whose double-heterozygous state was diagnosed based on family history. The three additional included patients were previously observed by the genetics department and/or had a relevant family history.

### 3.2. Clinical history

Six patients from five families underwent genetic consultation in the context of multiple cancers or early-onset disease, leading to the identification of two heterozygous PVs in the HR genes of each patient (see Table 2).

**Table 2.** Characteristics of the patients included in the study.

| | Family 1 | | Family 2 | Family 3 | Family 4 | Family 5 |
|---|---|---|---|---|---|---|
| | **Patient 1 II:5** | **Patient 2 III:2** | **Patient 3 IV:2** | **Patient 4 II:1** | **Patient 5 IV:3** | **Patient 6 III:2** |
| **Sex** | **Male** | **Female** | **Female** | **Male** | **Male** | **Female** |
| Age (years) | 67 | 34 | 28 | 65 | 58 | 29 |
| Medical history | Childhood asthma, gouty arthritis, hypercholesterolemia, and hypertrophic heart disease | None | None | Essential thrombocytopenia | Type 2 diabetes, volvulus | Glucose intolerance |
| Oncological history | Renal cancer at 50, prostate cancer at 51, and metastatic pancreatic cancer at 66 years | Breast cancer at 29 years (ER positive, PR positive, Ki67 60%, HER2 ++, SISH −) | Metastatic breast cancer at 28 years (ER positive, PR positive, Ki67 40%, HER2 ++ SISH −) | Prostate cancer at 49, gastric cancer at 60, and metastatic pancreatic cancer at 64 years | Metastatic pancreatic cancer at 57 years | Ductal breast cancer at 28 years (ER positive, PR negative, Ki67 50%, HER2 ++, SISH −) |
| Treatment | Renal cancer–surgery, prostate cancer–brachytherapy, pancreatic cancer–chemotherapy, and targeted therapy | Neoadjuvant chemotherapy, surgery, and radiotherapy | Chemotherapy | Prostate and gastric cancer–surgery, pancreatic cancer–chemotherapy | Chemotherapy | Surgery, adjuvant chemotherapy, radiotherapy, and hormonal therapy |
| Identified germline heterozygous pathogenic and likely pathogenic variants | | | | | | |
| *BRCA2* | c.3865_3868del p.(Lys1289Alafs*3) | c.3865_3868del p.(Lys1289Alafs*3) | c.5057T>A p.(Leu1686*) | c.4284dup p.(Gln1429Serfs*9) | c.8243G>A p.(Gly2748Asp) | c.537dup p(Ile180Tyrfs*3) |
| *ATM* | c.8494C>T p.(Arg2832Cys) | c.8494C>T p.(Arg2832Cys) | c.7516-2A>G | c.6326G>A p.(Trp2109*) | - | |
| *BRCA1* | - | - | - | - | c.1121del p.(Thr374Asnfs*2) | - |
| *CHEK2* | - | - | - | - | - | c.499G>A p.(Gly167Arg) |

ER—estrogen receptor; PR—progesterone receptor; SISH—silver in situ hybridization. Reference transcripts: *ATM* NM_000051.3, *BRCA1* NM_007294.3, *BRCA2* NM_000059.3, and *CHEK2* NM_007194.3.

The first family included a 67-year-old male with a medical history of multiple cancers whose daughter had been diagnosed with breast cancer (see Figure 1). The male patient presented kidney and prostate cancer and pancreatic adenocarcinoma at the ages of 50, 51, and 66, respectively.

The patient's daughter was diagnosed with breast cancer at 29 years. A tumorectomy showed grade 3 invasive ductal carcinoma with axillary and mediastinal lymph node extension (ypT1cN2aMx). Two years after the diagnosis, she presented a first relapse with one successfully treated bone metastasis. The subsequent relapses included liver metastasis, lymph node invasion, and finally brain metastasis in 2021.

In the second family, a 28-year-old female underwent an exploratory laparoscopy due to persistent non-specific abdominal pain with nausea and vomiting, showing endometriotic lesions and multiple hepatic lesions described as angiomas. A month later, after a week of hyperthermy and a positive COVID-19 test, the thoracoabdominal computed tomography scan demonstrated a large breast lesion with a highly suspicious right axillary lymph node, necrotic hepatic and bone lesions, and possibly-COVID-19-related pulmonary foci. A grade 3 infiltrating ductal carcinoma was diagnosed and treated.

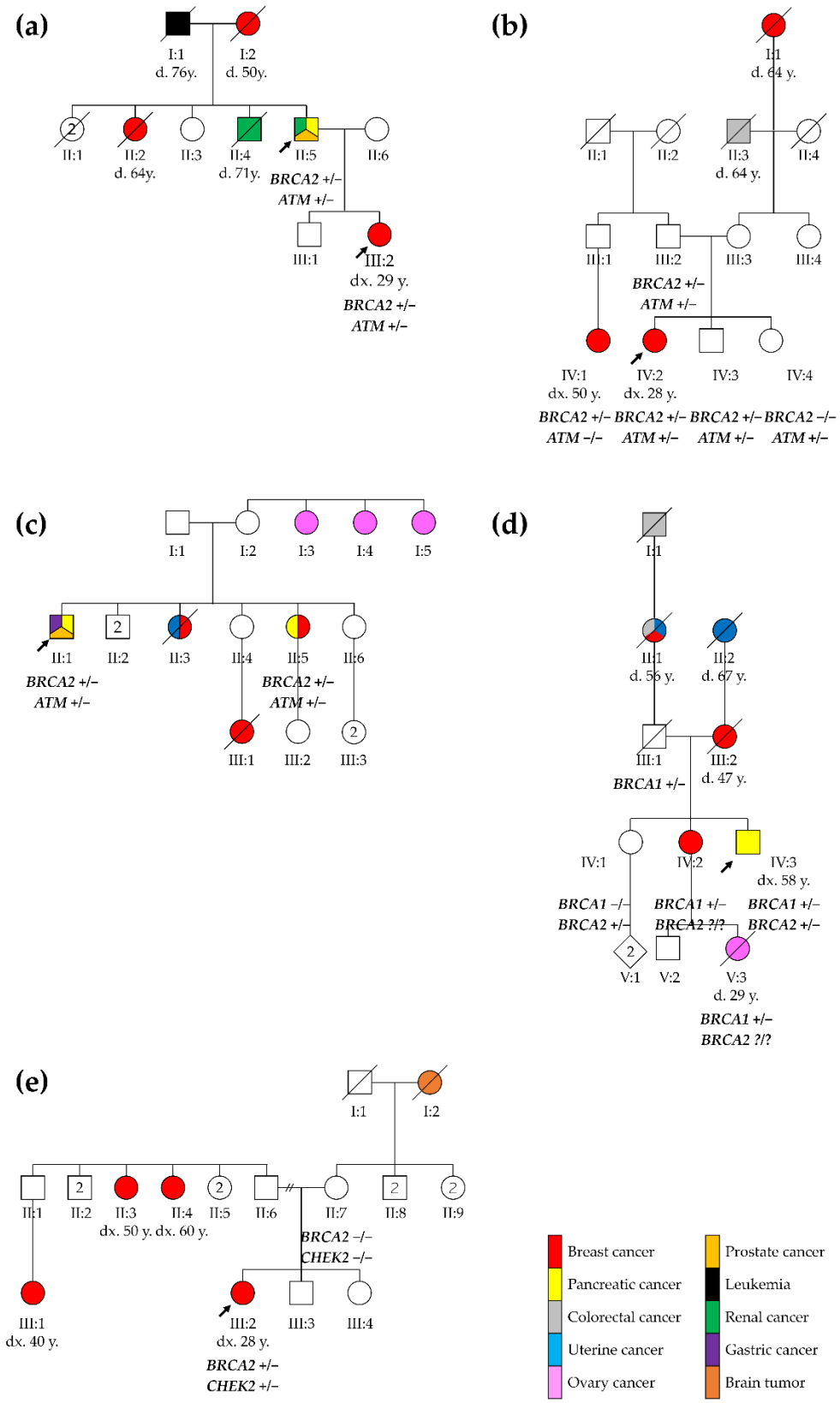


**Figure 1.** Pedigree of the five families reported: (**a**) family 1, (**b**) family 2, (**c**) family 3, (**d**) family 4, and (**e**) family 5. The probands are marked with arrows. ca., cancer; CRC, colorectal cancer; d., death; dx., diagnosis; y., years; +/–, heterozygous genotype; –/–, homozygous wild type genotype; ?/?, unknown genotype.

A 65-year-old male from the third family was diagnosed with a Gleason 3 pT2bNxM0 prostate adenocarcinoma at the age of 49, well-differentiated pT1N0M0 enteric adenocarcinoma one year later, and finally metastatic pancreatic cancer. His older sister was first diagnosed with breast cancer at 60, and then pancreatic cancer at 70 years old.

The fourth family included a 58-year-old male who presented a 15 kg weight loss, fatigue, nausea, and transfixing abdominal pain for 2 weeks. In a tomography, an isthmus pancreatic mass of 4 cm infiltrating peripancreatic fat with hepatic metastasis was discovered (CTxNxM1). In this patient, a familial *BRCA1* variant was found 15 years earlier at the time of a breast cancer diagnosis for his sister at the age of 35 (she developed a second breast cancer 15 years later, and pancreatic cancer at the age of 67). The male patient was known to carry this familial BRCA1 variant, inherited from their father. As the *BRCA1* familial variant was not sufficient to explain both pancreatic cancers, those in the patient and his sister, as well as their mother's breast cancer, we re-initiated a CPG analysis and this showed that he carried two pathogenic variants: the known familial *BRCA1* PV, and a *BRCA2* PV.

The 29-year-old female from the fifth family discovered three mobile, not painful masses in her right breast while performing self-palpation. The biopsy of one of the masses revealed a ductal breast adenocarcinoma (cT2N0M0). After a right mastectomy with sentinel ganglion, an infiltrating tubular adenocarcinoma (pT2mN1mi) was diagnosed. During genetic evaluation, a *BRCA2* and a *CHEK2* PV were identified in the patient. Both PVs were absent in the mother, while the father was not available for testing. The patient has two sisters, one of whom is underage and has not been tested.

### *3.3. Genetic characteristics*

In the patients from families 1 to 3, genetic analyses showed *BRCA2* and *ATM* PVs. The patients from family 4 and 5 carried PVs in *BRCA1*/*BRCA2* and *BRCA2*/*CHEK2*, respectively.

Three of the identified *BRCA2* nonsense variants were located in exon 11/27 (c.3865_3868del, c.5057T>A, c.4284dup), while the fourth was located in exon 7/27 (c.537dup), leading to the existence of a severely truncated or absent protein due to nonsense-mediated mRNA decay (NMD) [28]. *BRCA2* c.3865_3868del, c.5057T>A, and c.537dup variants were absent from the gnomAD (v2.1.1) and ALFA databases, while *BRCA2* c.4284dup had a frequency of 1 out of 244426 alleles in the total population of gnomAD (v2.1.1) and was absent from the ALFA database (see Table 3). *BRCA2* c.8243G>A had a frequency of 2/249060 in the total population of gnomAD (v2.1.1) and 1/25340 in ALFA. Various functional studies show a loss of function and/or protein stability linked to the *BRCA2* c.8243G>A variant [29,30]. All of the *BRCA2* variants were previously described as pathogenic [22,31—33].

The missense *ATM* c.8494C>T variant was located in exon 58 out of 63, was present in 7 out 236730 alleles in the total population in gnomAD (v2.1.1), and has been previously described as pathogenic and associated with an increased cancer risk [34]. The *ATM* c.7516-2A>G variant located in intron 50 out of 62 has not been previously reported, and was not present in the gnomAD (v2.1.1) or ALFA databases. However, the variant was located in a region of the gene where other variants have been described as pathogenic, affecting a conserved splice site [35]. *ATM* c.7516-2A>G in silico evaluation results showed splicing alteration by wild-type acceptor site breakage. The nonsense *ATM* c.6326G>A variant in exon 43 out of 63 was predicted to cause loss-of-function by premature protein truncation or NMD. This variant was not found in the gnomAD (v2.1.1) or ALFA databases and has been previously reported as pathogenic [36].

**Table 3.** Characteristics of the variants identified in the patients.

| Gene | Variant | Type | MAF | In Silico Predictors' Results | ACMG Classification |
|---|---|---|---|---|---|
| *BRCA2* | c.3865_3868del p.(Lys1289Alafs*3) | Deletion | gnomAD: - ALFA: - | F: not applicable | Pathogenic |
| *BRCA2* | c.5057T>A p.(Leu1686*) | Nonsense | gnomAD: - ALFA: - | F: not applicable | Pathogenic |
| *BRCA2* | c.4284dup p.(Gln1429Serfs*9) | Duplication | gnomAD: 0.0004% ALFA: - | F: not applicable | Pathogenic |
| *BRCA2* | c.8243G>A p.(Gly2748Asp) | Missense | gnomAD: 0.0008% ALFA: 0.0039% | F: deleterious | Pathogenic |
| *BRCA2* | c.537dup p(Ile180Tyrfs*3) | Duplication | gnomAD: 0.0004% ALFA: - | F: not applicable | Pathogenic |
| *ATM* | c.8494C>T p.(Arg2832Cys) | Missense | gnomAD: 0.0030% ALFA: - | F: deleterious | Pathogenic |
| *ATM* | c.7516-2A>G | Splicing | gnomAD: - ALFA: - | F: deleterious HSF: Site acceptor broken | Likely pathogenic |
| *ATM* | c.6326G>A p.(Trp2109*) | Nonsense | gnomAD: - ALFA: - | F: not applicable | Pathogenic |
| *BRCA1* | c.1121del p.(Thr374Asnfs*2) | Deletion | gnomAD: - ALFA: - | F: not applicable | Pathogenic |
| *CHEK2* | c.499G>A p.(Gly167Arg) | Missense | gnomAD: 0.0024% ALFA: 0.0030% | F: deleterious | Pathogenic |

F—Franklin by Genoox, MAF—Minor Allele Frequency, ACMG—American College of Medical Genetics and Genomics, HSF—Human Splicing Finder. Reference transcripts: *ATM* NM_000051.3, *BRCA1* NM_007294.3, *BRCA2* NM_000059.3, and *CHEK2* NM_007194.3.

Nonsense *BRCA1* c.1121del variant caused a frameshift with a predicted stop codon two amino acids after the deletion, which could result in loss of normal protein function through protein truncation or NMD. This variant was absent in gnomAD (v2.1.1) or ALFA, but was present in several individuals suffering from breast and/or ovarian cancer [37]. This variant was also known as c.1240delC in the literature.

Missense *CHEK2* c.499G>A variant leads to a substitution of a highly conserved amino acid. This variant was present in the total population of gnomAD (v2.1.1) in 6 out of 251424 alleles, and 3/100662 alleles in ALFA. Additionally, functional analysis showed a loss of function of the protein due to structural instability [38] or phosphorylation anomaly [39]. The in silico analysis of the variant predicted a deleterious effect on the protein, and CHEK2 loss-of-function variants are known to be pathogenic [40].

## 4. Discussion

PVs in *BRCA1*, *BRCA2*, *CHEK2*, and *ATM* increase the lifetime cancer risk of breast cancer [41]. In women carrying *BRCA1* and *BRCA2* PVs, the cumulative risk of breast cancer was 4% before the age of 30 for each gene, and reached 72% for *BRCA1* and 69% for *BRCA2* by age 80 [42]. For *ATM* variants, there was an estimated breast cancer relative risk of 2.8, and the absolute breast cancer risk reached 27% by 80 years. The *CHEK2* breast cancer risk was variable for different PVs. Common *CHEK2* truncating variants conferred a greater than twofold relative risk, while a less common I157T variant was associated with a 1.4-fold risk [43]. Similarly, in a study that included 65 057 women with breast cancer, the age of diagnosis of CHEK2 PV's carriers was 47.7 years [41]. However, there is a lack of epidemiological data on BC risk in patients carrying PVs in two of these genes. Our study indicates very precocious and even metastatic BC in women with PVs in *BRCA2* and *ATM* (patients 2 and 3) or *BRCA2* and *CHEK2*

genes (patient 6), while a previous study evaluating 17 double-heterozygous patients with breast cancers failed to demonstrate a younger age at presentation in this group [13]. A similar trend could be expected when PVs in *BRCA1* are associated with PVs in other CPGs.

The risks of other cancers are also elevated in *BRCA1*-, *BRCA2*-, and *ATM*-variant carriers. *BRCA1* and *BRCA2* PVs confer increased risks of prostate, pancreatic, and ovarian cancers [44], while moderate-to-high risks of pancreatic (OR 4.21), prostate (OR 2.58), and gastric (OR 2.97) cancers were estimated for *ATM*-variant carriers [24]. In our observations, two male patients were treated for a prostate cancer diagnosed at an early age, which might suggest that the *BRCA2*-linked risk is further increased by the presence of the *ATM* PV.

The reported pancreatic cancer risks in *BRCA1* and *BRCA2* carriers by the age of 70 years were 1.16% and 4.1% in men [44]. As BRCA1, BRCA2, and ATM proteins interact in the HR pathway, an additive effect on HR deficiency could be expected, giving a further increased risk of pancreatic cancer, as observed in patients 1, 4, and 5. Indeed, in a recent case report of a female patient carrying two heterozygous pathogenic variants in *BRCA2* and *ATM*, breast cancer was diagnosed at 34 and pancreatic cancer at 48 years [45]. This raises the question of whether the previously-described reported young women with breast cancer (family 1) will need additional monitoring for their pancreatic cancer risk.

Therefore, our observations suggest that patients carrying a PV in BRCA2 plus another HR gene should be carefully monitored for BC, pancreatic cancer, and prostate cancer. However, incomplete penetrance and variability of the age of onset of the disease are also observed in double-heterozygous patients. In the second reported family, the proband's father also carried both *BRCA2* and *ATM* PVs (see Figure 1) but did not have any history of cancer, indicating that both genetic and non-genetic factors can influence cancer risk in variant carriers [44], while in the third family, the proband´s sister developed cancer at an older age, supporting the variable expressivity of these mutations. Further studies and larger cohorts are thus of course needed to better define the cancer risk associated with having two PVs in HR genes.

PVs in *BRCA1* and *BRCA2* have frequencies of 0.21% and 0.31% in the European population [46], while the frequencies of *ATM* and *CHEK2* PVs reach 1% [47] and 1.4% [48]. These estimations, taken together, and given the scarcity of double-heterozygotes reports, indicate that the prevalence of digenic coinheritance is likely underestimated. Recently, even a patient with breast cancer and concurrent PVs in three cancer-related genes (*BRCA1*, *BRCA2*, and *CHEK2*) has been reported [49]. Therefore, given the high variability of phenotypes within families and between different families, when a cascade testing is performed after the identification of a familial PV, the assessment should not stop at the single known familial PV, at least in individuals with precocious breast, pancreatic, or prostate cancers; in those with multiple cancers; and in cases of cancers that are not frequently associated with the identified PV, as the possibility of co-segregation of another PV should not be neglected.

The size of the genetic panels used for cancer patients´ evaluation has progressively increased in recent years [50]. Consequently, the findings derived from these expanded panels are still in the preliminary stages, and it is impossible to directly compare the new data with previous results from shorter panels. Nonetheless, instances of double mutations are expected to remain relatively rare. After introducing multi-gene panel testing in 2014, by 2023, in the Fox Chase Cancer Center Risk Assessment Program Registry, 70 patients were found to carry at least two PVs in CPGs (excluding biallelic *MUTYH* PVs) [51]. In a review of 55,803 patients screened with a 25-gene hereditary cancer panel, 106 individuals (0.19%) showed PVs or likely pathogenic variants in two or more genes [52], a frequency of double heterozygotes very similar to that observed in the present study.

With the increase in patient numbers and the utilization of larger cohorts for analysis, more robust data will be available soon. Furthermore, the criteria for recommending genetic studies have undergone multiple revisions over time. Only recently has genetic testing for pancreatic and prostate cancer been included as part of the standard practice [53]. Consequently, the reports of larger cohorts of patients with diverse primary tumors will increase the likelihood of identifying cases with double mutations.

The small number of patients, the bias in recruitment, and the inability to evaluate the segregation in all of the families are the main limitations of our study. Additionally, we did not address the associated treatment strategies—platinum-based chemotherapy or PARP inhibitors—and the patients′ responses. With only six patients, we lack the data for meaningful comparisons or response-rate calculations. A larger study involving double-heterozygous patients is necessary to address these questions effectively.

Therefore, in young cancer patients from a family with a single known CPG PV, it could be useful to evaluate other genes to identify the potential transmission of several PVs and double-heterozygous carriers with a specific high cancer risk. Moreover, our data suggest that the surveillance of patients carrying two PVs in HR genes should include at least breast, pancreas, and prostate cancer screening, starting early. From our limited study, we would recommend starting a screening in those patients, at the latest, from the ages of 25, 40, and 50 for breast, prostate and pancreas cancer, respectively.

## 5. Conclusions

In conclusion, the early age of diagnosis and the development of multiple cancers in the reported patients indicate a very high risk of cancer in double-heterozygous patients associated with PVs in HR-related CPGs. Therefore, when a CPG PV is identified in a family, the usual cascade testing needs also to consider a study of other CPGs in patients with specific phenotypes, even distinct from other family members, either based on the age at diagnosis or the type of cancer.

**Supplementary Materials:** The following supporting information can be downloaded at: : https://www.mdpi.com/article/10.3390/jpm14060584/s1, Figure S1: Chromatograms of the variants identified in the patients; Table S1: DNA extraction; Table S2: Primer sequences used.

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**Data Availability Statement:** All the data relevant to the study is available in the manuscript.

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# Part 3. Multiple primary cancers

MPCs occur when a patient develops more than one cancer, either simultaneously (synchronous) or sequentially (metachronous). Definitions and identification criteria for MPCs vary among regulatory agencies, and the threshold for defining synchronous versus metachronous tumors also differs.

In recent years, advancements in screening tests and cancer therapies have significantly improved patient survival rates. Their risk of subsequent cancers is influenced by factors such as the type of first primary site, age at diagnosis, environmental exposure, and genetic factors. Patients with MPC are more likely to carry germline pathogenic variants in cancer-related genes.

The study aimed to identify novel genetic mechanisms associated with tumor development in MPC patients, particularly those without a germline molecular genetic diagnosis after initial evaluation.

Ten patients were included with a range of tumors including melanomas, seminomas, thyroid cancer, and gynecological tumors. The identified variants included missense, nonsense, and splicing SNVs, as well as CNVs. Most SNVs were variants of uncertain significance (VUS), with only two classified as pathogenic. Tumor sequencing revealed that all germline variants were present in the somatic samples, with no additional hits identified in the same genes. Mutational signature analysis highlighted common etiological factors like DNA mismatch repair alterations and chemotherapy-related signatures.

The study found that only two out of ten patients had clearly pathogenic SNVs. Previous studies showed similar results, indicating that undetected germline pathogenic variants could account for MPC in a small number of cases. The presence of multiple low penetrance SNVs suggests an oligogenic effect, where combinations of genetic events influence cancer risk.

The study suggests that multiple germline variants can increase cancer risk, and comprehensive genetic testing, including SNVs, CNVs, and chromosomal rearrangements, is essential for accurate risk assessment. Further research is needed to understand the interactions between genetic, epigenetic, and environmental factors in MPC. The limitations of this study included the small sample size and challenges in correlating VUS with disease, emphasizing the need for larger studies to explore these complex genetic interactions.

# Genetic evaluation of patients with multiple primary cancers

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**Abstract.** Regarding inherited cancer predisposition, single gene carriers of pathogenic variants (PVs) have been extensively reported on in the literature, whereas the oligogenic coinheritance of heterozygous PVs in cancer-related genes is a poorly studied event. Currently, due to the increased number of cancer survivors, the probability of patients presenting multiple primary cancers (MPCs) is higher. The present study included patients with MPCs aged ≤45 years without known PVs in common cancer predisposition genes. This study used whole exome sequencing (WES) of germline and tumoral DNA, chromosomal microarray analysis (CMA) of germline DNA (patients 1-7, 9 and 10), and a karyotype test of patient 8 to detect variants associated with the disease. The 10 patients included in the study presented a mean of 3 cancers per patient. CMA showed two microduplications and one microdeletion, while WES of the germline DNA identified 1-3 single nucleotide variants of potential interest to the disease in each patient and two additional copy number variants. Most of the identified variants were classified as variants of uncertain significance. The mapping of the germline variants into their pathways showed a possible additive effect of these as the cause of the cancer. A total of 12 somatic samples from 5 patients were available for sequencing. All of the germline variants were also present in the somatic samples, while no second hits were identified in the same genes. The sequencing of patients with early cancers, family history and multiple tumors is already a standard of care. However, growing evidence has suggested that the assessment of patients should not stop at the identification of one PV in a cancer predisposition gene.

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## Introduction

Multiple primary cancers (MPCs) are defined as the occurrence of more than one synchronous or metachronous cancer in a patient. For reporting purposes, the identification of MPCs is variable across the regulatory agencies. The Surveillance, Epidemiology and End Results (SEER) program considers the histology, site, laterality, and time since initial diagnosis of the tumor, while for the International Agency for Research on Cancer (IARC) only one tumor is registered for an organ, irrespective of time. The definition of synchronous and metachronous tumors also varies, having a threshold of 2 months in SEER and 6 months in IARC (1,2).

The development of screening tests and new cancer therapies improved cancer patient survival. By 2005, almost 900,000 of the 11 million cancer survivors were diagnosed with more than one cancer. The risk of developing subsequent cancers varies with regards to the type of first primary site, age at diagnosis, environmental exposure and genetic factors (3).

The identification of genes associated with hereditary cancer risk started 25 years ago with the discovery of BRCA pathogenic variants in families with breast and ovarian cancers. Since then, more than 80 cancer predisposition syndromes and 100 cancer-related genes were identified (4). For each syndrome, the most commonly associated cancers are defined but the exact risk profile remains often unknown. One common characteristic of these conditions is the young age at cancer diagnosis in most patients, although a considerable heterogeneity is observed. Additionally, it is widely stated that patients with multiple primary cancers are more likely to carry germline pathogenic variants in cancer-related genes (5).

For patients with MPCs without a germline molecular genetic diagnosis after initial evaluation, single-nucleotide variants (SNVs), copy number variants (CNVs), or de novo chromosomal rearrangements could be the cause of the disease. Another possibility could be the additive effect of multiple genetic events influencing a single biochemical mechanism. The aim of this study was to identify novel genetic mechanisms associated with risk of tumor development in patients with MPCs.

## Patients and methods

*Patient selection.* We selected patients with ≥2 tumors before 45 years old, who were evaluated by the genetic department of the CHU Liège. The routine genetic assessment and testing with targeted next-generation sequencing (NGS) failed to establish a germline molecular genetic diagnosis. Additionally, the patients lacked family history suggestive of a cancer predisposition syndrome. Tumors in the same tissue or organ were considered separate primary tumors if, in the case of paired organs, they presented bilaterally or if the clinical history clearly indicated that they were different. All participants gave informed consent to participate in the study.

*Data collection.* Patient sex, age, age at diagnosis of each of the tumors, personal and family history were extracted from the medical records. Data regarding cancer diagnosis and treatment was collected from the institution's database.

*Germline analysis.* Whole exome sequencing (WES, Novogene) and a high-resolution (180K) chromosomal microarray analysis reference (CMA, Agilent) were performed on the DNA extracted from the blood of the patients. The WES data was analyzed using our own Humanomics pipeline (as described in (6)) and two interpretation software (Illumina BaseSpace Variant Interpreter and Diploid Moon). The variants were filtered according to their quality (allele depth and genotype quality), population frequency, the effect on the gene and the classification of the variant in databases. Variant classification was performed according to ACMG Standards and Guidelines for the Interpretation of Sequence Variants (7). The identified variants were confirmed by Sanger sequencing.

CNV analysis was conducted on the BAM file (mapping with bwa *version v0.7.17* and cleaning with elprep *version v4.1.5*) arising from the identical sequencing batch using the Humanomics BED file, which enabled the detection of CNVs bigger than one whole exon. The depth of coverage was calculated specifically on exons within these files, and the normalization set comprised 400 patients (200 males and 200 females). The CNVs were detected by implementing of our in-house pipeline (StueckFinder v2.2) that integrates the well-established tool CANOES (8) and code modifications to enable CNV detection on the gonosomes. Variant classification was performed according to the ACMG/ClinGen technical standards for the interpretation and reporting of constitutional copy number variants (9). The detected CNVs were confirmed by nanopore sequencing.

*Tumor analysis.* WES of the tumoral DNA (using Mechanical Fragmentation and the Twist Universal Adapter System) was performed on the DNA extracted from the available Formalin-Fixed Paraffin-Embedded (FFPE) patient samples. The data analysis was performed using QIAGEN Digital Insight Software Genomics Workbench 21 for data preparation, mapping, variant calling and annotation. The tumoral WES results were analyzed separately and as a tumor-normal pair. Variant classification was performed according to ACMG Standards and Guidelines for the Interpretation of Sequence Variants in Cancer (10) and the ClinGen specification when available. Mutational signatures in the samples were analyzed Using Mutational Patterns R package (11) and COSMIC v2 signatures (12), taking only the somatic variants into account.

## Results

*Patients' characteristics.* Ten patients with multiple cancers were included in the study. The mean age was 40.7±5.4 years, 7 patients were female and 3 were male. None of the patients had family cancer history suggestive of a cancer predisposition syndrome. Before enrolment, by targeted NGS sequencing a *CHEK2* and *ATM* variant was identified in two patients, however they could not explain the full phenotype observed. The CMA results included two microduplications and one microdeletion (Table I).

We observed a mean of 3.2 tumors per patient. The tumors included melanomas, seminomas, thyroid cancer, gynecological tumors, and others (Table II).

*Germline WES results.* CNVs and missense, nonsense and splicing SNVs were identified in the patients included in the study (See Table III). The variants were classified according to the ACMG criteria and ClinGen specifications whenever available. Most of the identified SNVs were variants of uncertain significance (VUS), and only two variants were classified as pathogenic (See table IV). One of the pathogenic variants, an *ATM* splicing variant (c.8988-1G>A) that affected a canonical splice site, was not present in GnomAD, was predicted as deleterious by SIFT, Mutation taster, Provean, and the splicing in silico analysis. The second pathogenic variant in the *MUTYH* gene was previously described in patients with *MUTYH*-Associated Polyposis, an autosomal recessive disease that predisposes to colorectal cancer (13). Furthermore, heterozygous deleterious *MUTYH* variants were described as drivers in various types of cancer (adrenocortical carcinoma, esophageal carcinoma, sarcoma, prostate adenocarcinoma, and kidney renal clear cell carcinoma) (14).

Two CNVs were detected when evaluating WES data in the patients (Table III). One included an *MSR1* gene heterozygous deletion of exons 7-10. This CNV was classified as variant of unknow significance according to the ACMG/ClinGen criteria. The second was a heterozygous deletion of the whole *APOBEC3B* gene, which was classified as pathogenic as for a full gene deletion a pathogenic classification is warranted (15).

*Tumor WES results.* Twelve somatic samples were available for sequencing, a seminoma and thyroid cancer from patient 1, four melanomas from patient 3, an ovary cancer from patient 4, a thyroid cancer, dermatofibroma and dysplastic nevus from patient 6, and breast and thyroid cancer from patient 7. All the germline variants were also present in the somatic samples, no second hits were identified in the same genes. The mutational signature analysis aimed to identify common etiological factors for the development of the multiple cancers in the patients. The most frequently presented Single Base Substitution (SBS) signatures with a proposed etiology were the DNA mismatch repair alteration signature, and the signatures related to chemotherapy treatment (Table SI). The

Table I. Patient characteristics.

| Patient | Sex | Age, years | Family cancer history and age at diagnosis, years | Cancer type and age at diagnosis, years | Initial genetic evaluation | CMA results |
|---|---|---|---|---|---|---|
| P1 | M | 44 | Choroidal melanoma in father at 68 | Melanoma at 18, left seminoma at 21, right seminoma at 37, thyroid cancer at 38 | Kit BRCA HEREDITARY CANCER MASTR Plus: *BRCA1, BRCA2, PALB2, CHEK2, BARD1, BRIP1, RAD51C, RAD51D, TP53, MRE11A, RAD50, NBN, FAM175A, ATM, STK11, MEN1, PTEN, CDH1, MUTYH, BLM, XRCC2, MLH1, MSH6, PMS2* and *MSH2*, and the 3' UTR of *EPCAM* | arr[hg19] 2q13(111,408,390-113,098,686)x1 |
| P2 | F | 43 | Basal cell carcinoma carcinoma at 37 | Bilateral breast tumor at 23, melanoma | Kit BRCA HEREDITARY CANCER MASTR Plus: *BRCA1, BRCA2, PALB2, CHEK2, BARD1, BRIP1, RAD51C, RAD51D, TP53, MRE11A, RAD50, NBN, FAM175A, ATM, STK11, MEN1, PTEN, CDH1, MUTYH, BLM, XRCC2, MLH1, MSH6, PMS2* and *MSH2*, and the 3' UTR of *EPCAM* | arr[hg19] 16p13.11(14910205-16525348)x3 |
| P3 | F | 38 | Generalized cancer in half-sister at 35 | Melanoma at 23, 24, 30, 34 and 35 | Sanger sequencing of *CDK4* and *BAP1*, direct sequencing of c.1100delC in gene *CHEK2*. High-throughput sequencing: BRCA MASTR Dx kit (*BRCA1, BRCA2*), *PALB2, TP53* (exons 2-11), *ATM, CDKN2A* | arr[hg19] (1-22, X)x2 |
| P4 | F | 33 | Pancreatic cancer in paternal grandmother at 64 | Medulloblastoma at 10, ovary tumor at 26 | Sanger sequencing of *CDK4* and *BAP1*, direct sequencing of c.1100delC in gene *CHEK2*. High-throughput sequencing: BRCA MASTR Dx kit (*BRCA1, BRCA2*), *PALB2, TP53* (exons 2-11), *ATM, CDKN2A* | arr[hg19] (1-22, X)x2 |
| P5 | M | 42 | No history | Colorectal cancer at 37, melanoma | kit HNPCC MASTR Plus: *MLH1, MSH2, MSH6, PMS2*, and 3' UTR of *EPCAM*. Sequecing of *APC, MTYH, NTHL1, TP53, BAP1* | arr[hg19] (1-22)x2, (XY)x1 |
| P6 | F | 36 | Thyroid cancer in sister at unknown age, breast cancer in mother after the age of 50 and maternal grandmother. | Thyroid tumor at 26, melanoma at 28 | Kit SUREMASTR HEREDITARY CANCER: *BRCA1, BRCA2, PALB2, TP53, CHEK2, MLH1, MSH2, MSH6, ATM, BRIP1, RAD51C, RAD51D* | arr[hg19] (1-22, X)x2 |
| P7 | F | 44 | Ovarian cancer in mother 45, breast cancer in half-sister (same mother) at 37 | Breast tumor at 35, thyroid tumor at 36 | Kit BRCA HEREDITARY CANCER MASTR Plus: *BRCA1, BRCA2, PALB2, CHEK2, BARD1, BRIP1, RAD51C, RAD51D, TP53, MRE11A, RAD50, NBN, FAM175A, ATM, STK11, MEN1, PTEN, CDH1, MUTYH, BLM, XRCC2, MLH1, MSH6, PMS2* and *MSH2*, and the 3' UTR of *EPCAM* | arr[hg19] (1-22, X)x2 |
| P8 | F | 52 | Prostate cancer at 57, pheochromocytoma at 68 and paraganglioma in father, breast cancer in mother at 49 and grandmother at 60 | Breast tumor at 33, thyroid cancer at 38 | High-throughput sequencing: Kit SUREMASTR HEREDITARY CANCER (*BRCA1, BRCA2, PALB2, TP53, CHEK2, MLH1, MSH2, MSH6, ATM, BRIP1, RAD51C, RAD51D*). *PTEN* gene sequencing and MLPA | arr[hg19] (1-22, X)x2 |

Table I. Continued.

| Patient | Sex | Age, years | Family cancer history and age at diagnosis, years | Cancer type and age at diagnosis, years | Initial genetic evaluation | CMA results |
|---|---|---|---|---|---|---|
| P9 | F | 38 | Synovial sarcoma in mother at 35 | Melanoma at 31, thyroid cancer at 34 | Arrhytmia/Primary Electrical disease panel: *ABCB4, ABCC9, ACTN2, AKAP9, ANK2, CACNA1C, CACNA2D1, CACNB2, CALM1, CALM2, CALM3, CASO2, CAY3, CTNNA3, DES, DPP6, DSC2, DSG2, DSP, GJA1 (CX43), GJAS (CX40), GNB5, GPD1L, HCN4, JUP, KCNAS, KCND3, KCNE1, KCNE2, KCNE3, KCNE5 (KCNE1L), KCNH2, KCNJ2, KCNJ5 (GIRK4), KCNJ8, KCNKI7, KCNQI, LMNA, NKX2-5 (NKX2E), NOS1AP, NPPA, PKP2, PLN, PPA2, PRKAG2, RANGRF (MOG1), RRAD, RYR2, SCN1B, SCN2B, SCN3B, SCN4B, SCN5A, SCN10A, SLMAP, SNTA1, TGFB3, TMEM43, TRDN, TRPM4* | arr[hg19] (1-22, X)x2 |
| P10 | M | 37 | No history | Seminoma at 24, bladder cancer at 33, kidney cancer at 34 | High-throughput sequencing: Kit SUREMASTR HEREDITARY CANCER (*BRCA1, BRCA2, PALB2, TP53, CHEK2, MLH1, MSH2, MSH6, ATM, BRIP1, RAD51C, RAD51D*). *FH* gene sequencing. Hereditary pheochromocytoma and paraganglioma panel (*SDHA, SDHB, SDHC, SDHD, VHL, RET*). Hereditary non polyposic colorectal cancer panel (*MLH1, MSH2, MSH6*). MLPA of *BRCA1, BRCA2, FH, SDHB, SDHC, SDHD, VHL, MLH1, MSH2, MSH6* | arr[hg19] 2q13(110862477-110964737)x4 |

CMA, Chromosomal Microarray Analysis; F, female; M, male.

Table II. Tumor characteristics.

| Characteristic | First tumor (n=10) | Second tumor (n=10) | Third tumor (n=6) | Fourth tumor (n=4) | Fifth tumor (n=2) | Total (n=32) |
|---|---|---|---|---|---|---|
| Mean±SD age at presentation, years | 26.9±8.5 | 30.8±6.0 | 33.8±4.0 | 35.3±1.5 | 35±1.4 | |
| Tumor type, n (%) | | | | | | |
| Melanoma | 3 (30.0) | 3 (30.0) | 3 (50.0) | 2 (50.0) | 2 (100.0) | 13 (40.6) |
| Seminoma | 1 (10.0) | 1 (10.0) | 1 (16.7) | 0 (0.0) | 0 (0.0) | 3 (9.4) |
| Thyroid cancer | 1 (10.0) | 3 (30.0) | 0 (0.0) | 1 (25.0) | 0 (0.0) | 5 (15.6) |
| Breast tumor | 3 (30.0) | 1 (10.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 4 (12.5) |
| Ovarian tumor | 0 (0.0) | 1 (10.0) | 1 (16.7) | 0 (0.0) | 0 (0.0) | 2 (6.3) |
| Neuronal tumors | 1 (10.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (3.1) |
| Muscle tumors | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (25.0) | 0 (0.0) | 1 (3.1) |
| CRC | 1 (10.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (3.1) |
| Renal and urinary tract tumors | 0 (0.0) | 1 (10.0) | 1 (16.6) | 0 (0.0) | 0 (0.0) | 2 (6.3) |

CRC, colorectal cancers, SD, standard deviation

Table III. Genetic alterations identified in the patients.

| Patient | Gene | Variant | Type |
|---|---|---|---|
| P1 | *CHEK2* | c1100delC (p.Thr367Metfs*15) | Nonsense SNV |
| | *EME2* | c.964C>T (p.Gln322*) | Nonsense SNV |
| | *ATRIP* | c.1637T>G (p.Leu546Trp) | Missense SNV |
| | *BUB1* | arr[hg19] 2q13(111,408,390-113,098,686)x1 | CNV |
| P2 | *HNF1A* | c.92G>A (p.Gly31Asp) | Missense SNV |
| | *TSC2* | c.5383C>T (p.Arg1795Cys) | Missense SNV |
| | *MSR1* | c.(956+1_957-1)_ 3618del | CNV |
| P3 | *ATM* | c.8988-1G>A | Splicing SNV |
| | *APC* | c.295C>T (p.Arg99Trp) | Missense SNV |
| P5 | *ERCC2* | c.2260G>C (p.Glu754Gln) | Missense SNV |
| P6 | *RASA2* | c.865T>C (p.Tyr289His) | Missense SNV |
| P7 | *RIF1* | c.1475A>G (c.1475A>G) | Missense SNV |
| | *APOBEC3B* | g.(?_ 39378444)_( 39388168_?)del | CNV |
| P8 | *CHEK2* | c.434G>A (p.Arg145Gln) | Missense SNV |
| P9 | *ATM* | c.2057T>A (p.Leu686His) | Missense SNV |
| P10 | *MUTYH* | c.536A>G (p.Tyr179Cys) | Missense SNV |

Reference transcripts: APC NM_000038.6, APOBEC3B NC_000081.7, ATM NM_000051.3, ATRIP NM_130384.2, BRCA1 NM_007294.3, CHEK2 NM_007194.4, EME2 NM_001257370.2, ERCC2 NM_000400 HNF1A NM_000545.5, MSR1 NM_138715.2, MUTYH NM_012222.2, RASA2 NM_006506.5, RIF1 NM_018151.4, TSC2 NM_000548.5.4,

only obvious correlation between the patient´s signatures was observed between three of the four patient 3 melanomas.

## Discussion

In this study only two out of 10 patients with MPCs presented clearly pathogenic SNVs. Previous reports showed similar results. When performing a whole-genome sequencing (WGS) of a cohort of patients with MPC who had undergone genetic assessment, undetected germline pathogenic variants were identified in 15.2% cases (16). In another study, 21% of patients with MPC had at least one PV identified (17). The rest of our patients had one or more VUS that could potentially act together to cause the disease, supporting the hypothesis of an oligogenic effect, which has been described before (18).

The oligogenic effect of combinations of low penetrance SNVs in cancer-related genes has been suspected for a long time, including in young patients with breast and lung cancers. In a case-control study of 631 women with breast cancer diagnosed under the age of 53, it was proposed that SNVs in ten

Table IV. Single nucleotide variants identified in the patients.

| Patient | Gene | Variant | Effect | Predictors classifying the variant as deleterious | GnomAD frequency | Literature reports | Classification |
|---|---|---|---|---|---|---|---|
| P1 | *CHEK2* | c1100delC (p.Thr367Metfs*15) | Nonsense | CADD | 0.17% | Associated with higher risk of breast, prostate and colorectal cancer (12) | LPV |
| | *EME2* | c.964C>T (p.Gln322*) | Nonsense | Mutation taster, FATHMM MKL, SIPHY | 0.82% | Reported in individual with pancreatic ductal adenocarcinoma at 60 (13) | VUS |
| | *ATRIP* | c.1637T>G (p.Leu546Trp) | Missense | SIFT, PROVEAN, FATHMM MKL, SIPHY | 0.00% | - | VUS |
| P2 | *HNF1A* | c.92G>A (p.Gly31Asp) | Missense | Mutation taster, FATHMM, PROVEAN, SIPHY | 0.08% | Identified in a female with clear RCC and two chromophobe RCC at age 75. No second variant was found (14). Identified in person with MSS CRC at 58 (15) | VUS |
| | *TSC2* | c.5383C>T (p.Arg1795Cys) | Missense | SIFT, POLYPHEN, Mutation Taster, FATHMM, PROVEAN, SIPHY | 0.15% | Reported as probably neutral in functional study using HEK 293T cells (16). Reported in individual at high risk for breast and/or ovarian cancer (17) | VUS |
| P3 | *ATM* | c.8988-1G>A | Splicing | SIFT, Mutation Taster, PROVEAN, SIPHY, SPLICEAI AG, SPLICEAI AL, SPLICING ADA | 0% | A splicing variant at this position was proven to alter the splice acceptor site (18) | PV |
| | *APC* | c.295C>T (p.Arg99Trp) | Missense | SIFT, FATHMM, PROVEAN, FATHMM MKL, SIPHY | 0.04% | Detected in a person from a cohort with B-cell neoplasms (19). Identified in one of 40 unrelated patients with familial CRC without classical familial adenomatous polyposis coli (20) | VUS |
| P4 | - | - | - | - | - | - | - |
| P5 | *ERCC2* | c.2260G>C (p.Glu754Gln) | Missense | PROVEAN, SIPHY | 0.01% | - | VUS |
| P6 | *RASA2* | c.865T>C (p.Tyr289His) | Missense | SIFT, Mutation Taster, PROVEAN, FATHMM MKL, SIPHY | 0% | - | VUS |
| P7 | *RIF1* | c.1475A>G (c.1475A>G) | Missense | SIFT, Mutation Taster, PROVEAN, FATHMM MKL, SIPHY | 0% | - | VUS |
| P8 | *CHEK2* | c.434G>A (p.Arg145Gln) | Missense | SIFT, POLYPHEN, Mutation Taster, FATHMM, PROVEAN, FATHMM MKL, SIPHY | 0.00% | Reported in a female with breast cancer at 37(21). Found in one individual with CRC and in none of the cancer free controls (22). A yeast in vivo assay showed that the variant was benign (23) | VUS |

Table IV. Continued.

| Patient | Gene | Variant | Effect | Predictors classifying the variant as deleterious | GnomAD frequency | Literature reports | Classification |
|---|---|---|---|---|---|---|---|
| P9 | *ATM* | c.2057T>A (p.Leu686His) | Missense | Mutation taster, PROVEAN, FATHMM MKL, SIPHY | 0.00% | Reported in a female older than 40 with breast cancer (25) | VUS |
| P10 | *MUTYH* | c.536A>G (p.Tyr179Cys) | Missense | SIFT, Mutation taster, FATHMM, PROVEAN, FATHMM MKL, SIPHY | 0.15% | Observed in homozygous and compound heterozygous state in multiple individuals with MUTYH-Associated Polyposis (10) | PV |

CRC, colorectal cancer; LPV, likely pathogenic variant; MSS, microsatellite stable; RCC, renal cell carcinoma; PV, pathogenic variant; VUS, variant of uncertain significance. Reference transcripts: APC NM_000038.6, ATM NM_000051.3, ATRIP NM_130384.2, BRCA1 NM_007294.3, CHEK2 NM_007194.3, EME2 NM_001257370.1, ERCC2 NM_000400.4, HNF1A NM_000545.5, MUTYH NM_012222.2, RASA2 NM_006506.5, RIF1 NM_018151.4, TSC2 NM_000548.5.

genes with known or predicted roles in breast cancer interact to affect a woman's cancer risk in a way unpredictable from single gene effects (19). On the other hand, sets of germline SNVs were identified in young non-smokers with lung adenocarcinoma that underwent WES (20).

The additive effect of multiple genetic events influencing a few biochemical mechanisms was observed in patient 1, where the identified variants were mapped into the cancer-related pathways. The SNVs affected genes of the DNA repair pathways (double-strand break repair and Fanconi anemia: *CHEK2*, *ATRIP*) and cell cycle checkpoints (*EME2*), while the genes involved in the microdeletion were mapped into the cell cycle checkpoints (*BUB1*). The pathogenic variant observed in *CHEK2* could explain each of the cancers observed in the patient separately due to the reports that *CHEK2* can be associated with risk of melanoma (21), seminoma (22), and other cancers (23). However, each of these reported patients showed a narrower spectrum of cancer, therefore the contribution of other pathogenic variants cannot be ruled out to explain multiple cancers in a single patient. The study of this single patient with a very significant number of precocious malignant tumors thus suggests an oligogenic effect. This patient, now healthy at the age of 46, benefits from a regular clinical and imaging surveillance. However, genetic counselling for his 5 children is very difficult; so far, family testing was not proposed as patient 1 had his first diagnosis at the age of 18 (a melanoma), a clinical follow-up from the age of 13 should probably be proposed.

Patient 2 in addition to two SNVs presented a deletion of the four last exons in *MSR1* gene. Germline variants in these gene have been linked to prostate cancer (24) and esophageal carcinoma (25). The importance of this gene in breast cancer and melanoma is yet to be identified. The second CNV was a complete deletion of *APOBEC3B* gene in patient 7. Germline deletions of this gene have been associated with breast cancer risk (26). The CNVs identified in patient 2 and patient 7 were confirmed by nanopore sequencing.

Three of the patients presented microdeletions/microduplications that could alter micro-RNA expression. However, none of the micro-RNA included in the CNVs has been associated with germline risk of cancer.

The other patients from our cohort showed limited numbers of variants or not any identified variant (patient 4). Of course, we cannot exclude PVs in non-coding areas. Other explanations could include epigenetic or environmental factors. Interestingly, there were not any variants identified in patient 4, who presented with a medulloblastoma at an early age, and a bilateral mucinous ovarian borderline tumor later in life, while her monozygotic twin sister never had a tumor, which suggested a non-genetic cause or low penetrance factors that we could not detect.

In five patients, we could analyze the tumors to search for second hits and confirm the involvement of germline variants in tumor suppressor genes. We could not identify such somatic genetic events. However, for further studies, a simultaneous analysis of the germline and tumor DNA should be done, including epigenetic analysis of the tumor DNA.

On the tumors, we investigated the mutational signatures, as they could orient the investigation toward specific oncologic mechanisms, such as DNA repair defects. We could not identify a recurrent signature in the different tumors

from a single patient, except for a defective DNA mismatch repair (MMR) signature observed in the three cancers from patient 6, in which we have not identified any PV in the MMR genes. Interestingly, several tumors showed a signature indicating exposure to chemotherapy. Although these patients were not previously exposed to chemotherapy nor radiotherapy, exposure to therapeutic or environmental DNA-damaging agents could contribute significantly to cancer risk if there is any constitutional defect in DNA-repair pathways (27).

Our study thus suggests that the simultaneous presence of multiple germline variants can confer a significant cancer risk. We also observed three families with multiple early cancers in patients carrying BRCA2 and ATM mutations (28) and other reports showed similar data (29,30). Moreover, the polygenic risk scores can identify a small population with high cancer risk, as demonstrated for breast cancer (31). Finally, rare recessive conditions could also induce a significant cancer risk as indicated for MCM9 mutations associated with primary ovarian insufficiency and cancer risk (32). Taken together these studies indicate that: i) investigations should not be limited to single gene studies in patients/families with multiple and/or early cancers; ii) In order to identify all the genetic events that could be associated with cancer genes, SNVs, CNVs, and chromosomal rearrangements including gene fusions should be tested combining different techniques such as NGS and optical genome mapping; iii) further studies are needed to investigate the respective role of genetic and epigenetic events in these patients; iv) the exact role of the observed variants and their cumulative effect should be addressed by functional studies.

The limitations of the study include the small number of participants, the challenges of correlating VUS with the disease and the limited availability of somatic samples. The limited number of participants restricts the generalizability of the findings, and makes impossible a mutation frequency analysis to identify common mutation patterns or recurring PVs. At the moment, it will be not possible to reclassify the VUS into other categories, as the information on the variants is limited, these variants are not recurrent in several patients and consequently there is not enough evidence of pathogenicity to initiate functional validation of the variants. Further studies such as dynamic variant analysis, data integration and bioinformatic analysis can provide insights into the variant role in MPCs, and discover potential biomarkers, signaling pathways, and therapeutic targets. Furthermore, it will be necessary to evaluate the effect of gene-gene, gene-environment and protein-protein interactions, and the role of genetic modifiers and environmental factors in MPCs. Indeed, it will be a real challenge to study the role of oligogenic variants in cancer predisposition. Among possibilities, it could be envisioned to inactivate several genes in cell models and study specific biochemical pathways, controlling for instance cell proliferation, apoptosis or DNA repair mechanisms. In these cell models, and in human tumors studied in parallel, proteomic and transcriptomic studies will evaluate simultaneous loss of gene or protein expression as well as protein-protein interactions. In animal models, one could cross heterozygous or homozygous knock-out animals and evaluate spontaneous or induced cancer development.

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**Availability of data and materials**

The whole exome sequencing data generated in the present study may be found in the SRA database under PRJNA1127072 or at the following URL: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1127072. The chromosomal microarray analysis data generated in the present study may be found in the Gene Expression Omnibus database under accession number GSE271498 or at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE271498.

**Authors' Contributions**

VB conceptualized the present study. MF, MM and VB collected and analyzed the data for the present study. MF was in charge of the formal analysis of the study. VB performed the funding acquisition. MF, MM, RT, MA, KD, LHe, MD, KS, NL, AL, JG, LHa and CF performed the investigation. MF, MM, RT, CJ and LP defined the methodology. VB administered the project and acquired the resources. CJ, AL, LP and VB checked and validated the data analysis. MF and VB wrote the original draft. MF, RT, MM, MA, KD, LHe, CF, MD, AL, NL, KS, JG, LHa, LP, CJ and VB reviewed and edited the manuscript. MF, JG and MD confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

**Ethics approval and consent to participate**

This study was approved by the Comité d'Éthique Hospitalo-Facultaire Universitaire de Liège (approval no. 2019/245). All subjects provided written informed consent.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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# General Discussion

Genetic counseling for cancer patients presents numerous challenges, particularly with the rapid advancements in genomic technologies. This project sought to understand how novel genetic findings from NGS could enhance our knowledge of cancer predisposition and improve patient management. With the shift from single-gene testing to more comprehensive methods such as panel sequencing, WES, and WGS, testing became more affordable and faster. Our primary goal was to investigate patients with MPCs and to evaluate the role of monogenic or oligogenic predisposition in these patients. This led to observation about rare cancers in monogenic conditions, such as CRCs linked with BRCA1 pathogenic variants, the evaluation of tumor risks associated with digenic inheritance involving known CPGs, and uncovering novel genetic mechanisms in patients with MPCs diagnosed before age 45. The methodology involved selecting cancer patients based on specific inclusion criteria, collecting clinical and genetic data from medical charts, and performing both germline and somatic DNA evaluations using various genomic techniques.

Key findings from this study underscore the significance of *BRCA1* germline pathogenic variants in the development of CRC through HRD, advocating for *BRCA1* testing in young patients with microsatellite stable CRC to enable personalized treatment with PARPi. Additionally, the early onset and multiplicity of cancers in double-heterozygous patients with PVs in HR-related CPGs highlight a substantial cancer risk. This necessitates comprehensive cascade testing in families with identified PVs in CPGs, considering the evaluation of additional CPGs in patients exhibiting specific phenotypes. The data also suggest that the presence of multiple germline variants significantly increases cancer risk. Collectively, these results advocate for broader genetic investigations beyond single-gene studies in patients or families with early or MPCs, employing techniques such as NGS and optical genome mapping to detect SNVs, CNVs, and chromosomal rearrangements.

In a recent study, in individuals with a broad spectrum of non–breast or ovarian malignancies and germline pathogenic variants in BRCA1 and BRCA2, BRCA1/2 deficiency and genomic instability features were found in 27% and 23% of patients, respectively. These malignancies had a higher genomic instability score than BRCA1- or BRCA2-proficient malignancies ($P < 0.001$ in both cases) . In tumors with a confirmed absence of a wild-type allele, the effectiveness of PARPi should be evaluated (353).

The population frequency of the PVs in certain genes can play a role in their identification in rare cancers and digenic inheritance. In a cancer patient cohort, germline PVs in certain genes, primarily associated with breast cancer and other common cancers, such as *BRCA2*, *ATM*, and *PALB2*, were found in a broad range of patients with rare tumors (309). On the other hand, in another study double hits were particularly frequent in the *TP53* and *FA/BRCA* pathways. However, it is not clear yet to what extent such functional perturbations of key cancer pathways by at least two co-inherited heterozygous digenic mutations are translated into the clinics (354).

## *BRCA1* germline variants can be involved in CRC development through HRD

Evidence linking *BRCA1* germline pathogenic variants to an increased risk of CRC is limited. Two large studies indicate that *BRCA1* variants may confer a fivefold increased risk for CRC, especially in young, high-risk patients (355,356). Among three recent meta-analyses, only one identified an increased CRC risk with *BRCA1* variants (odds ratio = 1.49, 95% CI = 1.19 to 1.85, $P < 0.001$) (121), while the other two did not (357,358). Another study suggested that *BRCA1* or *BRCA2* variant carriers, mostly of Ashkenazi ancestry, might be prone to anal carcinoma and left-sided mucinous CRC (359). A large study found that men with *BRCA1* variants had a slightly higher risk of CRC compared to those with *BRCA2* variants, suggesting a potential increased risk (360).

The presence of *BRCA1* germline variants in tumors was confirmed in our study and HRD signatures like those in ovarian cancers with BRCA variants were found. This indicated that *BRCA1* variants may drive CRC development or progression in some cases, making these patients potential candidates for PARPi. Clinical trials have demonstrated the efficacy of PARPi and platinum-based chemotherapy in BRCA-mutated and HRD-positive cancers (361).

## Double heterozygous patients with PVs in HRD-related CPGs have a very high risk of cancer

PVs in *BRCA1*, *BRCA2*, *CHEK2*, and *ATM* significantly elevate the lifetime risk of breast cancer. There is limited epidemiological data on breast cancer risk in individuals with PVs in two genes. However, this study and others suggest an increased risk of early-onset and metastatic breast cancer in such cases (362,363).

*BRCA1*, *BRCA2*, and *ATM* variant carriers also face higher risks of other cancers. *BRCA1* and *BRCA2* PVs are linked to elevated risks of prostate, pancreatic, and ovarian cancers, while *ATM* variants are associated with increased risks of pancreatic (OR 4.21), prostate (OR 2.58), and gastric (OR 2.97) cancers (364,365). This study suggests that the combination of *BRCA2* and *ATM* PVs might further heighten these risks, as seen in the two male patients with early-onset prostate cancer. Pancreatic cancer risks for *BRCA1* and *BRCA2* carriers are reported to be 1.16% and 4.1% by age 70, respectively (365). Given the interaction of *BRCA1*, *BRCA2*, and *ATM* proteins in the HR pathway, an additive effect on HR deficiency—and consequently, an increased pancreatic cancer risk—could be expected. This was observed in several of the patients, raising the question of additional monitoring for young breast cancer patients with these PVs.

Double heterozygous PVs may go unnoticed in clinical settings when each PV is inherited from a clinically unaffected parent. However, it remains uncertain to what extent this phenomenon could initiate or alter malignant transformation. The likelihood of such events occurring randomly depends heavily on the mutation frequency within the population and may vary among different populations and genes (306).

The present findings indicate that patients with PVs in *BRCA2* and another HR gene should be closely monitored for breast, pancreatic, and prostate cancers. Despite the rarity of double heterozygotes, as genetic testing becomes more comprehensive, the detection of multiple PVs will likely increase.

Moreover, when a single PV in a CPG is identified in a family, geneticists propose a cascade testing, searching for this single PV, starting with the index case first degree relatives. Our study indicates that, in case of very precocious or unexpected cancers, the possibility of another variant should be considered and investigated.

## The simultaneous presence of multiple germline variants can confer a significant cancer risk

In the study of patients with MPCs, only two out of ten individuals were found to have clearly pathogenic SNVs. This aligns with previous research, which identified undetected germline PVs in 15.2% of MPC cases via WGS and found pathogenic variants in 21% of MPC patients in another study (366,367). Most patients exhibited several VUS, suggesting the hypothesis of an oligogenic effect, where combinations of low-penetrance SNVs in cancer-related genes may collectively cause disease. It remains unclear whether these VUS represent hypomorphic mutations with potential additive effects or merely benign

variants. Functional studies are required to clarify this distinction; however, conducting such studies is particularly challenging in the case of hypomorphic mutations.

The mapping of the identified variants into cancer-related pathways suggested that multiple genetic factors influencing a few biochemical mechanisms might influence cancer risk. Despite identifying various SNVs and CNVs, some patients showed no detectable genetic variants, suggesting potential roles for non-coding mutations, epigenetic, or environmental factors. This study highlights the importance of comprehensive genetic testing and of further research into the combined effects of multiple germline variants, their functional impacts, and the roles of genetic and epigenetic events in cancer susceptibility.

## Genetic cancer predisposition is a spectrum

Genetic cancer predisposition can be conceptualized as a continuous spectrum, beginning with PRS and progressing through oligogenic, digenic, and monogenic inheritance patterns (See Figure 15). At the broadest end of this spectrum, PRS involves the contribution of hundreds or even thousands of genetic variants. Each variant typically has a relatively high allele frequency but exerts minimal individual effect on cancer risk. Collectively, however, these variants can significantly elevate an individual's susceptibility to cancer.

Among the common low penetrance variants implicated by WES in the risk of several cancer types are 5p15 (*TERT-CLPTM1L*) (368), 6p21 (*HLA*) (369,370), 8q24 (371), and other loci. Various studies have investigated pleiotropy in these regions and characterized cross-cancer susceptibility variants (372,373). The rare pleiotropic variant HOXB13 G84E has been described as more strongly associated with MPC risk development than with single cancer risk (374). Multiple studies have demonstrated that PRS can generate informative predictions for heritable traits and diseases (375), however, there is not any “universal” PRS for any cancer risk prediction.

Moving along the spectrum, we encounter oligogenic inheritance, which generally includes 3 to 20 genetic loci combinations. The allele frequencies of these loci can vary widely, and they typically have moderate effect sizes. These genetic loci may interact in different ways, such as a dominant gene being modified by other loci, or multiple genes having additive effects on the same pathway (303).

Further along the continuum is digenic inheritance, where an individual carries two low-frequency variants with high effect sizes. These variants are in two distinct CPGs. The interaction of these two variants substantially increases the risk or severity of cancer compared to any single variant alone.

At the opposite end of the spectrum is monogenic inheritance. Here, the predisposition to cancer is driven by highly uncommon variants with very high effect sizes in a single CPG. These variants are strong enough to predispose an individual to developing a tumor or a specific combination of tumors. However, it is important to note that some patients might develop cancer types that are atypical for the particular gene involved, suggesting a complex interplay of genetic and environmental factors.

This continuous spectrum model underscores the multifaceted nature of genetic cancer predisposition, ranging from the subtle influences of numerous common variants to the profound impacts of rare, high-effect mutations. Moreover, the combination of PVs and PRS can have an even higher effect on the MPC risk than any of those factors separately. The cancer predisposition spectrum also highlights the importance of considering the entire genetic landscape when assessing cancer risk, rather than focusing solely on single genes or variants.

## Genetic counseling for multiple primary cancers

Individuals with hereditary cancer syndromes often face an elevated risk of developing cancer at a young age and may be susceptible to more than one type of cancer. An estimated 5–10% of all cancers are hereditary, and about 20% of primary care patients have family histories indicating a higher risk of developing a hereditary cancer (376–379).

To address these risks, both the National Comprehensive Cancer Network (NCCN) and the American College of Medical Genetics (ACMG) have developed sets of hereditary cancer clinical practice guidelines. These guidelines based on family history were designed to aid medical professionals in assessing hereditary cancer risks (57,380).

The NCCN and ACMG guidelines share overlapping criteria, but there are key differences between them. One major distinction is that the NCCN guidelines are continuously updated, whereas the ACMG guidelines have not been revised since their publication in 2015. Additionally, the ACMG guidelines include criteria for several cancers that are not covered by the NCCN guidelines. These differences present both strengths and weaknesses in the effectiveness of identifying patients at risk. Frequent updates to the NCCN guidelines ensure that the criteria remain current, while the ACMG guidelines may

identify at-risk patients for cancers not addressed by the NCCN (381). However, a significant gap exists in clinical guidelines for patients and families dealing with MPCs.

Efforts to improve hereditary cancer risk assessment have included reinterpreting the phenotypic spectra of well-characterized hereditary cancer predisposition syndromes (382–384), defining high and moderate cancer risk genes through case-control studies (385–387) and pedigree analysis (388), and using WES/WGS to discover novel genes (389,390). As a result, genetic testing guidelines now include multigene panel testing for hereditary cancer in clinical practice.

For instance, the NCCN Guidelines provide information on cancer risks and management recommendations for various genes included in multigene panel tests. Despite significant progress in understanding the clinical relevance and implications of multiple CPGs, testing criteria remain largely limited to those associated with historically established cancer syndromes, such as *BRCA1/2*, *TP53*, and mismatch repair genes (380). Precision oncology studies on patients with common cancers have shown that these restrictive criteria result in about half of the patients with LP/P germline variants remaining undiagnosed (391–393).

Clinician preference for broader panels of genes is increasing, even though explicit testing criteria for genes recently associated with cancer risk and cancers falling outside the traditional phenotypic spectra for established hereditary syndromes are lacking (391). This is particularly relevant for patients with MPCs, who can fall outside the phenotypic spectra of established hereditary syndromes. The guideline gap necessitates clinicians to rely on empirical data and personal judgment when counseling these patients. The absence of specific genetic counseling guidelines for MPCs highlights the need for ongoing research and updates to ensure comprehensive risk assessment and management of these patients.

## Limitations and future directions

### Sample size

The main common limitations of the three studies were the recruitment bias and the small number of individuals. Even though approximately 5–10% of cancers are hereditary, these studies included very specific and rare populations — patients with uncommon tumor-gene associations, double heterozygous mutations, and individuals with MPCs without P/LP variants in common cancer predisposition genes. The limited number of patients caused a lack of data to make meaningful

comparisons, and these limitations highlighted the need for more studies with larger and more diverse populations to draw definitive conclusions and develop effective screening and treatment strategies.

**Segregation evaluation**

Another common limitation was the inability to evaluate the segregation of the identified variants in all the patients, due to limited access to the genetic material of the proband´s relatives. Assessing the family allows the determination of inheritance patterns and the potential for recurrence risk among other relatives. Utilizing a progenitor-proband (trio) approach is essential because parents may appear clinically unaffected due to factors like phenotypic variability, incomplete penetrance, gender-specific cancer risks, and environmental exposures. Furthermore, recognizing familial predispositions provides the chance for early cancer surveillance in at-risk relatives (306).

**Functional validation of variants of unknown significance**

The studies on uncommon tumor-gene association and digenic inheritance included patients with P/LP variants, whereas the multiple cancer study did not. Following the evaluation of MPCs patients, the high number of identified VUS posed challenges in correlating these VUS with the disease. Since no VUS was common among MPCs, the functional validation of the variants was not justified. However, future functional studies could potentially help to link VUS to MPCs. The identified VUS that could explain the patient´s phenotype, without a functional validation, cannot be used for genetic counseling and family predictive testing.

**Availability of somatic samples**

Another significant limitation was the limited availability of tumor samples from the patients. Understanding the impact of germline PVs in CPGs requires distinguishing between their causal role in cancer development and their coincidental presence. A thorough analysis of individual tumors along with germline sequencing should be performed to make this distinction. Key steps include identifying a second hit such as somatic mutation, LOH, or epigenetic silencing, leading to biallelic inactivation of the gene harboring the germline PV; assessing tumor characteristics such as HRD, MSI, and tumor mutational burden; and analyzing somatic mutation patterns using mutational signatures, among other methodologies (354). These comprehensive analyses are essential for elucidating the precise role of germline PVs in tumorigenesis. However, they are limited if the tumor samples are not available.

**Treatment evaluation**

The studies did not address the patients' treatment strategies because of an insufficient sample size to draw meaningful conclusions, and the studies' focus on genetic analysis rather than treatment approaches.

## Concluding remarks

In conclusion, this project highlights the importance of comprehensive genetic testing in understanding and managing cancer risks. Key findings show the significance of *BRCA1* variants in CRC, the higher cancer risk in double heterozygous patients with HR-related gene mutations, and the additive effect that multiple germline variants can have. These results suggest that integrating advanced genomic techniques into clinical practice can detect a broad spectrum of genetic anomalies. Despite limitations such as small sample sizes and recruitment bias, the study emphasizes the need for larger, more diverse research to improve cancer risk assessment and personalized treatments. Ultimately, the evolving genetic counseling guidelines must adapt to encompass the full spectrum of genetic predisposition, ensuring comprehensive risk assessment and management for individuals with hereditary cancer syndromes.

# Perspectives

### Sample size

The sample size was the main limitation in all the studies. Including a larger population through international cooperation in future research could enable more definitive conclusions and the development of effective screening and treatment strategies for cancer patients.

### Variants of unknown significance

Correlating VUS with disease is a challenge that requires functional studies to reclassify these variants into the LP/P or benign/likely benign category. While nonsense variants are typically considered pathogenic, the significance of frequent missense variants may often be unclear, potentially occurring by chance in cancer pathways containing numerous genes. For example, the Exome Aggregation Consortium (ExAC) database includes a substantial number of missense variants in key *FA/BRCA* pathway genes—567 in *BRCA1*, 1186 in *BRCA2*, 46 in *RAD51*, and 385 in *PALB2*—detected among 60,000 healthy individuals. Interestingly, some missense variants may even exert more harmful effects than truncating variants, particularly if they produce an additional dominant-negative effect (394). Therefore, rigorous functional validation of the identified variants is essential but presents significant challenges in clinical practice, especially since not all changes in protein function result in complex clinical conditions (306).

The development of databases containing functional analyses of variants in genes associated with cancer pathways is expected to address this issue in the future. A notable example of ongoing efforts in this field is the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) consortium, an international collaboration of researchers focused on evaluating the clinical significance of sequence variants in genes such as *BRCA1*, *BRCA2*, and other known or suspected breast and ovarian cancer predisposition genes. Through its contributions to global database and classification initiatives, ENIGMA provides expert insights and explores effective strategies for communicating this critical information to both healthcare providers and patients (395).

Over time, it is essential to reevaluate the VUS identified in the patients because new information is continually published, and some VUS may be reclassified into benign or pathogenic categories.

**Negative test results**

In the MPC cohort, there were patients without any identified germline variant. The possible explanations include the limitation of the current analysis methods (CMA and WES). These might be addressed by using other techniques, such as WGS, for future patient evaluation. Additionally, other pathogenic mechanisms should be considered, including an evaluation of epigenetics, regulatory elements, non-coding regions, and microRNAs. Negative genetic tests should also be reanalyzed every few years because new genes are constantly being associated with disease and variants reclassified.

**Absence of identified somatic second hits**

The MPC study evaluated both germline and somatic DNA. In the pathogenesis of hereditary cancer predisposition LOH or a second somatic hit are expected. However, no second hits were observed in the somatic samples from the cohort. Epigenetic silencing of the wild-type allele in the tumor has been described as a second hit. This opens the possibility of evaluating in the future the tumoral epigenetics.

**Future patient evaluation strategies**

Technological advancements should be leveraged to capture the full spectrum of genetic events associated with cancer. Combining NGS with optical genome mapping and other advanced techniques can detect SNVs, CNVs, and chromosomal rearrangements, including gene fusions. This comprehensive approach will enhance our understanding of the genetic underpinnings of cancer.

Furthermore, exploring the interplay between genetic and epigenetic events is crucial. Future studies should investigate how epigenetic modifications contribute to cancer development and progression, alongside genetic mutations. Understanding these interactions may reveal new therapeutic targets and strategies for cancer prevention.

**Functional genetics evaluation in oligogenic inheritance**

Addressing the observed variants' cumulative effects through robust functional studies will be essential. Such research could provide insights into the combined impact of multiple genetic alterations, paving the way for more personalized and effective treatment options for cancer patients. By addressing these future perspectives, the field can move towards more comprehensive and accurate cancer genetics research, ultimately improving patient outcomes.

**Treatment perspectives**

The digenic and oligogenic inheritance of PVs in the same pathway holds significant clinical implications for the patients and their families, including early diagnosis, cancer risk assessment, and surveillance. In the era of advancing precision medicine, which encompasses early tumor detection, immunoprevention, and innovative approaches such as analyzing circulating cell-free DNA, molecular markers for premalignant lesions, and deep sequencing for clonal hematopoiesis, these considerations are particularly relevant (396). Recent years have witnessed the approval of new FDA drugs and vaccines for cancer prevention, alongside the development of combinatorial chemopreventive strategies. These advancements suggest future opportunities for enhanced cancer prevention and detection methods, including surveillance programs (306).

**Potential of polygenic risk scores**

PRS have potential implications for improving the understanding of the shared mechanisms of carcinogenesis. With further replication, they may also enable prevention (e.g., smoking cessation) and screening strategies that prioritize individuals at risk for developing additional cancers (102). Accurate population-calibrated estimates of lifetime risks are needed for incorporation of PRS into risk-based screening. In patients with tumors of suspected genetic origin and without PVs that could explain the disease, PRS could potentially explain the observed phenotype.

# Conclusions

In conclusion, this project aimed to explore how the use of NGS could deepen our understanding of cancer predisposition and enhance patient care. The objectives included clarifying the role of *BRCA1* in CRC, assessing tumor risks linked to digenic inheritance involving known CPGs, and uncovering new genetic mechanisms in patients with MPCs diagnosed before age 45. The methodology involved selecting cancer patients based on specific inclusion criteria, gathering clinical and genetic data from medical records, and conducting both germline and somatic DNA analyses using various genomic techniques.

Key findings from this study emphasize the importance of *BRCA1* germline pathogenic variants in the development of CRC through HRD. This supports *BRCA1* testing in young patients with microsatellite stable CRC to enable personalized treatment with PARPi. Additionally, the early onset and multiplicity of cancers in double-heterozygous patients with PVs in HR-related CPGs indicate a significant cancer risk. In families with identified PVs in CPGs comprehensive cascade testing is needed, and additional CPGs should be considered in patients with specific phenotypes. The data also showed that the presence of multiple germline variants significantly increased cancer risk. Collectively, these results advocated for broader genetic investigations beyond single-gene studies in patients or families with early-onset or MPCs, using techniques like NGS and optical genome mapping to detect SNVs, CNVs, and chromosomal rearrangements.

The clinical conclusions applicable for patient management included:

- BRCA1 testing should be considered in young patients with a personal history of microsatellite stable CRC
- When a CPG PV is identified in a family, additionally to the usual cascade testing, we should also consider a study of other CPGs in patients with specific phenotypes, even distinct from other relatives, either based on the age at diagnosis or the type of cancer
- Investigations should not be limited to single gene studies in patients or families with multiple and/or early cancers

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# Annexes

## Digenic Inheritance of Mutations in Homologous Recombination Genes in Cancer Patients. Supplementary data

### DNA extraction methods

**STARlet**

Germline DNA was extracted from 200 µl of peripheral blood using the NucleoMag Blood kit (Macherey-Nagel) according to the manufacturer's instructions. The NucleoMag® Blood 200 µL procedure is based on reversible adsorption of nucleic acids to paramagnetic beads under appropriate buffer conditions.

Finally, highly purified DNA was eluted with low-salt Elution Buffer MBL5 (100 µl) and directly used for downstream applications. The protocol was automated on a Starlet (Hamilton) robot.

**QIAcube**

Germline DNA was extracted from 200 µl of peripheral blood using the QIAamp DNA Blood Mini Kits (QIAGEN) according to the manufacturer's instructions. The QIAamp DNA Blood Kit provided silica-membrane-based DNA purification. During the DNA purification procedure, DNA bound specifically to the QIAamp silica-gel membrane while contaminants passed through. PCR inhibitors, such as divalent cations and proteins, were completely removed in two efficient wash steps, leaving pure DNA to be eluted in water (100 µl). The protocol was automated on a QiaCube (Qiagen) robot.

### Genes´ accession numbers

| Gene | SureMASTR | Twist |
|---|---|---|
| *BRCA2* | NM_000059.3 | NM_000059.4 |
| *BRCA1* | NM_007294.3 | NM_007294.4 |
| *ATM* | NM_000051.3 | NM_000051.4 |
| *CHEK2* | NM_007194.3 | NM_007194.4 |

### Software used

SeqPilot V4.3.1 (JSI, Ettenheim, GmbH, Germany) was used for Sanger and MLPA.

SeqPilot, module SeqNext v4.3 (JSI, Ettenheim, GmbH, Germany) was used for NGS with Agilent technologies.

ALISSA Interpret v5.4 (Agilent technologies, Santa Clara, CA, USA) was used for NGS with Twist Bioscience.

**Table S1.** DNA extraction.

| | **Patient 1** | | **Patient 2** | **Patient 3** | | **Patient 4** | **Patient 5** | | **Patient 6** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Sample 1** | **Sample 2** | **Sample 1** | **Sample 1** | **Sample 2** | **Sample 1** | **Sample 1** | **Sample 2** | **Sample 1** | **Sample 2** |
| Type of sample | Blood | Blood | Blood | Blood | Blood | Blood | Blood | Blood | Blood | Blood |
| DNA extraction method | QIAcube | STARlet | QIAcube | STARlet | QIAcube | QIAcube | QIAcube | STARlet | STARlet | QIAcube |
| Spectroscopic analysis | NanoDrop | NanoDrop | NanoDrop | NanoDrop | NanoDrop | NanoDrop | NanoDrop | NanoDrop | NanoDrop | NanoDrop |
| Purity (260/280 ratio) | 1.62 | 2.00 | 1.80 | 1.98 | 1.77 | 1.62 | 1.79 | 2.13 | 1.91 | 1.71 |
| Concentration (ng/ul) | 25.45 | 67.76 | 52.95 | 208.98 | 89.13 | 43.36 | 56.70 | 95.46 | 80.45 | 50.58 |
| Sequencing kit | SureMASTR Hereditary Cancer Agilent kit, 26 genes | Sanger | SureMASTR Hereditary Cancer Agilent kit, 12 genes + MLPA. Sanger | SureMASTR Hereditary Cancer Agilent kit, 12 genes + MLPA | Sanger | SureMASTR Hereditary Cancer Agilent kit, 12 genes + MLPA. Sanger | High-throughput Breast cancer panel 26 genes + MLPA | Sanger | 13-gene Breast cancer panel + MLPA | Sanger |
| Panel manufacturer | Agilent technologies, Santa Clara, CA, USA | - | Agilent technologies, Santa Clara, CA, USA | Agilent technologies, Santa Clara, CA, USA | - | Agilent technologies, Santa Clara, CA, USA | Twist Bioscience, San Francisco, CA, USA) | - | Twist Bioscience, San Francisco, CA, USA) | - |
| Sequenced genes | *BRCA1, BRCA2,* | *BRCA2, ATM* | *BRCA1, BRCA2,* | *BRCA1, BRCA2,* | *BRCA2, ATM* | *BRCA1, BRCA2,* | *BRCA1, BRCA2,* | *BRCA1, BRCA2* | *BRCA1, BRCA2,* | BRCA2, *CHEK2* |

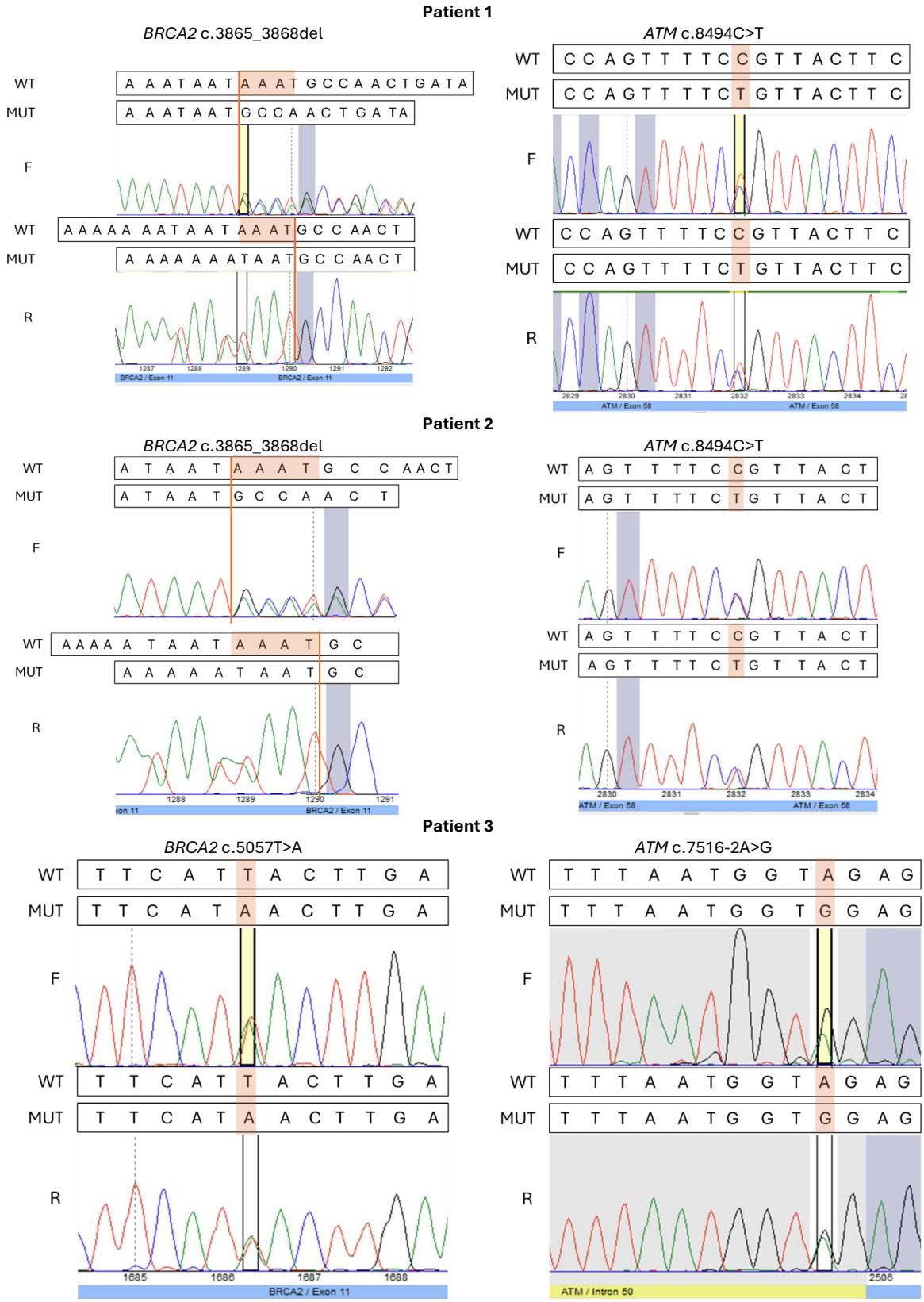
| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *PALB2, CHEK2, BARD1, BRIP1, RAD51C, RAD51D, TP53, MRE11A, RAD50, NBN, FAM175A, ATM, STK11, MEN1, PTEN, CDH1, MUTYH, BLM, XRCC2, MLH1, MSH6, PMS2* and *MSH2* and the 3' UTR of *EPCAM* | | *PALB2, TP53, CHEK2, MLH1, MSH2, MSH6, ATM, BRIP1, RAD51C and RAD51D* | | *PALB2, TP53, CHEK2, MLH1, MSH2, MSH6, ATM, BRIP1, RAD51C and RAD51D* | | *PALB2, TP53, CHEK2, CDH1, STK11, PTEN, MLH1, MSH2, MSH6, MTYH, EPCAM, MEN1, BRIP1, RAD51C, RAD51D, ATM, BARD1, RAD50, BLM, FAM175A, MRE11A, NBN, XRCC2, PMS2* | | *PALB2, TP53, CHEK2, MLH1, MSH2, MSH6, ATM, BRIP1, RAD51C, RAD51D, BARD1* | |
| MLPA | - | - | *BRCA1, BRCA2* | *BRCA1, BRCA2* | - | *BRCA1, BRCA2* | *BRCA1, BRCA2, MLH1, MSH2, MSH6* | - | *BRCA1, BRCA2, CHEK2* c.1100del | - |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Amplicon or enrichment method | Amplicon | - | Amplicon | Amplicon | - | Amplicon | Enrichment method | - | enrichment method | - |
| Reference genome | hg19 | hg19 | hg19 | hg19 | hg19 | hg19 | hg19 | hg19 | hg19 | hg19 |
| Bioinformatic pipeline | Inhouse demultiplexing pipeline | - | Inhouse demultiplexing pipeline | Inhouse demultiplexing pipeline | - | Inhouse demultiplexing pipeline | Inhouse Humanomics pipeline | - | Inhouse Humanomics pipeline | - |

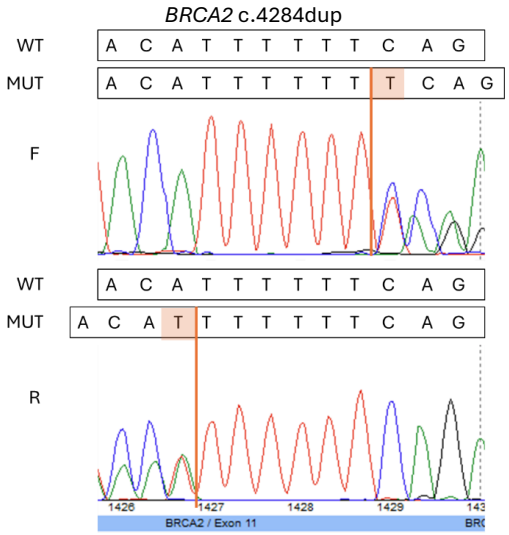
**Table S2.** Primer sequences used.

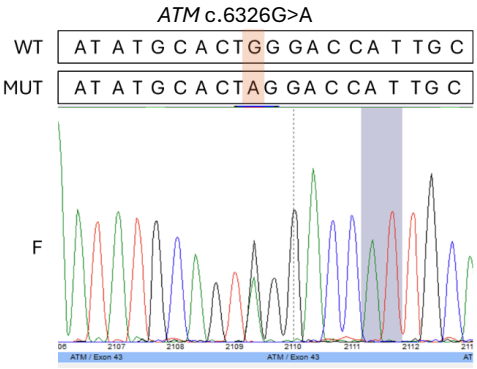
| Patient | Gene | Exon | Forward primer sequence | Reverse primer sequence |
|---|---|---|---|---|
| Patient 1 | *BRCA2* | 11-05 | AAGTGCCTGAAAACCAGATG | CAACAAAAGTGCCAGTAGTCA |
| | *ATM* | 58 | GCTTCCCTGTCCAGACTGTT | CACTATCATCCCCCTGCAAC |
| Patient 2 | *BRCA2* | 11-05 | AAGTGCCTGAAAACCAGATG | CAACAAAAGTGCCAGTAGTCA |
| | *ATM* | 58 | GCTTCCCTGTCCAGACTGTT | CACTATCATCCCCCTGCAAC |
| Patient 3 | *BRCA2* | 11-08 | TGAGACCATTGAGATCACAGC | TAGTCACAAGTTCCTCAACGCA |
| | *ATM* | 51 | TGCATTAATCTAGAGTACCCATTAG | GAAATCCTAGGCCTCCCA |
| Patient 4 | *BRCA2* | 11-07 | TTGTCAGATTTAACTTTTTTGGAAG | CAACTGGGACACTTTCTTTCAG |
| | *ATM* | 43 | TCAAACTCCTGGGCTCAAGT | CAGTTGTTGTTTAGAATGAGGAGAGA |
| Patient 5 | *BRCA1* | 10.3-4 (11-03) | GGGCTGGAAGTAAGGAAACAT | ACGCTCTTGTATTATCTGTGG |
| | *BRCA2* | 18 | CAGTGGAATTCTAGAGTCAC | GAAAGATCTCTGGACCTCC |
| Patient 6 | *BRCA2* | 7 | GCAATTCAGTAAACGTTAAGTG | GTCAGTTACTAACACACTTATC |
| | *CHEK2* | 4 | GGAGAGCTGGTAATTTGGTCA | CGCCTCAGCCTCCCAAAG |

**Figure S1.** Chromatograms of the variants identified in the patients.

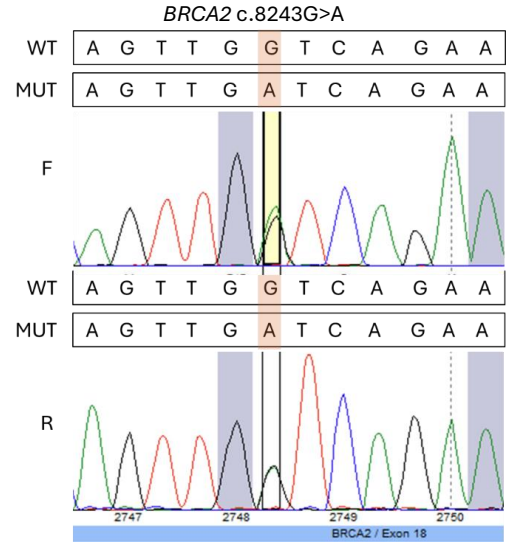


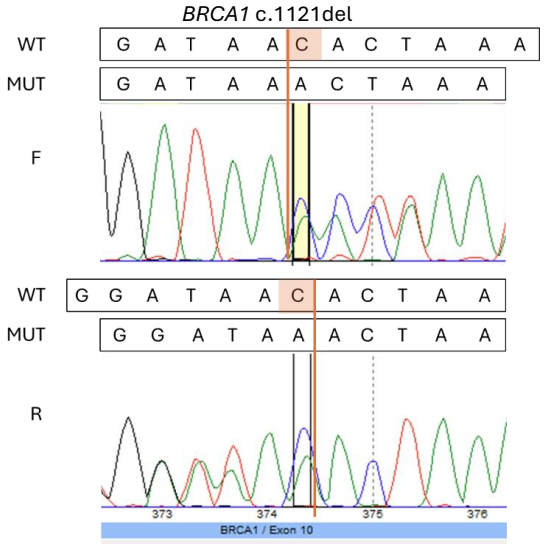
**Patient 4**



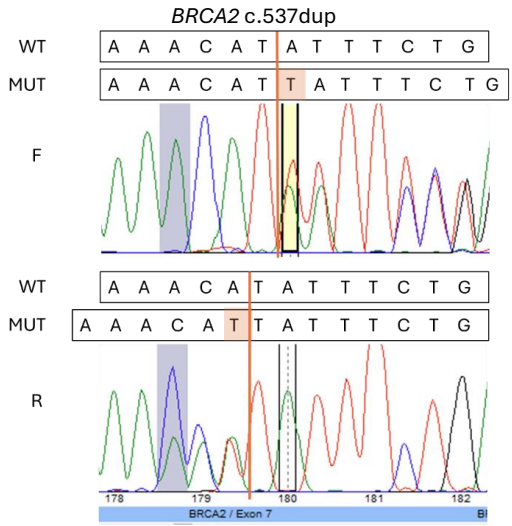



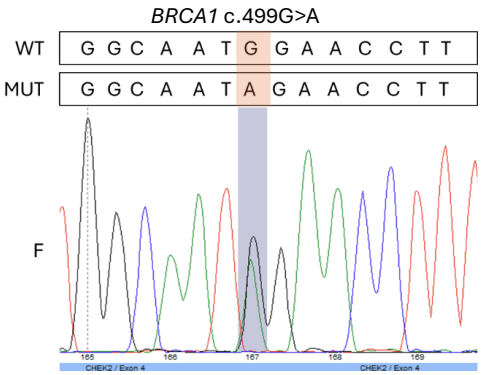

**Patient 5**




**Patient 6**




F – forward primer, MUT – Mutant, R – reverse primer WT – wild type

# Genetic evaluation of patients with multiple primary cancers. Supplementary data

**Supplementary Table I.** Mutational signatures analysis of the tumors

| Signature | P1 Seminoma | P1 Thyroid cancer | P3 Melanoma | P3 Melanoma | P3 Melanoma | P3 Melanoma | P4 Ovarian cancer | P6 Thyroid cancer | P6 Dermatofibroma | P6 Dysplastic nevus | P7 Breast cancer | P7 Thyroid cancer | Proposed etiology |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SBS1 (%) | 3.23 | 3.7 | 0 | 0.25 | 0 | 0.5 | 0.43 | 2.05 | **9.79** | 0 | **5.4** | 4.4 | Clock-like |
| SBS5 (%) | 1.96 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **8.56** | 0 | 0 | 0 | Clock-like |
| SBS4 (%) | 3.39 | 0 | **18.8** | **13.4** | 1.7 | **19** | **21.7** | 4.48 | 0 | **24.9** | 2.4 | 3.2 | Tobacco smoking |
| SBS7b (%) | 1.75 | 2.77 | **6.01** | 4.54 | 3.4 | 3.6 | **6.76** | 1.65 | 3.79 | **7.25** | 0.1 | 0 | UV light |
| SBS6 (%) | **10.8** | 3.74 | **12** | **8.57** | 2.4 | **5.7** | **11.2** | **13** | **12.5** | **11.8** | 2.1 | 7.3 | Defective DNA mismatch repair |
| SBS43 (%) | 4.05 | 3 | **6.94** | **6.57** | 4 | **5.8** | 3.39 | 4.39 | 1 | 4.62 | 3.7 | 4.4 | Defective DNA mismatch repair |
| SBS26 (%) | 3.51 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.75 | 0 | **5.2** | 4.9 | Defective DNA mismatch repair |
| SBS18 (%) | 0 | 0 | 0 | 0 | **5.5** | 0.1 | 0 | 0 | 0 | 0 | 0 | 0 | Damage by ROS |
| SBS30 (%) | 3.35 | 2.15 | 0 | 0 | 0.2 | 1.3 | 0 | 0 | 0 | 0.44 | **7.8** | **5.3** | Defective BER |
| SBS84 (%) | 1.73 | **11.8** | 0.25 | 2.28 | 1.4 | 1.7 | 0 | 2.86 | 0 | 0.37 | 0 | 1.7 | Activity of activation-induced cytidine deaminase |
| SBS24 (%) | 0 | 0 | 1.51 | 0.15 | **5.8** | 3.5 | **5.08** | 0 | 1.73 | 4.56 | 1.7 | 0 | Aflatoxin exposure |
| SBS25 (%) | 4.47 | **6.34** | 0.44 | **6.77** | **5.2** | 4.7 | 1.38 | 0 | 0 | 0.72 | **6.5** | **5.8** | CT treatment |
| SBS31 (%) | **5.13** | 3.82 | 3.74 | 4.81 | 0.9 | 1.4 | 0.54 | **6.29** | 1.67 | 1.05 | 2.2 | **5.4** | Platinum CT treatment |
| SBS87 (%) | **7.82** | 4.83 | **9.47** | **6.89** | 3.7 | 3.8 | **8.76** | **10.9** | **14.2** | **7.19** | **8** | **8.6** | Thiopurine CT treatment |

BER – base excision repair, CT – chemotherapy, ROS – reactive oxygen species. Mutational signatures with values >5% are in bold.

## Gene panel. Cancer-related genes

| | | | | |
|---|---|---|---|---|
| *A1CF* | *ARHGEF10* | *BCL2* | *CALR* | *CD79B* |
| *ABI1* | *ARHGEF10L* | *BCL2L12* | *CAMTA1* | *CDC73* |
| *ABL1* | *ARHGEF12* | *BCL3* | *CANT1* | *CDH1* |
| *ABL2* | *ARID1A* | *BCL6* | *CARD11* | *CDH10* |
| *ACKR3* | *ARID1B* | *BCL7A* | *CARS* | *CDH11* |
| *ACSL3* | *ARID2* | *BCL9* | *CASP3* | *CDH17* |
| *ACSL6* | *ARNT* | *BCL9L* | *CASP8* | *CDK12* |
| *ACVR1* | *ASPSCR1* | *BCLAF1* | *CASP9* | *CDK4* |
| *ACVR2A* | *ASXL1* | *BCOR* | *CBFA2T3* | *CDK6* |
| *AFDN* | *ASXL2* | *BCORL1* | *CBFB* | *CDKN1A* |
| *AFF1* | *ATF1* | *BCR* | *CBL* | *CDKN1B* |
| *AFF3* | *ATIC* | *BIRC3* | *CBLB* | *CDKN2A* |
| *AFF4* | *ATM* | *BIRC6* | *CBLC* | *CDKN2C* |
| *AKAP9* | *ATP1A1* | *BLM* | *CCDC6* | *CDX2* |
| *AKT1* | *ATP2B3* | *BMP5* | *CCNB1IP1* | *CEBPA* |
| *AKT2* | *ATR* | *BMPR1A* | *CCNC* | *CEP89* |
| *AKT3* | *ATRX* | *BRAF* | *CCND1* | *CHCHD7* |
| *ALDH2* | *AXIN1* | *BRCA1* | *CCND2* | *CHD2* |
| *ALK* | *AXIN2* | *BRCA2* | *CCND3* | *CHD4* |
| *AMER1* | *B2M* | *BRD3* | *CCNE1* | *CHEK2* |
| *ANK1* | *BAP1* | *BRD4* | *CCR4* | *CHIC2* |
| *APC* | *BARD1* | *BRIP1* | *CCR7* | *CHST11* |
| *APOBEC3B* | *BAX* | *BTG1* | *CD209* | *CIC* |
| *AR* | *BAZ1A* | *BTK* | *CD274* | *CIITA* |
| *ARAF* | *BCL10* | *BUB1B* | *CD28* | *CLIP1* |
| *ARHGAP26* | *BCL11A* | *C15orf65* | *CD74* | *CLP1* |
| *ARHGAP5* | *BCL11B* | *CACNA1D* | *CD79A* | *CLTC* |

| | | | | |
|---|---|---|---|---|
| *CLTCL1* | *CYLD* | *ELF4* | *FAM131B* | *FLCN* |
| *CNBD1* | *CYP2C8* | *ELK4* | *FAM135B* | *FLI1* |
| *CNBP* | *CYSLTR2* | *ELL* | *FAM47C* | *FLNA* |
| *CNOT3* | *DAXX* | *ELN* | *FANCA* | *FLT3* |
| *CNTNAP2* | *DCAF12L2* | *EML4* | *FANCC* | *FLT4* |
| *CNTRL* | *DCC* | *EP300* | *FANCD2* | *FNBP1* |
| *COL1A1* | *DCTN1* | *EPAS1* | *FANCE* | *FOXA1* |
| *COL2A1* | *DDB2* | *EPHA3* | *FANCF* | *FOXL2* |
| *COL3A1* | *DDIT3* | *EPHA7* | *FANCG* | *FOXO1* |
| *COX6C* | *DDR2* | *EPS15* | *FAS* | *FOXO3* |
| *CPEB3* | *DDX10* | *ERBB2* | *FAT1* | *FOXO4* |
| *CREB1* | *DDX3X* | *ERBB3* | *FAT3* | *FOXP1* |
| *CREB3L1* | *DDX5* | *ERBB4* | *FAT4* | *FOXR1* |
| *CREB3L2* | *DDX6* | *ERC1* | *FBLN2* | *FSTL3* |
| *CREBBP* | *DEK* | *ERCC2* | *FBXO11* | *FUBP1* |
| *CRLF2* | *DGCR8* | *ERCC3* | *FBXW7* | *FUS* |
| *CRNKL1* | *DICER1* | *ERCC4* | *FCGR2B* | *GAS7* |
| *CRTC1* | *DNAJB1* | *ERCC5* | *FCRL4* | *GATA1* |
| *CRTC3* | *DNM2* | *ERG* | *FEN1* | *GATA2* |
| *CSF1R* | *DNMT3A* | *ESR1* | *FES* | *GATA3* |
| *CSF3R* | *DROSHA* | *ETNK1* | *FEV* | *GLI1* |
| *CSMD3* | *DUX4L1* | *ETV1* | *FGFR1* | *GMPS* |
| *CTCF* | *EBF1* | *ETV4* | *FGFR1OP* | *GNA11* |
| *CTNNA2* | *ECT2L* | *ETV5* | *FGFR2* | *GNAQ* |
| *CTNNB1* | *EED* | *ETV6* | *FGFR3* | *GNAS* |
| *CTNND1* | *EGFR* | *EWSR1* | *FGFR4* | *GOLGA5* |
| *CTNND2* | *EIF1AX* | *EXT1* | *FH* | *GOPC* |
| *CUL3* | *EIF3E* | *EXT2* | *FHIT* | *GPC3* |
| *CUX1* | *EIF4A2* | *EZH2* | *FIP1L1* | *GPC5* |
| *CXCR4* | *ELF3* | *EZR* | *FKBP9* | *GPHN* |

| | | | | |
|---|---|---|---|---|
| *GRIN2A* | *IDH2* | *KEAP1* | *LSM14A* | *MLLT11* |
| *GRM3* | *IGF2BP2* | *KIAA1549* | *LYL1* | *MLLT3* |
| *H3F3A* | *IGH* | *KIF5B* | *LZTR1* | *MLLT6* |
| *H3F3B* | *IGK* | *KIT* | *MACC1* | *MN1* |
| *HERPUD1* | *IGL* | *KLF4* | *MAF* | *MNX1* |
| *HEY1* | *IKBKB* | *KLF6* | *MAFB* | *MPL* |
| *HIF1A* | *IKZF1* | *KLK2* | *MALAT1* | *MRTFA* |
| *HIP1* | *IL2* | *KMT2A* | *MALT1* | *MSH2* |
| *HIST1H3B* | *IL21R* | *KMT2C* | *MAML2* | *MSH6* |
| *HIST1H4I* | *IL6ST* | *KMT2D* | *MAP2K1* | *MSI2* |
| *HLA-A* | *IL7R* | *KNL1* | *MAP2K2* | *MSN* |
| *HLF* | *IRF4* | *KNSTRN* | *MAP2K4* | *MTCP1* |
| *HMGA1* | *IRS4* | *KRAS* | *MAP3K1* | *MTOR* |
| *HMGA2* | *ISX* | *KTN1* | *MAP3K13* | *MUC1* |
| *HMGN2P46* | *ITGAV* | *LARP4B* | *MAPK1* | *MUC16* |
| *HNF1A* | *ITK* | *LASP1* | *MAX* | *MUC4* |
| *HNRNPA2B1* | *JAK1* | *LATS1* | *MB21D2* | *MUTYH* |
| *HOOK3* | *JAK2* | *LATS2* | *MDM2* | *MYB* |
| *HOXA11* | *JAK3* | *LCK* | *MDM4* | *MYC* |
| *HOXA13* | *JAZF1* | *LCP1* | *MDS2* | *MYCL* |
| *HOXA9* | *JUN* | *LEF1* | *MECOM* | *MYCN* |
| *HOXC11* | *KAT6A* | *LEPROTL1* | *MED12* | *MYD88* |
| *HOXC13* | *KAT6B* | *LHFPL6* | *MEN1* | *MYH11* |
| *HOXD11* | *KAT7* | *LIFR* | *MET* | *MYH9* |
| *HOXD13* | *KCNJ5* | *LMNA* | *MGMT* | *MYO5A* |
| *HRAS* | *KDM5A* | *LMO1* | *MITF* | *MYOD1* |
| *HSP90AA1* | *KDM5C* | *LMO2* | *MLF1* | *N4BP2* |
| *HSP90AB1* | *KDM6A* | *LPP* | *MLH1* | *NAB2* |
| *ID3* | *KDR* | *LRIG3* | *MLLT1* | *NACA* |
| *IDH1* | *KDSR* | *LRP1B* | *MLLT10* | *NBEA* |

| | | | | |
|---|---|---|---|---|
| *NBN* | *NTRK3* | *PICALM* | *PRKCB* | *RECQL4* |
| *NCKIPSD* | *NUMA1* | *PIK3CA* | *PRPF40B* | *REL* |
| *NCOA1* | *NUP214* | *PIK3CB* | *PRRX1* | *RET* |
| *NCOA2* | *NUP98* | *PIK3R1* | *PSIP1* | *RFWD3* |
| *NCOA4* | *NUTM1* | *PIM1* | *PTCH1* | *RGPD3* |
| *NCOR1* | *NUTM2B* | *PLAG1* | *PTEN* | *RGS7* |
| *NCOR2* | *NUTM2D* | *PLCG1* | *PTK6* | *RHOA* |
| *NDRG1* | *OLIG2* | *PML* | *PTPN11* | *RHOH* |
| *NF1* | *OMD* | *PMS1* | *PTPN13* | *RMI2* |
| *NF2* | *P2RY8* | *PMS2* | *PTPN6* | *RNF213* |
| *NFATC2* | *PABPC1* | *POLD1* | *PTPRB* | *RNF43* |
| *NFE2L2* | *PAFAH1B2* | *POLE* | *PTPRC* | *ROBO2* |
| *NFIB* | *PALB2* | *POLG* | *PTPRD* | *ROS1* |
| *NFKB2* | *PATZ1* | *POLQ* | *PTPRK* | *RPL10* |
| *NFKBIE* | *PAX3* | *POT1* | *PTPRT* | *RPL22* |
| *NIN* | *PAX5* | *POU2AF1* | *PWWP2A* | *RPL5* |
| *NKX2-1* | *PAX7* | *POU5F1* | *QKI* | *RPN1* |
| *NONO* | *PAX8* | *PPARG* | *RABEP1* | *RSPO2* |
| *NOTCH1* | *PBRM1* | *PPFIBP1* | *RAC1* | *RSPO3* |
| *NOTCH2* | *PBX1* | *PPM1D* | *RAD17* | *RUNX1* |
| *NPM1* | *PCBP1* | *PPP2R1A* | *RAD21* | *RUNX1T1* |
| *NR4A3* | *PCM1* | *PPP6C* | *RAD51B* | *S100A7* |
| *NRAS* | *PDCD1LG2* | *PRCC* | *RAF1* | *SALL4* |
| *NRG1* | *PDE4DIP* | *PRDM1* | *RALGDS* | *SBDS* |
| *NSD1* | *PDGFB* | *PRDM16* | *RANBP2* | *SDC4* |
| *NSD2* | *PDGFRA* | *PRDM2* | *RAP1GDS1* | *SDHA* |
| *NSD3* | *PDGFRB* | *PREX2* | *RARA* | *SDHAF2* |
| *NT5C2* | *PER1* | *PRF1* | *RB1* | *SDHB* |
| *NTHL1* | *PHF6* | *PRKACA* | *RBM10* | *SDHC* |
| *NTRK1* | *PHOX2B* | *PRKAR1A* | *RBM15* | *SDHD* |

*SEPT5*
*SEPT6*
*SEPT9*
*SET*
*SETBP1*
*SETD1B*
*SETD2*
*SETDB1*
*SF3B1*
*SFPQ*
*SFRP4*
*SGK1*
*SH2B3*
*SH3GL1*
*SHTN1*
*SIRPA*
*SIX1*
*SIX2*
*SKI*
*SLC34A2*
*SLC45A3*
*SMAD2*
*SMAD3*
*SMAD4*
*SMARCA4*
*SMARCB1*
*SMARCD1*
*SMARCE1*
*SMC1A*
*SMO*
*SND1*
*SNX29*
*SOCS1*
*SOX2*
*SOX21*
*SPECC1*
*SPEN*
*SPOP*
*SRC*
*SRGAP3*
*SRSF2*
*SRSF3*
*SS18*
*SS18L1*
*SSX1*
*SSX2*
*SSX4*
*STAG1*
*STAG2*
*STAT3*
*STAT5B*
*STAT6*
*STIL*
*STK11*
*STRN*
*SUFU*
*SUZ12*
*SYK*
*TAF15*
*TAL1*
*TAL2*
*TBL1XR1*
*TBX3*
*TCEA1*
*TCF12*
*TCF3*
*TCF7L2*
*TCL1A*
*TEC*
*TENT5C*
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*TET2*
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*TRB*
*TRD*
*TRIM24*
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*TSC1*
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*USP44*
*USP6*
*USP8*
*VAV1*
*VHL*
*VTI1A*
*WAS*
*WDCP*
*WIF1*
*WNK2*
*WRN*
*WT1*
*WWTR1*
*XPA*
*XPC*
*XPO1*
*YWHAE*
*ZBTB16*
*ZCCHC8*
*ZEB1*
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*ZMYM2*
*ZMYM3*
*ZNF331*
*ZNF384*
*ZNF429*
*ZNF479*
*ZNF521*
*ZNRF3*
*ZRSR2*
*ABRAXAS1*
*ACVRL1*
*AIP*
*AKT1*
*ALK*
*ALKBH2*
*ALKBH3*
*ANKRD26*
*APC*
*APEX1*
*APEX2*
*APLF*

| | | | | |
|---|---|---|---|---|
| *APTX* | *CHK1* | *ETV6* | *GTF2H1* | *MGMT* |
| *ATM* | *CLK2* | *EXO1* | *GTF2H2* | *MITF* |
| *ATR* | *CTNNA1* | *EXT1* | *GTF2H3* | *MLH1* |
| *ATRIP* | *CYLD* | *EXT2* | *GTF2H4* | *MLH3* |
| *AXIN2* | *DCLRE1A* | *EZH2* | *GTF2H5* | *MMS19* |
| *BAP1* | *DCLRE1B* | *FAAP20* | *H2AFX* | *MNAT1* |
| *BARD1* | *DCLRE1C* | *FAAP24* | *HELQ* | *MPG* |
| *BLM* | *DDB1* | *FAM111B* | *HLTF* | *MRE11* |
| *BMPR1A* | *DDB2* | *FAN1* | *HNF1A* | *MSH2* |
| *BRAF* | *DDX41* | *FANCA* | *HNF1B* | *MSH3* |
| *BRCA1* | *DICER1* | *FANCB* | *HOXB13* | *MSH4* |
| *BRCA2* | *DIS3L2* | *FANCC* | *HRAS* | *MSH5* |
| *BRIP1* | *DMC1* | *FANCD2* | *HUS1* | *MSH6* |
| *BUB1B* | *DKC1* | *FANCE* | *IKZF1* | *MUS81* |
| *CASR* | *DSS1* | *FANCF* | *KIF1B* | *MUTYH* |
| *CBL* | *DUT* | *FANCG* | *KIT* | *NBN* |
| *CCNH* | *EFL1* | *FANCI* | *KITLG* | *NABP2* |
| *CD70* | *EGFR* | *FANCL* | *KRAS* | *NEIL1* |
| *CDC73* | *ELANE* | *FANCM* | *LIG1* | *NEIL2* |
| *CDH1* | *EME1* | *FEN1* | *LIG3* | *NEIL3* |
| *CDK4* | *EME2* | *FH* | *LIG4* | *NF1* |
| *CDK7* | *ENDOV* | *FLCN* | *LZTR1* | *NF2* |
| *CDKN1B* | *EPCAM* | *GALNT12* | *MAP2K1* | *NHEJ1* |
| *CDKN1C* | *ERCC1* | *GATA2* | *MAP2K2* | *NRAS* |
| *CDKN2A* | *ERCC2* | *GDNF* | *MAX* | *NSD1* |
| *CEBPA* | *ERCC3* | *GEN1* | *MBD4* | *NSUN2* |
| *CEP57* | *ERCC4* | *SLX1A* | *MC1R* | *NTHL1* |
| *CETN2* | *ERCC5* | *SLX1B* | *MDC1* | *NUDT1* |
| *CHAF1A* | *ERCC6* | *GPC3* | *MEN1* | *OGG1* |
| *CHEK2* | *ERCC8* | *GREM1* | *MET* | *PALB2* |

*PARP1*
*PARP2*
*PARP3*
*PAX5*
*PCNA*
*PDGFRA*
*PER1*
*PHOX2B*
*PIK3CA*
*PMS1*
*PMS2*
*PMS2L3*
*PNKP*
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*POLD1*
*POLE*
*POLG*
*POLH*
*POLI*
*POLQ*
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*SDHAF2*
*SDHB*
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*SDHD*
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*SHOC2*
*SHPRH*
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*SMUG1*
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*SOS2*
*SPO11*
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*TDG*
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*TDP2*
*TERC*
*TERT*
*TGFBR2*
*TINF2*
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*TP53*
*TP53BP1*
*TREX1*
*TREX2*
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*TSC1*
*TSC2*
*MPLKIP*
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*UBE2B*
*UBE2V2*
*UBE2N*
*UNG*
*UVSSA*
*VHL*
*WRN*
*WT1*
*XAB2*
*XPA*
*ERCC3*
*XPC*
*ERCC2*
*DDB2*
*ERCC4*
*ERCC5*
*POLH*
*XRCC1*
*XRCC2*
*XRCC3*
*XRCC4*
*XRCC5*
*XRCC6*

## Posters

**Poster 1.** Genetics of multiple primary cancers.

Presented at the Télévie seminar in 2022. U Liège, Liège, Belgium.

Presented at the GIGA-Cancer: Evaluation by the SAB in 2023. CHU Liege, Liège, Belgium.

Presented at the Cancer Genetics and Epigenetics Gordon Research Seminar in 2023. Lucca, Italy.

Presented at the Cancer Genetics and Epigenetics Gordon Research Conference in 2023. Lucca, Italy.

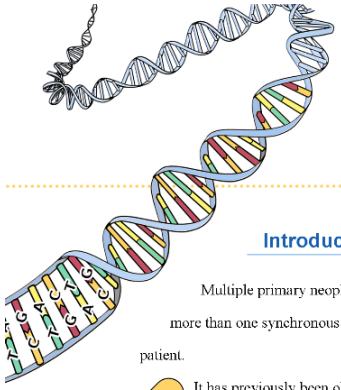
**Poster 2.** Genetics of multiple primary cancers. Presented at the European Society of Human Genetics conference in 2023. Glasgow, Scotland, UK.

**Poster 3.** Coinheritance of pathogenic variants in ATM and BRCA2 in families with multiple cancers: a case series.

Presented at the Télévie seminar in 2023. UCLouvain, Ottignies-Louvain-la-Neuve, Belgium.

Presented at the Belgian Society of Human Genetics in 2023. Charleroi, Belgium.

**Poster 4.** Aggressive HRD-positive colorectal cancer related to a *BRCA1* germline mutation in young patients. Presented at GIGA-Cancer Day in 2021. ULiège, Liège, Belgium.



# Genetics of Multiple Primary Cancers

Maria Valeria Freire, Claire Josse and Vincent Bours. GIGA Research Centre – University of Liège and CHU Liège, Belgium

## Introduction

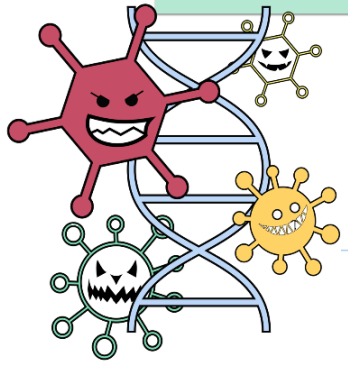
Multiple primary neoplasms (MPN) are the occurrence of more than one synchronous or metachronous cancer in the same patient.

It has previously been observed that genetic susceptibility may be an important cause of MPN.[1]

DNA repair pathways detect and repair genetic damage, therefore, various cancer-related genes are included in them.

There's no clear association between mutations in various genes from the same pathway and MPN predisposition.

**Objective:** Identification of novel genetic mechanisms associated with risk of tumour development.



**Hypotheses:**

1.Existence of de novo single dominant mutations

2.Presence of an additive effect of multiple genetic events influencing a single pathway

## Methods

We included patients with ≥2 cancers diagnosed before 45 years and no mutations in cancer predisposition genes.

We used whole exome sequencing (WES) of germline and tumoral DNA, and chromosomal microarray (CMA) on germline DNA to detect variants associated with the disease.

All identified variants were confirmed by Sanger sequencing and classified according to the ACMG criteria.

## Results and discussion

Currently, 12 out of 20 planned patients were recruited. The patients presented a mean of 3 cancers including melanomas, seminomas, thyroid cancers, gynecological and central nervous system tumors.

No family history suggestive of cancer predisposition syndromes was reported. CMA results were normal in most of the patients.

| Patient | Age (years) | SNVs before enrollment | CMA results |
|---|---|---|---|
| P1 | 44 | CHEK2: c.1100delC | 2q13x1 |
| P2 | 46 | | 7q21.12q21.13x3 |
| P3 | 43 | | 16p13.11x3 |
| P4 | 39 | BRCA1: c.-19-33G>T | Normal |
| P5 | 33 | | Normal |
| P6 | 42 | | Normal |
| P7 | 36 | | Normal |
| P8 | 44 | | Normal |
| P9 | 53 | | Normal |
| P10 | 39 | NF1: c.6788C>T | 8p22x3 |
| P11 | 38 | | Normal |
| P12 | 37 | | 2q13x3 |

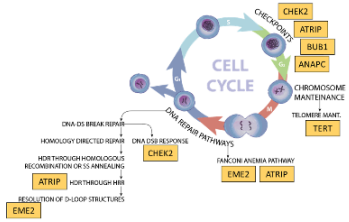
WES of the germline DNA identified 1-3 variants possibly related to the disease in each patient.

| Patient | Gene | Variant | Type | Classification |
|---|---|---|---|---|
| P1 | EME2 | c.964C>T | Nonsense | LP |
| | ATRIP | c.1637T>G | Missense | VUS |
| P3 | NIIF1A | c.92G>A | Missense | VUS |
| | TSC2 | c.5383C>T | Missense | VUS |
| | TP53 | c.1100+24G>C | Splicing | VUS |
| P4 | ATM | c.8988-1G>A | Splicing | VUS |
| | APC | c.295C>T | Missense | VUS |
| P7 | RASA2 | c.865T>C | Missense | VUS |
| P8 | FLCN | c.1333G>A | Missense | VUS |
| | RIF1 | c.1475A>G | Missense | VUS |
| P9 | CHEK2 | c.434G>A | Missense | VUS |
| | NF1 | c.6851C>T | Missense | VUS |
| P10 | POLD1 | c.46A>G | Missense | VUS |
| | SETD6 | c.811C>G | Missense | VUS |

LP—Likely pathogenic; VUS –Variant of Uncertain significance

The mapping of the variants into their pathways showed a possible additive effect of these as the cause of the cancer

**Figure 1.** Variants identified in patient 1




## Conclusions

No single culprit has been identified yet.

There are clear suggestions of additive effects of multiple genes.

Therefore, the possibility of an oligogenic effect as the cause of the cancer was considered in the patients.

We can not recommend a systematic exome sequencing in young patients with multiple cancers without pathogenic variants in known cancer predisposition genes.

## Bibliography

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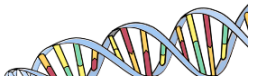
2. Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA. DNA repair pathways as targets for cancer therapy. Nat Rev Cancer. 2008 Mar;8(3):193–204.



**Poster 2.** Genetics of multiple primary cancers

Poster presentation number:
P13.007.C

# Genetics of Multiple Primary Cancers

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## Introduction

Multiple primary neoplasms (MPN) are the occurrence of more than one synchronous or metachronous cancer in the same patient.

It has previously been observed that genetic susceptibility may be an important cause of MPN.[1]

DNA repair pathways detect and repair genetic damage, therefore, various cancer-related genes are included in them.

There's no clear association between mutations in various genes from the same pathway and MPN predisposition.

**Objective:** Identification of novel genetic mechanisms associated with risk of tumour development.

## Methods

We included patients with ≥2 cancers diagnosed before 45 years and no mutations in cancer predisposition genes.

The routine genetic assessment and testing with targeted next-generation sequencing failed to establish a germline molecular genetic diagnosis

We used whole exome sequencing (WES) of germline and tumoral DNA, and chromosomal microarray (CMA) on germline DNA to detect variants associated with the disease.

## Results and discussion

Eleven patients were recruited. The patients presented a mean of 3 cancers including melanomas, seminomas, thyroid cancers, gynecological and central nervous system tumors.

No family history suggestive of cancer predisposition syndromes was reported. CMA results were normal in most of the patients.

**Table 1.** Patient characteristics

| | Sex | Age (years) | Cancer type and age at diagnosis | CMA results |
|---|---|---|---|---|
| **P1** | M | 44 | Melanoma at 18, left seminoma at 21, right seminoma at | arr[hg19] 2q13x1 |
| **P2** | F | 43 | Bilateral breast tumor at 23, melanoma | arr[hg19] 16p13.11x3 |
| **P3** | F | 38 | Melanoma at 23, 24, 30, 34 and 35 | arr[hg19] (1-22, X)x2 |
| **P4** | F | 33 | Medulloblastoma at 10, ovary tumor at 26 | arr[hg19] (1-22, X)x2 |
| **P5** | M | 42 | Colorectal cancer at 37, melanoma | arr[hg19] (1-22)x2, (XY)x1 |
| **P6** | F | 36 | Thyroid tumor at 26, melanoma at 28 | arr[hg19] (1-22, X)x2 |
| **P7** | F | 44 | Breast tumor at 35, thyroid tumor at 36 | arr[hg19] (1-22, X)x2 |
| **P8** | F | 52 | Breast tumor at 33, thyroid cancer at 38 | arr[hg19] (1-22, X)x2 |
| **P9** | F | 38 | Schwannoma at 33, hamartoma at 35, leiomyoma at 35 | arr[hg19] 8p22x3 |
| **P10** | F | 38 | Melanoma at 31, thyroid cancer at 34 | arr[hg19] (1-22, X)x2 |
| **P11** | M | 37 | Seminoma at 24, bladder cancer at 33, kidney cancer at 34 | arr[hg19] 2q13x4 |

CMA – Chromosomal Microarray Analysis; F – female; M – male

WES of the germline DNA identified 1-3 variants possibly related to the disease in each patient.

**Table 2.** Single nucleotide variants identified in the patients

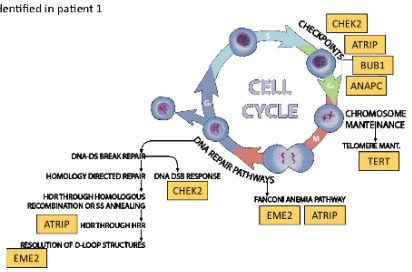
| Patient | Gene | Variant | Effect | Classification |
|---|---|---|---|---|
| **P1** | *CHEK2* | c.1100delC (p.Thr367Metfs*15) | Nonsense | LPV |
| | *EME2* | c.964C>T (p.Gln322*) | Nonsense | VUS |
| | *ATRIP* | c.1637T>G (p.Leu546Trp) | Missense | VUS |
| **P2** | *HNF1A* | c.92G>A (p.Gly31Asp) | Missense | VUS |
| | *TSC2* | c.5383C>T (p.Arg1795Cys) | Missense | VUS |
| **P3** | *ATM* | c.8988-1G>A | Splicing | PV |
| | *APC* | c.295C>T (p.Arg99Trp) | Missense | VUS |
| **P4** | - | - | - | - |
| **P5** | *ERCC2* | c.2260G>C (p.Glu754Gln) | Missense | VUS |
| **P6** | *RASA2* | c.865T>C (p.Tyr289His) | Missense | VUS |
| **P7** | *RIF1* | c.1475A>G (c.1475A>G) | Missense | VUS |
| **P8** | *CHEK2* | c.434G>A (p.Arg145Gln) | Missense | VUS |
| **P9** | *NF1* | c.6851C>T (p.Thr2283Ile) | Missense | VUS |
| | *POLD1* | c.46A>G (p.Lys16Glu) | Missense | VUS |
| | *SETD6* | c.811C>G (p.His271Asp) | Missense | VUS |
| **P10** | *ATM* | c.2057T>A (p.Leu686His) | Missense | VUS |
| **P11** | *MUTYH* | c.536A>G (p.Tyr179Cys) | Missense | PV |

CRC – colorectal cancer, LPV – likely pathogenic variant, MSS – microsatellite stable, RCC – renal cell carcino-

Reference transcripts: APC NM_000038.6, ATM NM_000051.3, ATRIP NM_130384.2, BRCA1 NM_007294.3, CHEK2 NM_007194.3, EME2 NM_001257370.1, ERCC2 NM_000400.4, HNF1A NM_000545.5, MUTYH NM_012222.2, NF1 NM_000267.3, POLD1 NM_002691.3, RASA2 NM_006506.5, RIF1 NM_018151.4, SETD6 NM_001160305.1, TSC2 NM_000548.5

The mapping of the variants into their pathways showed a possible additive effect of these as the cause of the cancer

**Figure 1.** Variants identified in patient 1




Fourteen somatic samples were available for sequencing. All the germline variants were also present in the somatic samples, no second hits were identified in the same genes.

**Table 3.** Mutational signatures analysis of the tumors

| Signature | P1 Seminoma | P1 Thyroid cancer | P3 Melanoma | P3 Melanoma | P3 Melanoma | P3 Melanoma | P4 Ovarian cancer | P6 Thyroid cancer | P6 Dermatofibroma | P6 Dysplastic nevus | P7 Breast cancer | P7 Thyroid cancer | P9 Schwannoma | P9 Hamartoma | Proposed etiology |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SBS1 (%) | | | | | | | | | | | | | | | Clock-like |
| SBS5 (%) | | | | | | | | | | | | | | | Clock-like |
| SBS4 (%) | | | | | | | | | | | | | | | Tobacco smoking |
| SBS7b (%) | | | | | | | | | | | | | | | UV light |
| SBS6 (%) | | | | | | | | | | | | | | | Defective DNA mismatch repair |
| SBS43 (%) | | | | | | | | | | | | | | | Defective DNA mismatch repair |
| SBS26 (%) | | | | | | | | | | | | | | | Defective DNA mismatch repair |
| SBS18 (%) | | | | | | | | | | | | | | | Damage by ROS |
| SBS30 (%) | | | | | | | | | | | | | | | Defective BER |
| SBS84 (%) | | | | | | | | | | | | | | | Activity of activation-induced CDA |
| SBS24 (%) | | | | | | | | | | | | | | | Aflatoxin exposure |
| SBS25 (%) | | | | | | | | | | | | | | | CT treatment |
| SBS31 (%) | | | | | | | | | | | | | | | Platinum CT treatment |
| SBS87 (%) | | | | | | | | | | | | | | | Thiopurine CT treatment |

0 1-3 3-6 6-9 9-12 12-15 18-21 21-24

**Conclusions:** Investigations should not be limited to single gene studies in patients/families with multiple and/or early cancers.

The genetic analyses should include large gene panels and CNV análisis.

Further studies are needed to investigate the respective role of genetic and epigenetic events in these patients.

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COI disclosure: the authors declare no conflict of interest.

**Poster 3.** Coinheritance of pathogenic variants in *ATM* and *BRCA2* in families with multiple cancers: a case series.

# Coinheritance of pathogenic variants in *ATM* and *BRCA2* in families with multiple cancers: a case series

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## Introduction

The coinheritance of heterozygous pathogenic variants (PVs) in two cancer-related genes has been restricted to small case series and single case reports[1].

*BRCA2* and *ATM* are part of the HR pathway[2].

PVs in *BRCA2* and *ATM* predispose to breast, prostate, and pancreatic cancer[3].

Gastric cancer was associated to *ATM*[3], while ovarian cancer was described in *BRCA2* PVs carriers[4].

### Objective

To describe four patients from three families with multiple cancers who coinherited PVs in *BRCA2* and *ATM* genes.

## Methods

Patient (P) 1 was analyzed using the SureMASTR Hereditary Cancer Agilent kit (26 genes).

In P2 the PVs identified in P1 were confirmed.

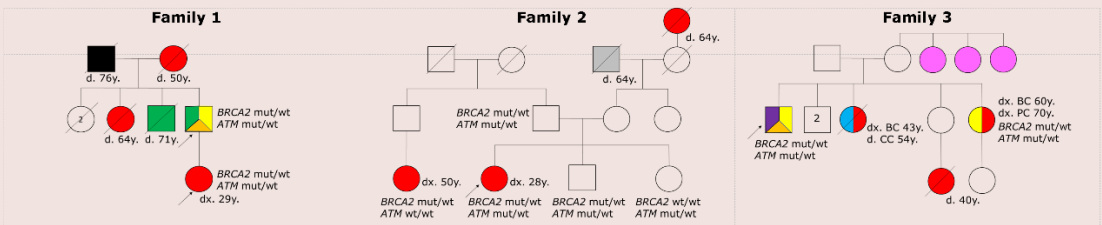
In P3 12 genes were sequenced using the Sure-MASTR Hereditary Cancer Agilent kit.

P4 was tested for the family PVs using Hereditary Cancer Solution Sophia Genetics kit (35 genes).

All the PVs were confirmed by Sanger sequencing.

## Results

Four patients from three families underwent genetic consultation in the context of multiple cancers or early-onset disease, leading to the identification of two heterozygous PVs in each patient, one in *BRCA2* and one in *ATM* gene.




**Identified variants**

**Family 1**
*BRCA2* c.3865_3868del p.(Lys1289Alafs*3)
*ATM* c.8494C>T p.(Arg2832Cys)
**Family 2**
*BRCA2* c.5057T>A p.(Leu1686*)
*ATM* c.7516-2A>G
**Family 3**
*BRCA2* c.4284dup p.(Gln1429Serfs*9)
*ATM* c.6326G>A p.(Trp2109*)

***BRCA2* variants**

Nonsense-mediated mRNA decay
Previously described as pathogenic
GnomAD:
- c.3865_3868del – 0%
- c.5057T>A – 0%
- c.4284dup – 0.0004%

***ATM* variants**

c.8494C>T – GnomAD 0.0014%, previously described as pathogenic
c.7516-2A>G – GnomAD 0%, located in a region where variants were described as pathogenic, affects a conserved splice site
c.6326G>A – GnomAD 0%, lof, NMD, previously reported as pathogenic

## Discussion

The development of the cancers at an early age may be the result of a combined effect of the PVs in *BRCA2* and *ATM* genes.

As *BRCA2* and *ATM* interact in the HR pathway, an additive effect on HR deficiency could be expected.

During cascade testing the assessment should not stop at a single PV.

Genetic or non-genetic factors can influence cancer risk in variant carriers.

### Conclusion

The risk of cancer in double heterozygous patients carrying PVs in the *BRCA2* and *ATM* genes is very high.

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XXX

# Aggressive HRD-positive colorectal cancer related to a *BRCA1* germline mutation in young patients

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## Introduction

*BRCA1* is a tumor suppressor gene that coordinates DNA repair, transcriptional regulation, cell-cycle control, centrosome duplication, and apoptosis.[1]

Mutations in *BRCA1* gene have been associated with familial risk of breast and ovarian cancers (OMIM: 604370).[2]

Women with a history of gynecological cancers presented a statistically significant but small risk for subsequent CRC, suggesting the existence of common etiologic factors for the development of these tumors.[3]

Patients carrying a germline *BRCA1* variant can develop a sporadic tumor, independently of *BRCA1* loss of function, highlighting the need to demonstrate the causal role of the variant in cancer development.[4]

**Aim:**

to gain insight into the role of known *BRCA1* mutations in the development of CRC in young patients.

### HRD evaluation in somatic WES results

Loss of BRCA1 function in a tumor results in HRD. The HRD can be evaluated through an HRD score or through specific mutational signatures.

We performed paired germline and tumoral DNA exome WES .

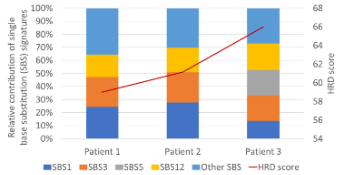
HRD score
- Loss of heterozygosity
- Telomeric-allelic imbalance
- Large-scale state transitions

Positivity threshold of HRD score ≥33

We used sequenza to detect and quantify CNV and estimate tumor cellularity and ploidy.

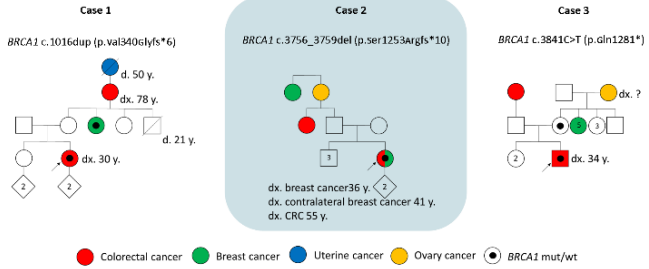
We used MutationalPatterns R package and COSMIC v2 signatures to analyze Mutational signatures in the samples.

Single base substitution (SBS) signatures with a relative contribution ≥ 15% of the global pattern and HRD score results



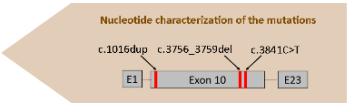

The three tumors showed high HRD scores and a SBS3, demonstrating a BRCA1 loss-of-function

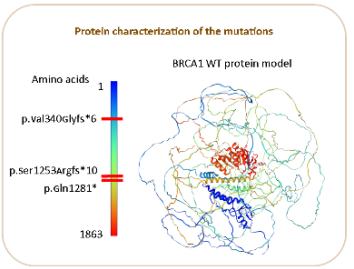
## Case report

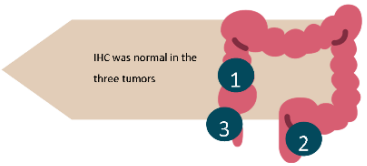



### Tumor characteristics

| | Case 1 | Case 2 | Case 3 |
|---|---|---|---|
| **Dx.** | Right colon moderately differentiated | Well to moderately differentiated rec- | Mucinous appendix adenocarcinoma |
| **TNM** | pT4aN2aM1a | cT3N1M1b | pT4bN0M0 at diagnosis, peritoneal re- |
| **TIR** | 50% | 30% | 20% |
| **CMA** | Partial gains and losses on chr 1-3, 5- 9, 12, 13, 15-20 and X | Entire and partial gains and losses on chr 1, 7, 8, 12, 13 and 18-20 | Normal |




## Discussion

Existing data on CRC risk remain contradictory, not allowing to recommend screening for CRC in *BRCA1* variants heterozygotes, or to consider the variants as a factor predisposing to familial CRC.

We not only confirmed that the *BRCA1* germline variants were still present in the tumor, but we also demonstrate HRD scars in the three tumors.

Germline *BRCA1* variants may, in a small proportion of carriers, play a driver role in CRC development or progression and these patients might benefit from a treatment with PARPi.

The small number of patients and the bias in recruitment are the main limitations of our study, precluding to justify any specific surveillance or screening program in the absence of a personal or family history.

## Conclusion:

*BRCA1* germline mutations can be involved in CRC development through HRD. Thus, *BRCA1* testing should be considered in young patients with a personal history of microsatellite stable CRC.

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**Abbreviations** CRC colorectal cancer; PARPi poly (ADP-ribose) polymerase-inhibitors; CMA chromosomal microarray; chr chromosome; d. death; dx. diagnosis; HRD homologous recombination deficiency; IHC immunohistochemistry; TIR tumor infiltration rate; WES whole exome sequencing.