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Comprehensive Genomic Profiling of Male Breast Cancer Patients with Multiple Primary Tumors through Matched Germline and Somatic Whole Exome Sequencing

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SUMMARY

INTRODUCTION

MALE BREAST CANCER

Epidemiology and risk factors

Male breast cancer (MBC) is often classified as a rare disease, corresponding to less than 1% of all cases of breast cancer (BC) and of all cancers in men (Ottini, 2014; Campos *et al*, 2021).

The annual incidence of MBC is estimated about one per 100,000 men (Ly *et al*, 2013); however, over the past decades, the annual incidence of MBC continued to arise, with consequent increasing attention. This is partly related to the higher rates of obesity, a well-known risk factor for BC, and partly related to the increasing longevity of the population (Rizzolo *et al*, 2013; Howlader *et al*, 2014; Campos *et al*, 2021).

In particular, in the last 30 years a notable increase of about 40% in MBC incidence has been registered, in contrast with the increase of about 25% in female BC (FBC) (Al Assaad *et al*, 2024).

In Italy, lifetime incidence rate is about one case per 629 men and around 500 men with BC are estimated to be diagnosed in 2019 (AIRTUM, 2020). Overall, MBC incidence varies greatly in different geographical areas and ethnic groups. In particular, MBC accounts for about 0.5% of male cancers in Western countries, while African populations have a higher percentage (about 5%), of cases compared with Caucasian or Asian counterparts (Miao *et al*, 2011; Hong *et al*, 2016; Howlader *et al*, 2016; Siegel *et al*, 2021).

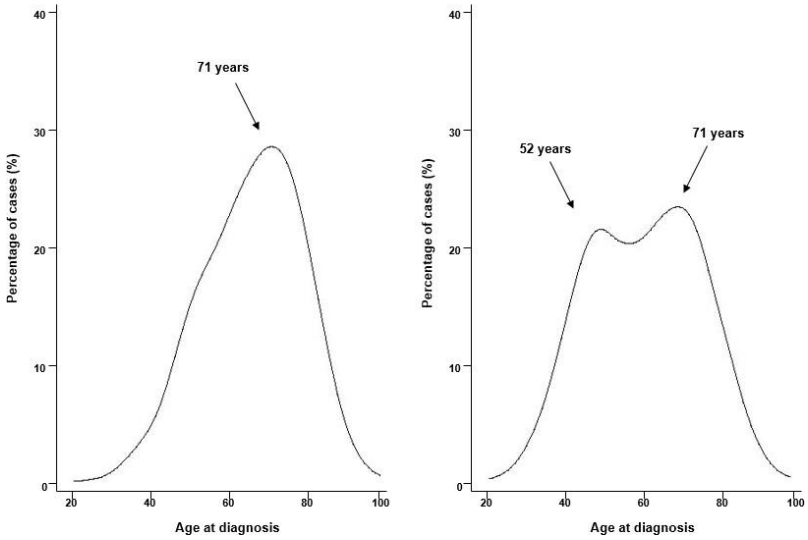
The worldwide variation of MBC resembles that of the female counterpart, with higher rates in North America and Europe, and lower rates in Asia (Ottini *et al*, 2010).

At the present, morbidity and mortality still represent a serious concern in MBC patients, probably because of its occurrence in advanced age and delayed diagnosis (Ottini, 2014; Łukasiewicz *et al*, 2021).

As shown in **Figure 1**, in contrast with the bimodal age distribution observed in FBC, BC incidence in men increases together with age, with a peak incidence in the sixth decade (Anderson *et al*, 2010; Ottini, 2014; Ishii *et al*, 2020).

Moreover, the mean age of BC presentation in males is mostly in late 60s, which is about 5-10 years greater than in female patients (Ferzoco & Ruddy, 2016).

Figure 1. Age-specific incidence rates for male and female BC (Rizzolo *et al*, 2012).



Even if BC mortality and survival rates have improved significantly over time for both sexes, some differences emerged in MBC and FBC prognosis. In fact, compared to FBC, MBC cases (MBCs) are diagnosed at a more advanced age and with a more severe clinical presentation than in women, with the consequence of a poor prognosis (Ottini, 2014; Tedaldi *et al*, 2020).

Moreover, even if at present in women BC awareness have increased, men still fail to seek attention early due lack of awareness. Although MBC often presents similarly with a lump, it has been suggested that men are more likely to dismiss the pathology as there is no associated pain and they view breasts merely as a vestigial anatomy (Co *et al*, 2020).

Overall survival rates are lower also for a delay or non-appropriate utilization of adjuvant therapies (Rizzolo *et al*, 2013; Abdelwahab, 2017; Wang *et al*, 2019; Valentini *et al*, 2024).

From an epidemiological point of view, MBC resembles postmenopausal FBC and generally MBC treatment follows the same indications as postmenopausal FBC (Khan *et al*, 2015). However, increasing evidence indicates that MBC may be different, according to clinical and pathological characteristics and molecular features, suggesting sex-specific differences in terms of biological and clinical behavior (Callari *et al*, 2011; Johansson *et al*, 2014; Piscuoglio *et al*, 2016; Gucalp *et al*, 2019; Valentini *et al*, 2024). To date, there is an urgent need to better understand the tumor subtypes of MBC, which would be crucial to improve our assessment of prognosis and our treatment recommendations for male patients (Leone *et al*, 2015).

A well assessed peculiarity of MBCs can be considered the high expression levels of hormone receptors (Losurdo *et al*, 2017). If compared with FBC, MBCs are more likely to be positive for estrogen receptor (ER+ >90% vs 76% in FBC) and progesterone receptor (PR+ >75% vs 60% in FBC) (Deb *et al*, 2016) **(Table 1)**. Similar to FBC, the percentage of men with ER+ BC significantly increases together with patient age (Rizzolo *et al*, 2012).

Information on other hormonal receptors and growth factors of interest, as the HER2 and AR, are sparser and more variable. In particular, the inconsistency of data about HER2 expression in MBC is principally due to not standardized technical approaches (Losurdo *et al*, 2017). However, most recent studies report HER2 expression in a range between 0-9% of MBCs (Shaaban *et al*, 2012; Humphries *et al*, 2017; Cardoso *et al*, 2018) (**Table 1**).
In a recent study including 144 Italian MBCs, the three-tiered categorization of HER2 (HER2-0, HER2-low, and HER2-positive) was applied, according to immunohistochemistry (IHC) results. Notably, in this study the category HER2-low was considered (Silvestri *et al*, 2024).
Studies like this are important as in clinical studies utilizing this new compound, males are either absent or represent a percentage lower than 1% (Gampenrieder *et al*, 2021; Won *et al*, 2022; Park *et al*, 2023; Peiffer *et al*, 2023).
Overall, over-expression of HER2 is less likely to be present in MBC than in FBC, where is considered a negative prognostic factor (Losurdo *et al*, 2017). Even if in men a positive association has been found between HER2 over-expression, more aggressive features, and worse outcomes, data about association between HER2 expression and survival are still lacking (Rizzolo *et al*, 2012; Esposito *et al*, 2023).

The expression of AR in MBC ranges from 39% to 95% according to various reports, while the positive rate for AR expression in FBC ranges from 57% to 65%, where the majority of FBC cases AR-positive are diagnosed in postmenopausal women and AR expression is related to ER+/PR+ status (Kornegoor *et al*, 2012; Rizzolo *et al*, 2012; Shaaban *et al*, 2012; Cardoso *et al*, 2014; Biserni *et al*, 2018) (**Table 1**). Overall, AR expression seems to be related to low malignancy in BC in both sexes (Rizzolo *et al*, 2012; Shaaban *et al*, 2012).

Ki67/MIB1 is a proliferating cell nuclear antigen, present exclusively in dividing cells. To date, the lack of standardization of assay reagents, procedures, scoring, and of a widely accepted cut-off score, with a consequent urgent need for standardization are still existing. Nevertheless, Ki67/MIB1 has been largely used as a prognostic marker in early BC and has

been part of the biological parameters used to determine surrogate definitions of intrinsic subtypes of BC (Goldhirsch *et al*, 2013; Bustreo *et al*, 2016, Finkelman *et al*, 2023). According to more recent studies, using a 20% cut-off, high expression of Ki67/MIB1 was reported in about 25-51% of MBCs (Masci *et al*, 2015; Cardoso *et al*, 2018; Ander *et al*, 2024) (**Table 1**). Moreover, most studies reported that high Ki67/MIB1 values can be associated with worse prognosis, even if data regarding the sensitivity to adjuvant chemotherapy connected to high Ki67/MIB1 are still more mixed (Jung *et al*, 2009; Liu *et al*, 2013; Varga *et al*, 2019; Finkelman *et al*, 2023). However, the possible value of Ki67/MIB1 as prognostic marker for MBC is strictly linked to the standardization of methodology and studies in larger case series are needed to establish if Ki67/MIB1 could be used to identify biological differences in MBC cases (Losurdo *et al*, 2017).

According to IHC markers, BC were divided into four molecular classes: luminal A (ER+ and/or PR+, HER2−), luminal B (ER+ and/or PR+, HER2+), basal-like/triple negative (ER−, PR−, HER2−, CK5/6+) and HER2 enriched-like (ER−, PR−, HER2+) (Perou *et al*, 2000; Kaufmann & Pusztai, 2011). Overall, according to most recent studies, the luminal A subtype is the most common profile (60-98%) in MBC, followed by the luminal B subtype that is observed in 17-25% of MBCs. Basal-like and HER2 enriched-like MBCs emerged as rarer, with a frequency of 2-4% and <1% respectively (Ge *et al*, 2009; Kornegoor *et al*, 2012; Ottini *et al*, 2012; Shaaban *et al*, 2012; Yu *et al*, 2013; Rizzolo *et al*, 2019; Ortega-Lozano *et al*, 2022) (**Table 1**). Notably, the HER2 enriched-like subtype associates with a worse prognosis in FBC, while basal-like subtype has the worst prognosis among MBCs. This may raise questions about prognosis, as well as in response to therapy, of BC in both sexes, also considering that frequency of BC subtypes seems to be different (Ottini *et al*, 2010; Ortega-Lozano *et al*, 2022).

Few years ago, one study reported the first results of the EORTC 10085/TBCRC/BIG/NABCG Male International Breast Cancer Program, a retrospective analysis of the clinical-pathologic characteristics of MBCs diagnosed between 1990 and 2010. This study represents the largest focused collection of MBC clinical characteristics and biological samples, with a total of 1483 MBCs collected. Notably, the authors found that MBC is usually ER, PR and AR-positive, Luminal B-like/HER2-negative (Cardoso *et al*, 2018). These results are consistent with those reported in a previous study, that observed HER2 over-expression in 5% of MBCs (Cardoso *et al*, 2014).

Notably, Johansson *et al*, proposed a microarray-based gene expression classification of MBCs. This analysis has revealed two molecular subgroups that do not resemble any of the intrinsic subgroups reported in FBC. These two novel subgroups of BC, luminal M1 and luminal M2, occurring exclusively in men, may consequently require novel treatment approaches specific for men (Johansson *et al*, 2012).
In addition, available data indicate that, in contrast to FBC, ERβ (the more recently identified ER type) is highly expressed in MBC, and this difference may explain the divergence of hormonal regulatory system between the two sexes (Shaaban *et al*, 2012).
Overall, these results suggest that several different hormonal dependencies in BC ER+ could exist between male and female, with the appearance of a gender-related landscape in hormone receptor pathways.

The histology of MBC is generally similar to that occurring in the female counterpart, even if the distribution of the histological subtypes is different. This difference is probably due to the anatomical structure of male mammary gland. In fact, female breast is predominantly composed of ducts, glandular epithelium organized in lobules and non-adipose stroma, while the male breast as a rudimentary structure consists of subcutaneous adipose tissue, remnant ductal tissue, and small nipple-areolar complex (Vandenberga *et al,* 2013; Fox *et al*, 2021).
The predominant histological type of BC in men is represented by invasive ductal carcinoma, which consists in about 80-90% of all male breast tumors (Fentiman *et al*, 2006; Shaaban *et al*, 2012; Bradley *et al*, 2014; Iorfida *et al*, 2014). The lobular histotype accounts only for about 1.5% of invasive cancers in males, as terminal lobules in the normal male breast are absent, unless it is exposed to high doses of endogenous and/or exogenous estrogens (Rizzolo *et al*, 2012) (**Table 1**).
However, the lobular histotype has been reported in association with Klinefelter's syndrome and, rarely, in genotypically normal men with no gynecomastia or previous history of estrogen exposure (Ottini *et al*, 2009; Fentiman, 2016; Fox *et al*, 2021).

Table 1. Summary of literature data on the main MBC pathologic features.

Histology	% Males
Invasive ductal carcinoma	80-90
In situ ductal carcinoma	10
Lobular carcinoma	1.5
Other (invasive papillary, medullary, Paget's)	7.5
Hormonal receptors and growth factors	
ER+	>90
PR+	>75
HER2+	0-9
AR+	39-95
Ki67/MIB1	25-51%
Molecular classes	
Luminal A	60-98
Luminal B	17-25
Basal-like/triple negative	2-4
HER2 enriched-like	<1

It is well known that BC is likely to be caused by the concurrent effects of different risk factors in both sexes, including hormonal, environmental and genetic risk factors (Ottini, 2014). However, the absence of reproductive components makes MBC a better model for studying some of these factors. MBC risk factors are summarized in **Table 2**.

Table 2. Summary of literature data on risk factors for MBC.

Risk Factors	Hormonal	Environmental	Genetic
Well-established	● Increased estrogen exposure ● Deficiency of testosterone ● Testicular disease	● Radiation	● BC family history ● *BRCA1/ BRCA2* ● *PALB2* ● Klinefelter's syndrome
Possible	● Obesity ● Diabetes ● Liver damage	● Exposure to electromagnetic fields	● *ATM* ● *CHEK2* ● SNPs
Suspected	● Gynecomastia	● Alcohol ● Tobacco	● Genetic syndromes (Cowden, Li-Fraumeni)

The abnormal estrogen production, due to imbalance of estrogen/androgen ratio, represents one of the major risk factors related to MBC. This imbalance may occur endogenously due to clinical disorders including testicular abnormalities or lesions, obesity, liver disease (in

particular cirrhosis) and Klinefelter's syndrome, or due to exogenous estrogen intake (Ferzoco & Ruddy, 2016; Fox *et al*, 2021).

Different testicular abnormalities and dysfunctions, mainly undescended testes, orchitis and congenital inguinal hernia have been shown to increase the risk of MBC in some studies (Giordano, 2005; Thomas, 2010).

Obesity, associated with an increased risk of postmenopausal FBC, is associated in men with decreased testosterone and sex hormone binding globulin levels, but increased estrogen levels (Masala *et al,* 2006; Rizzolo *et al*, 2012; Brinton *et al*, 2014; Ferzoco & Ruddy, 2016). Obesity might also affect risk through its correlation with gynecomastia, which relevance as an etiologic factor for MBC has been questioned (Brinton *et al,* 2014; De Blok *et al*, 2019).

Patients with Klinefelter's syndrome, characterized by a rare chromosomal abnormality (karyotype 47 XXY) and significant hormonal alterations, including high levels of estrogens and low levels of testosterone, have been shown to have over 20 to 50-fold increases in MBC risk. Men affected by this syndrome often have gynecomastia and received BC diagnosis earlier, with an average age at diagnosis of 58 years (Weiss *et al,* 2005; Ferzoco & Ruddy, 2016, Fox *et al*, 2021).

Long-term use of antiandrogens and estrogens in the treatment of prostate cancer, exogenous administration of estrogen to trans-sexual or abuse of steroids for physical performances have also been implicated as causative factors for MBC, since conditions increasing exposure to estrogen or decreasing exposure to androgen (Karamanakos *et al,* 2004; De Blok *et al*, 2019; Valentini *et al,* 2024).

Occupational activity and occupational exposure to heat and electromagnetic radiation have been considered as possible causal cofactors in the etiology of BC in both sexes (Ottini *et al*, 2010). In particular, several studies showed elevated MBC risks among men exposed to either high temperatures or polycyclic aromatic hydrocarbons. Exposure to high temperatures seems to cause damage to the testicles, that lead to altered androgens and estrogens circulating levels (Hansen, 2000; Villeneuve *et al,* 2010; Sun *et al*, 2013).

Moreover, MBC risk seems to be higher in men belonging to professional groups exposed to exhaust gases, in particular subjects carrying pathogenic variants (PVs) in BC susceptibility genes, such as *BRCA1* and *BRCA2* (*BRCA*) (Palli *et al,* 2004).

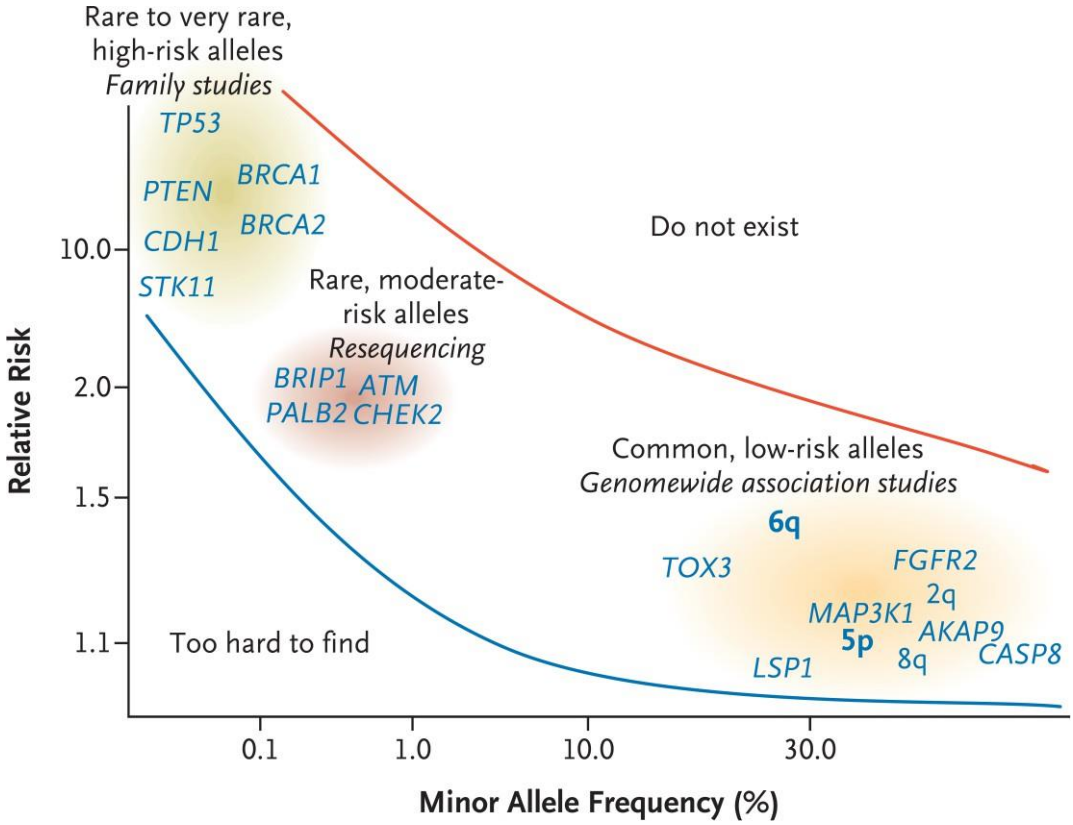
Some lifestyle factors have also been possibly involved in the etiology of MBC, including heavy alcohol consumption, that seems to represent a risk factor for the development of BC in both sexes, closely related to liver disease that can lead to hyperestrogenism (Speirs and Shaaban, 2008; Ottini *et al*, 2010).
In addition, one study reported that MBC patients with smoking habits seemed to have significantly reduced survival, which was worse by intensity of smoking, in comparison with never-smokers (Padron-Monedero *et al*, 2015).

Family history seems to play key role in MBC genetic susceptibility. A family history of BC, particularly in first-degree relatives, is considered the major MBC predisposition factor (Rosenblatt *et al*, 1991; Giordano *et al*, 2002; Korde *et al*, 2010; Brinton *et al*, 2008; Deb *et al*, 2016; Reddington *et al*, 2020). Specifically, a positive family history of BC in first-degree relatives, reported in about 20% of MBCs, even if, to date, screening mammography is not recommended for men with family history of BC (Ottini, 2014; Deb *et al*, 2016; Ferzoco & Ruddy, 2016; Reddington *et al*, 2020, Zheng & Leone, 2022). About 2% of patients with MBC develop a second primary BC and more than 20% of patients develop a second non-breast tumor, mainly prostate, colon and genitourinary cancers (Rizzolo *et al*, 2019; Sarmiento *et al*, 2020; Silvestri *et al*, 2020).
In addition to this, a high percentage of MBC patients without *BRCA* PVs was shown to have a positive family history of BC (Calip *et al*, 2021; Valentini *et al*, 2024). Studies enriched for high-risk MBC cases (*i.e.*, early onset BC, and/or cases with a family history of BC) showed a higher *PALB2* PV frequency. Overall, a higher frequency of *PALB2* PVs in high-risk MBC cases than in high-risk FBC cases was observed (4% vs. 1%) (Silvestri *et al*, 2017; Valentini *et al*, 2024).

Genetic landscape of male breast cancer

Genetic risk factors play a key role in MBC susceptibility. Genetic architecture of BC is similar between the two sexes. As shown in **Figure 2,** by their mutation frequency and the magnitude of their impact in BC susceptibility, susceptibility genes can be divided into high-moderate-, and low-penetrance genes (Rizzolo *et al,* 2013). Variants in high-penetrance genes are rarely found in general population (about 0.7%) and are associated with a higher risk of BC than rare variants in moderate-penetrance genes; low-penetrance variants are considered as polymorphism in general population and can help explaining the genetic component of sporadic tumors (Abul-Husn *et al*, 2019).

Figure 2. Breast cancer genetic susceptibility (Foulkes, 2008).



BRCA1 and *BRCA2* are the most important high-penetrance BC susceptibility genes.

PVs in *BRCA2* gene are estimated to be responsible for 60%–76% of MBCs occurring in high-risk BC families, whereas PV *BRCA1* frequency ranges from 10% to 16% (Frank *et al,* 2002; Rizzolo *et al*, 2013).

The role of *BRCA* PVs in BC susceptibility is significantly different between two sexes. To date, *BRCA1* resulted mainly involved in females while *BRCA2* in males (Rizzolo *et al*, 2013; Silvestri *et al*, 2020; Li *et al*, 2022). PVs in *BRCA2* are often found in patients with MBC who have family history of multiple cases of BC/ovarian cancer (OC), but they have also been found in patients with MBC without family history (Ottini, 2014; Valentini *et al*, 2024).

Overall, about 15% of all MBCs unselected for family history are caused by inherited germline PVs in *BRCA* genes. In the Italian population, a frequency of 13% and 2% of all MBCs has been observed for PVs in *BRCA2* and *BRCA1*, respectively (Ottini et al, 2012; Rizzolo et al, 2013).

The estimated lifetime risk of MBC is about 4% in *BRCA2* and about 0.4% in *BRCA1* PV carriers, compared with a risk of 0.1% of developing MBC in the general population (Li *et al*, 2022; Valentini *et al*, 2024). Furthermore, the median age at BC diagnosis among male *BRCA2* PV carriers is earlier (median, 58.8 yrs) than in MBCs *BRCA2*-negative (median, 67.9 yrs) (Rizzolo *et al*, 2012; Silvestri *et al*, 2020).

Male *BRCA1* and *BRCA2* mutation carriers are also at increased risk of developing several cancer types, including gastric, prostate and pancreatic carcinomas (Rizzolo *et al*, 2012; Li *et al*, 2022).

In MBC cases not selected for family history, *BRCA2* PVs are reported in a range of 4-40% of all cases.

In particular, highest frequencies are observed in specific genetically isolated populations, where mutations are descendent from a single founder. Founder mutations may also explain variability in BC incidence rates among countries (Frank *et al*, 2002; Liede *et al*, 2004; Deb *et al*, 2012; Tung *et al*, 2015; Susswein *et al*, 2016). On the contrary, *BRCA1* mutations are generally quite rare in unselected MBC cases, being more frequent in specific populations in which a founder effect is known to occur (Giordano, 2005).

BRCA large-scale rearrangements, including insertions, deletions, or duplications of more than 500 kb of DNA, have been also identified in both male and female BC patients (Karhu *et al*, 2006; Capalbo *et al*, 2007; Hansen *et al*, 2009; Sluiter & van Rensburg, 2011). Interestingly, large genomic rearrangements in *BRCA2* are more frequent in families with MBC (Tournier *et al*, 2004; Hansen *et al*, 2009) and, on the other hand, both *BRCA1* and

BRCA2 rearrangements are infrequent in MBC cases unselected for family history (Falchetti *et al*, 2008).

It is now well established that FBC *BRCA*-associated tend to manifest specific genotype–phenotype correlations (Honrado *et al*, 2006; Bane *et al*, 2006; Da Silva & Lakhani, 2010; Vargas *et al*, 2011; Mavaddat *et al*, 2012).

Notably, it was demonstrated that *BRCA2*-related MBCs represent a subgroup of tumors with specific phenotypic characteristics, not identified in the female counterpart, indicative of aggressive behavior, mainly characterized by higher grade (Silvestri *et al*, 2016).

Notably, a high percentage of BC patients without *BRCA* PVs was shown to have a positive family history of BC, suggesting the existence of other susceptibility factors (Calip *et al*, 2021).

The wide use of Next Generation Sequencing (NGS) in molecular oncology has allowed the simultaneous sequencing of several genes through personalized multigene panels, in many patients, at a cost comparable to that of a single gene test. This has taken to the discovery of numerous genes previously unknown involved in the onset and development of cancer, allowing the identification of new possible predictive, prognostic and therapeutic targets for patients (Walsh *et al*, 2010; Walsh *et al*, 2011; Catana *et al*, 2019; Neben *et al*, 2019; Rizzolo *et al*, 2019; Valentini *et al*, 2023).

To date, many studies make use of NGS multigene panels to identify genetic predisposition in patients with different types of cancer, or in individuals with family history of hereditary cancer syndromes not yet affected (Easton *et al*, 2015; Rey *et al*, 2017; Dong *et al*, 2018; Paulo *et al*, 2018).

To the best of our knowledge, literature is ample on surveillance and other risk reduction strategies for women with high risk of developing BC (Amir *et al*, 2010; Kleibl & Kristensen, 2016; Mouelle *et al*, 2023).

To date, a few numbers of studies with multigene panel approach have been conducted on MBCs, with the aim to to identify other genes associated with MBC predisposition in order to better define the genetic susceptibility landscape in addition to *BRCA* genes, even if men affected by BC may be in the same genetic condition (Pritzlaff *et al*, 2017; Fostira *et al*, 2018; Rizzolo *et al*, 2019; Scarpitta *et al*, 2019; Tedaldi *et al*, 2020; Rolfes *et al*, 2022; Barnes *et al*, 2022; Bucalo *et al*, 2023).

The studies conducted on MBC patients have made it possible the identification of PVs in a range of 5-8% MBCs in different genes, mainly including *PALB2, ATM* and *CHEK2* genes. These studies included from 70 to 767 MBC cases and from 10 to 94 genes. (Pritzlaff *et al*, 2017; Neben *et al*, 2019; Rizzolo *et al*, 2019; Tedaldi *et al*, 2020; Bucalo *et al*, 2023). In line with this, one our recent study highlighted the importance of developing case-control studies with cases and controls taken from the same geographical area, with the aim to obtain more precise estimates of MBC risk associated with PVs in specific populations (Bucalo *et al*, 2023).

CHEK2 and *PALB2* are considered the most important moderate-penetrance MBC susceptibility genes.

PALB2 mutations in MBC cases gave been investigated through several studies, showing a mutation frequency between 0.9% and 16% (Silvestri *et al*, 2010; Adank *et al*, 2011; Ding *et al*, 2011; Vietri *et al*, 2015; Silvestri *et al*, 2017; Rizzolo *et al*, 2019; Scarpitta *et al*, 2019; Tedaldi *et al*, 2020; Rolfes *et al*, 2022; Bucalo *et al*, 2023). These studies highlighted the importance of *PALB2* in MBC genetic susceptibility, suggesting that *PALB2* may be the third gene involved in MBC hereditary susceptibility, after *BRCA* genes (Antoniou *et al*, 2014; Silvestri *et al*, 2017; Bucalo *et al*, 2023).

Even if *PALB2* mutations were found in families with both female and male BCs, some study reported a higher frequency of *PALB2* PVs in high-risk MBC cases than that observed in high-risk FBC cases (4% vs 1%) (Rahman *et al*, 2007; Garcia *et al*, 2009; Silvestri *et al*, 2017). Moreover, *PALB2* heterozygotes had a 4-fold increased risk to have a male relative with BC (Casadei *et al*, 2011).

In addition to BC, *PALB2* mutations were frequently observed in families with cases of melanoma, pancreatic, prostate, lung, and stomach cancer (Adank *et al*, 2011; Ding *et al*, 2011; Blanco *et al*, 2012; Lu *et al*, 2015; Thompson *et al*, 2015; Silvestri *et al*, 2017).

One study reported that MBC relative risk related to *PALB2* PVs was about 7-fold increased if compared to general population, with an absolute risk of developing MBC to age 80 years of 0.9% (Yang *et al*, 2020).

In addition, *CHEK2* is also associated with MBC risk. In particular, *CHEK2* variant c.1100delC has been initially shown to confer approximately a 10-fold increase of BC risk in men without *BRCA* mutations, estimating to account for 9% of familial high-risk MBC

cases (Meijers-Heijboer *et al,* 2002; Rizzolo *et al,* 2013). However, this association is not so evident in other studies comprehending MBCs unselected for family history, suggesting that the contribution of *CHEK2* variant c.1100delC to MBC predisposition may variate by ethnic group and/or from country to country, with a decreased frequency of the c.1100delC allele from North to South orientation observed in Europe (Syrjäkoski *et al,* 2004; Falchetti *et al,* 2008; Wasielewski *et al,* 2009; Hallamies *et al,* 2017; Bucalo *et al,* 2023).

PVs in other genes have been identified in a small group of families with MBCs, but their contribution to MBC susceptibility remains to be assessed.

Among these genes are identified: *ATM,* gene involved in DNA repair (Hall *et al,* 2021); *FANCM* and *RECQL,* identified in whole-exome sequencing (WES) analysis of MBCs, are genes involved in the mechanism of protection from DNA damage, (Kiiski *et al,* 2014; Silvestri *et al,* 2018; Rizzolo *et al,* 2019); *BRIP1, RAD51C* and *RAD51D,* which seems play an important role in genetic susceptibility to OC (Silvestri *et al,* 2011a; Silvestri *et al,* 2011b; Rizzolo *et al,* 2019).

Furthermore, one study that evaluated the association between MBC and PVs in the *MUTYH* gene (involved in DNA repair), showed that *MUTYH* could be considered a low/moderate risk gene for MBC (Rizzolo *et al,* 2019).

MBC cases have been observed in subjects with Li-Fraumeni, Cowden and Lynch syndromes, caused by PVs in *TP53, PTEN* and mismatch repair genes respectively (Boyd *et al,* 1999; Fackenthal *et al,* 2001; Ottini *et al,* 2009; Pritzlaff *et al,* 2017). However, due to the rarity of these cancer syndromes, the correlation between these diseases and MBC needs to be further investigated, also considering that lifetime risk in these syndromes (at the present 40-80%) could be overestimated due to rarity of these conditions (Boyd *et al,* 1999; Fackenthal *et al,* 2001; Ottini *et al,* 2009; Imyanitov *et al* 2023).

Generally, it is assumed that about 15-20% of all MBC cases can be attributed to germline PVs in know genes involved in MBC susceptibility (Silvestri *et al,* 2017; Bucalo *et al,* 2023). To date, about 80-85% of MBCs are not explained by known genetic alterations, with this missing heritability that needs to be further investigated.

To explain the contribution of the genetic component in MBCs not characterized by specific PVs in BC susceptibility genes, a polygenic model, already used for other tumors, has been proposed. According to this model, in many genes are present polymorphic variants that give a low risk for MBC development if acting individually, but which constitute a greater risk if they are present in combination (Pharoah *et al*, 2002). This hypothesis, confirmed by Genome Wide Association Studies (GWAS), partially explained MBC cases not currently associated with PVs in known cancer susceptibility genes (BCAC, 2006; Easton *et al*, 2007; Turnbull *et al*, 2010; Ghoussaini *et al*, 2012). GWAS have identified over 300 single nucleotide polymorphisms (SNPs), common genetic variants associated with BC in both sexes (Fanale *et al*, 2012; Orr *et al*, 2012, Maguire *et al*, 2021). Some studies have evaluated the role of low-penetrance variants in MBC susceptibility through GWAS or through genotyping of candidate SNPs (Orr *et al*, 2011; Orr *et al*, 2012; Ottini *et al*, 2013; Silvestri *et al*, 2015; Lecarpentier *et al*, 2017; Maguire *et al*, 2021; Barnes *et al*, 2022). Recently, a Polygenic Risk Score (PRS) including 313 SNPs, has been developed and validated for the estimation of BC risk in European women (Mavaddat *et al*, 2019). PRS models developed for FBC have also been evaluated for BC in males, revealing similar distribution compared to FBC cases that suggest a genetic architecture of BC shared in both sexes (Lecarpentier *et al*, 2017; Maguire *et al*, 2021; Barnes *et al*, 2022). Specifically, PRS at 313-SNP was associated with the risk for both female and male BC of carrying PVs in *BRCA* genes, suggesting that PRS-based genetic profiling can provide additional stratification of individual cancer risk in an already high-risk population, with implications for their clinical management (Lecarpentier *et al*, 2017; Barnes *et al*, 2022).

Somatic landscape of male breast cancer

Considering their characteristics, NGS technologies can be used in several applications, including discovery of somatic alterations in oncology (McCarthy *et al*, 2013; Valentini *et al*, 2023).

The development of NGS technologies has produced a large amount of research data about genomic alterations in a huge variety of cancers, including BC (Vestergaard *et al*, 2021).

While therapies based on NGS findings have become a new standard of care for treating a variety of cancers (Karapetis *et al*, 2008; Chapman *et al*, 2011; Douillard *et al*, 2013; Magliacane *et al*, 2015), only a few studies have been performed to comprehensively characterize tumor profiles in MBC cases, with a consequent lack of data on specific molecular biomarkers in MBC. Due to this, associations among new potential molecular biomarkers and clinical data remain unclear in MBC, cutting out male patients from new targeted treatments (Piscuoglio *et al*, 2016; Moelans *et al*, 2019; Campos *et al*, 2021; Castaneda *et al*, 2021; Valentini *et al* 2023).

To date, different studies reported *PIK3CA* as the most frequently mutated gene, in the frame of MBCs targetable somatic alterations (Castaneda *et al*, 2021; Valentini *et al*, 2023).

In order to potentially improve the identification of molecular biomarkers in male breast tumors, one strategy could be studying a precise group of MBC patients, with personal history (PH) of multiple primary tumors. In this context, the use of NGS techniques for the detection of somatic variants in cancer, has great potential to improve patient diagnosis, prognosis, and treatment based on identified tumor variants (Misyura *et al*, 2016).

Moreover, characterization of the molecular profile tumor-specific allows to customize the therapies in order to choose the best therapeutic approach using selective biological drugs that act on the single altered molecules, through the inhibition of specific oncogenic pathways (targeted therapy) (Valentini *et al*, 2023). An extensive molecular tumor profiling, exploring not only MBC landscape, but also multiple primary malignancies arising in MBC patients, will facilitate the identification of possible new actionable somatic alterations and predictive genomic signatures. This could help in finding molecular biomarkers predictive of response to innovative treatments, for a more effective clinical management of MBC patients (van Vugt & Parkes, 2022).

WHOLE EXOME SEQUENCING

Since the early 2000s, NGS instruments have begun a more valid DNA sequencing technology compared to Sanger sequencing (Sanger *et al*, 1977), thanks to the improvement of the speed and the efficiency of DNA sequencing (Feliubadalo *et al*, 2013; Castéra *et al*, 2014; Yeo *et al*, 2014; Trujillano *et al*, 2015). Different technological platforms based on this new sequencing system have been developed, implementing a real revolution in the field of nucleic acid sequencing in terms of quantity of data produced, with a consequent reduction in the sample processing costs and, due to this, production of significant technical advances in molecular biology (Metzker, 2010).

Considering their characteristics, NGS technologies can be used in several applications, with the main goal of helping the biomedical and biological research (Lee *et al*, 2013).

Considering the different type of applications, we could use different NGS approaches. Indeed, we can use gene panel approach to analyze specific genomic regions of interest, WES or whole-genome sequencing.

Focusing on WES, it represents a useful high-throughput technology that allows the sequencing of the 1% to 2% protein-coding subset of the human genome and is considered to be a powerful tool for medical genetic studies (Ng *et al*, 2009).

WES is considered a significant breakthrough in the field of human genetics, with its large contribute to the identification of new disease-causing genes. Different exome sequencing projects have not only provided crucial information on variant frequencies in different populations but have also highlighted the role of the about 100 genuine loss-of-function variants found in the human genome, completely inactivating around 20 genes (MacArthur *et al*, 2012; Lek *et al*, 2016; Narasimhan *et al*, 2016).

To date, WES and genome sequencing techniques are now involved worldwide in the field of molecular diagnosis (Matthijs *et al*, 2016; Hartman *et al*, 2019; Marshall *et al*, 2020).

As an example, WES already has led to the detection of mutations that can be involved in some rare familial syndromes (Danielsson *et al*, 2014; Lapunzina *et al*, 2014; Rabbani *et al*, 2014; Zhang, 2014). Moreover, WES has proven to be very performing in the identification of genes that cause rare Mendelian diseases (Ku *et al*, 2011; Snape *et al*, 2012).

Whole exome sequencing in cancer

Recently, WES has emerged as a powerful tool for exploring the extent to which rare mutations may explain the heritability of complex diseases, including several types of cancer (Snape *et al*, 2012; Sokolenko *et al*, 2015; Silvestri *et al*, 2017).

To date, even if multigene panel studies have allowed a step forward in the field of MBC with the identification of additional MBC susceptibility genes and accurate risk estimates, there is still a fraction of MBC patients that deserve to be further investigated in order to proceed with the gender-specific precision prevention approach.

According to this, in the field of cancer research, during the last years WES technology allowed the discovering of previously unknown cancer-related genes (Stadler *et al*, 2014), or potentially new roles of known genes as, for example, *PALB2*, a well-known BC gene, that was discovered in a WES study involved also in familial aggregation of pancreatic cancer (Jones *et al*, 2009). In support of this, one study performing whole-genome sequencing on multiple primary cancers allowed the identification of a novel germline *PALB2* structural variant, reported also at somatic level (Schrader *et al*, 2016).

During last years, some studies have used WES to identify genetic predisposition in patients with different types of cancer, including BC in both sexes (Silvestri *et al*, 2017; Felicio *et al*, 2021; Hao *et al*, 2021). To date, some studies have used WES also to identify somatic mutations on tumor samples in several types of cancer, also on multiple tumors arising in the same patient (Wang *et al*, 2020; Xue *et al*, 2020).

One recent study applied an in-house pipeline on WES datasets with familial/hereditary BC patients without *BRCA* germline mutations, in order to try to explain the about 75% of familial BC cases still unexplained at the genetic level (Bianchi *et al*, 2024).

Even if WES technique applied on cancer research is giving promising results, further studies are still necessary to give a better understanding of BC etiology, especially in male patients. With this in mind, some studies evidenced the importance of performing sequencing of both germline and tumor samples of the same patient. The matched germline and tumor sequencing could be helpful in pinpointing genetic bases of suspected predisposition to BC in patients without PVs in established cancer-genes (Van Marcke *et al*, 2020). This approach acquired increasing importance especially for rare diseases as MBC, for which the current lack of knowledge could also be associated to the difficult to have large- sample-size studies. If, with the advent of NGS technologies, germline PV testing

gave information genetic susceptibility in many different cancer types, at the same time somatic tumor testing in oncology with the purpose of guiding therapeutic decisions for targeted therapies was also extensively performed. The hypothesis that an integrated approach may be the best approach for the optimal management of patients with different cancers is currently arising.

Moreover, if tumor-only sequencing was performed, without the match with the correspondent blood sample sequencing, the identification of both somatic and germline variants will be displayed together. One the other hand, a simultaneous sequencing of both normal and tumor tissues is capable to discriminate germline from somatic alterations, through the subtraction of the first form the second one (Van Marcke *et al*, 2020; Yap *et al*, 2023).

AIMS

Inherited PVs in *BRCA* genes account for approximately 15% of all MBC cases. Recent studies have shown that PVs in non-*BRCA* genes contribute an additional 5% of MBC cases. The personal and family histories of tumors in non-*BRCA* MBC cases suggest a "missing heritability", that warrants further investigation.

WES of multiple primary tumors from the same MBC patient offers a powerful approach to uncover potential genetic susceptibilities not only to MBC but also to other cancer types. Additionally, applying WES to explore the somatic mutational landscape of MBC and other cancers arising in the same patient may provide valuable insights into shared somatic alterations. The combined characterization of both germline and somatic mutations can refine our understanding of MBC genetic susceptibility while also profiling the distinct molecular features of different tumors.

Based on this rationale, the present study aims to match germline and tumor exome sequencing data from MBC cases with multiple primary tumors in order to:

- Identify novel candidate genes potentially associated with MBC genetic susceptibility, yielding a comprehensive understanding of genetic alterations across multiple malignancies.
- Characterize the somatic mutational landscape of MBC and other cancers, exploiting as new, actionable molecular targets for therapeutic intervention.

METHODS

STUDY POPULATION

The present study was focused on selected series of MBC patients with at least one additional primary tumor, tested negative by gene-panel analysis, selected as carrying an important risk of genetic susceptibility. All MBC cases were enrolled in the frame of the Italian Multicenter Study on MBC (Rizzolo *et al,* 2019; Bucalo *et al,* 2023).

Each MBC case had at least one additional tumor among eight cancer types, including stomach, prostate, colon, kidney, lung, skin, tongue, and urinary bladder cancer.

Blood sample was available for all cases, as multiple tumor sample; male breast tumor sample was available for the majority of MBC cases (8/13, 61.5%).

A total of 13 blood samples and 22 tumor samples (including eight MBC, four stomach and four prostate cancer, and one colon, kidney, lung, skin, tongue, and urinary bladder cancer, each) were collected from the 13 MBC cases, for a total of 35 samples (**Table 3)**. For each study participant, informed consent together with samples of blood and 10 μm-thick macroscopically dissected formalin fixed paraffin-embedded (FFPE) tumor sections were obtained.

All cases have been characterized for the main clinical features, including age at diagnosis, family history and PH of cancer. For breast tumors, histological type, stage (TNM classification), grade, estrogen and progesterone receptor (ER/PR), and HER2 expression were also available. In particular, the expression of ER and PR was scored based on the percentages of positive nuclei (positive if >10%) over the total number of counted cancer cell nuclei, whereas HER2 positivity was defined as a score of 3+ using IHC test, or amplification shown by fluorescence in situ hybridization, in equivocal cases (Ottini *et al* 2003; Bianchi *et al* 2006). Additional information on lifestyle habits, as working occupation, smoking and alcohol consumption, was collected.

Table 3. Distribution of multiple tumors among the series included in this study.

Sample ID	Multiple tumor(s) site
#1	Stomach
#2	Stomach
#3	Stomach, Kidney
#4	Stomach
#5	Prostate
#6	Prostate
#7	Prostate
#8	Prostate
#9	Colon
#10	Skin
#11	Urinary bladder
#12	Lung
#13	Tongue

DNA EXTRACTION FROM BLOOD AND FFPE SAMPLES

DNA extraction from blood and FFPE samples was performed using commercial kits. The kits use chromatographic columns containing a resin that acts as a separation matrix to which DNA binds. This method allows separation from other cellular components without the use of phenol or chloroform.

The procedure is performed at room temperature, using gloves, filter tips and DNAse-free plastic (contained in the kit). DNA extraction was performed in a room fully equipped in order to avoid contamination. Extracted DNA can be stored at -20°C for several months.

DNA extraction from blood samples

Before DNA extraction, blood samples were thoroughly mixed for at least 10 minutes in a rotisserie shaker at room temperature.

DNA from blood was extracted using ReliaPrep Blood gDNA Miniprep System (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. No ethanol was used in the protocol, eliminating downstream problems caused by ethanol carryover.

One 200 µl aliquot of blood sample was used for extraction. To these aliquots, 20 µl of Proteinase K (PK) Solution and 200 µl of Cell Lysis Buffer (CLD) were added for the lysate preparation. Subsequently, 250 µl of Binding Buffer (BBA) was added, then the contents of the tubes were transferred to special separation columns (ReliaPrep™ Binding Column) provided by the kit, for the DNA purification from the lysate. This was followed by a series of steps involving column washes using Column Wash Solution (CWD), steps repeated several times to remove any impurities. Finally, DNA elution was performed by the addition of 50 µl of Nuclease-Free Water to the column to obtain 50 µl of eluted volume. After DNA extraction, the DNA concentration of each sample was quantified with QubitFlex 4.0 using the Qubit dsDNA BR Assay Kit Fluorometer (Life Technologies, Carlsbad, California, USA).

For all samples enough quantity and good quality of DNA were available to perform molecular analysis.

DNA extraction from FFPE samples

10 µm-thick FFPE tumor sections were used for DNA extraction. After deparaffination of the sections with xylene, DNA from tumor samples was extracted using QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) respectively, according to the manufacturer's instructions.

The deparaffinised sample, immersed in 180 µl of Buffer ATL, was placed in a test tube to which 20 uL of proteinase K is added. The samples were first placed at 56°C overnight and the next day at 90°C to dissolve the paraffin. This is followed by the addition of 200 ul of Buffer AL and 200 ul of 96-100% ethanol. Subsequently, the contents of the tubes were transferred to special separation columns (QIAamp MinElute column) provided by the kit for the DNA purification. This was followed by a series of steps involving the addition of Buffers AW1 and AW2 repeated several times to remove any impurities. Finally, DNA elution is performed by the addition of 20-30 ul of Buffer ATE to the column to obtain 20-30 ul of eluted volume. After DNA extraction, the DNA concentration of each sample was quantified with QubitFlex 4.0 using the Qubit dsDNA BR Assay Kit Fluorometer (Life Technologies, Carlsbad, California, USA). For all samples enough quantity and good quality of DNA were available to perform molecular analysis.

NGS ANALYSIS

Library preparation and whole exome sequencing

Starting from a minimum of 50 ng input purified genomic DNA, indexed libraries were prepared using the (size 45Mb) kit (Illumina).

Library preparation workflow is represented in **Figure 3.**

Figure 3. DNA Prep with Enrichment with Exome Panel Workflow.

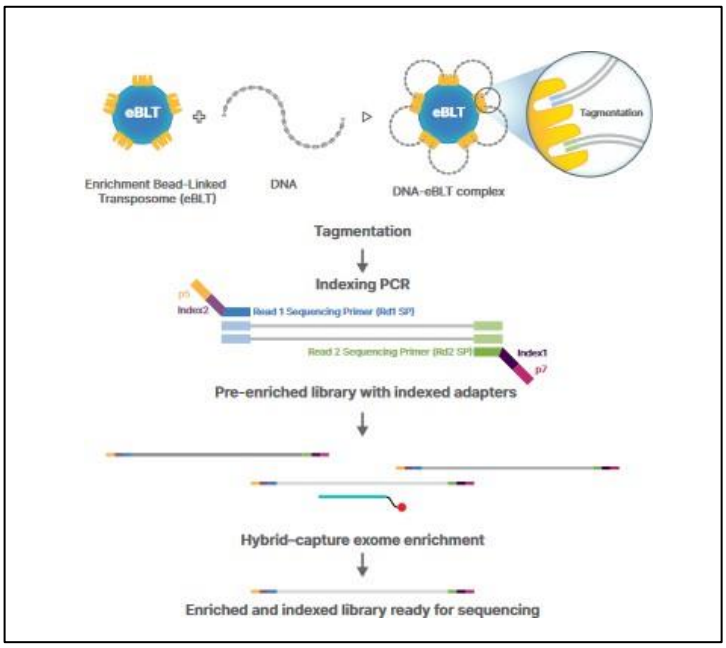
The Exome Panel-based library preparation generated adapter-tagged libraries thanks to a process of tagmentation of DNA, by transposomes, that simultaneously fragmented and tagged DNA with adapter sequences without the need for mechanical shearing.

After a cleanup step to purify the tagmented DNA from the transposomes, that can otherwise bind to DNA ends and interfere with downstream processes, tagmented DNA was amplified and index adapters were added using PCR program.

A further cleanup step to purify the DNA library and remove unwanted products, was performed after the amplification. Integrated sample barcodes allowed the pooling of these adapter ligated sample libraries into a single, hybridization based, pulldown reaction.

The pooled libraries were then denatured into single-stranded DNA and biotin-labeled probes, complementary to the targeted regions, are used for the hybridization. Streptavidin beads, which bind to the biotinylated probes that are hybridized to the targeted regions, were then added and the magnetic pulldown of the streptavidin beads enriched the targeted regions that were hybridized to biotinylated probes (**Figure 4**). Two heated washes to remove nonspecific binding from the beads were also performed. Two cleanup steps to purify the library before and after the last PCR amplification were performed.

Figure 4. Tagmentation and hybridization of biotinylated probes to targeted regions and capture using streptavidin beads.

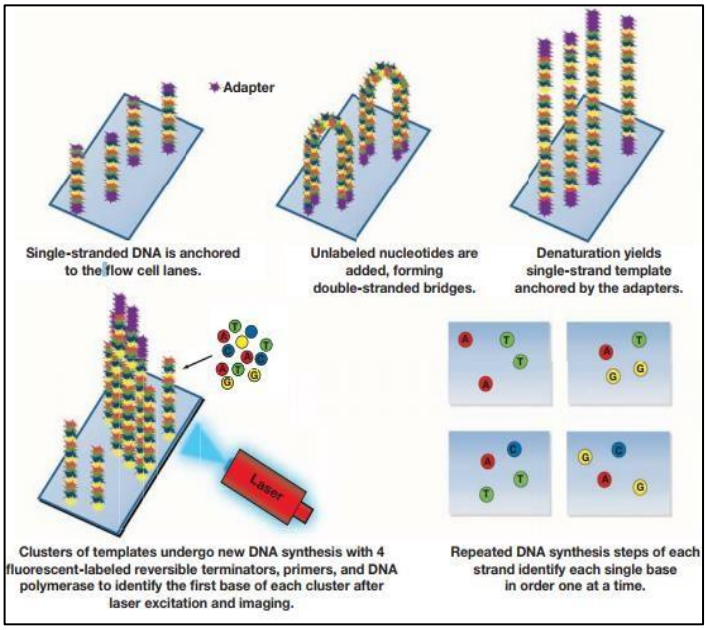


In order to assess the yield, all pooled libraries were quantified using the TapeStation 4200 and QubitFlex fluorometer, then pooled such that each index-tagged sample was present in equimolar amounts, with final concentration of the pooled samples of 5nM. In each experiment, 500µl of pooled libraries were loaded into a specific Reagent Cartridge, in turn loaded into the NovaSeq 6000 system (Illumina) for sequencing.

The pooled samples were subject to cluster generation and sequencing using a Novaseq6000 System (Illumina) in a 2x150 paired-end format and achieved approximately 50X for germline samples and 100X coverage for somatic samples.

Illumina sequencing technology leverages a process of clonal amplification, called bridge PCR, and a system of sequencing by synthesis with reversible terminators for rapid and accurate large-scale sequencing (**Figure 5**). Target DNA fragments, as single-stranded templates, were randomly immobilized on flow cell surface, and unlabelled nucleotides and enzyme to initiate solid-phase bridge amplification were added. The enzyme incorporated nucleotides to build double-stranded bridges on the solid-phase substrate that were then denaturated leaving single-stranded templates anchored to the substrate. This type of amplification allowed to obtain clusters of up to 1000 identical copies of each single template molecule in close proximity. Sequencing by synthesis technology used fluorescently labelled nucleotides to sequence all clusters on the flow cell surface in parallel. During each sequencing cycle, the labelled nucleotides, primers and DNA polymerase were added. The nucleotide label served as a terminator for polymerization, so after each incorporation and laser excitation, the fluorescent dye was imaged to identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide. The sequencing cycles were repeated to determine the sequence of bases in a fragment, one base at a time. Base calls are made directly from signal intensity measurements during each cycle. Thus, the Illumina sequencing approach is built around a massive quantity of sequence reads in parallel.

Figure 5. Illumina sequencing technology.



Germline and somatic variant calling, annotation and classification

The raw sequence files generated (.fastq files) were analyzed with DRAGEN Enrichment pipeline (Illumina) that is an accurate and efficient end-to-end (FASTQ to VCF) secondary analysis solution for whole exome NGS data; it can be used for both germline and somatic variant calling.

Paired-end reads were aligned to the NCBI reference sequence (GRCh37/hg19), and these results were then refined by running full Smith-Waterman alignments on the locations with the highest density of seed matches.

For somatic samples, in order to avoid calls at sites with germline variants and allow sensitive detection of low-frequency alleles, tumor/normal pairs analysis was further performed using DRAGEN Somatic pipeline.

The generated .vcf files for both germline and somatic samples were annotated using OpenCRAVAT tool (https://www.opencravat.org/).

For variant classification, an *ad hoc* in-house filtering pipeline was used to identify germline pathogenic variants and somatic driver variants. In particular, to identify candidate germline PVs, all PASS variants were filtered to include exonic non-synonymous or splice site variants with variant allele frequency between 30% and 70%, total read depth ≥ 20 and a global allele frequency < 1% in the gnomAD database. For the detection of driver somatic variants, the filters applied to germline PVs identification were used, with exceptions of variant allele frequency between 5% and 90%, total read depth ≥ 40, variants classified as somatic in the Catalogue Of Somatic Mutations In Cancer (COSMIC) database (https://cancer.sanger.ac.uk/cosmic), and variants with a REVEL score ≥ 0.6. COSMIC database was interrogated to determine if the identified driver somatic variants could be clinically actionable. Variants were named according to Human Genome Variation Society nomenclature (HGVS, https://www.hgvs.org/).

Shared molecular pathways characterization and statistical analysis

To perform enriched-pathway analysis intra-patient, the mutated genes identified in different tumors arisen in the same MBC patient were compared. To perform this type of analyses, we considered all the rare heterozygous variants identified in somatic samples.

Enriched pathway-based analysis based on mutated genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) in order to determine the biological relevance of mutated genes within the considered groups. To understand the function of hub genes in the key modules, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were used. Only the terms considered as the most representative and interesting among the significant ones are reported.

For enriched pathway-based analysis the p-values were corrected to false discovery rate (FDR) following the Benjamini-Hochberg method, considering FDR < 0.05 as a statistically significant difference.

RESULTS

CLINICAL-PATHOLOGIC CHARACTERISTICS AND LIFESTYLE HABITS OF MALE BREAST CANCER CASES

The present study included a well-characterized series of 13 MBC cases, all enrolled in the frame of the ongoing Italian multicenter study on MBC (Bucalo *et al*, 2023).

The main clinical and pathologic features are reported in **Table 4**.

Overall, mean age at BC diagnosis was 67.2 years (range 52-91 yrs, ±10.7). On the contrary, mean age at second primary tumor diagnosis was 72.5 years (range 56-91 yrs, ±10.0).

One case out of 13 (7.7%, case #3) had two additional primary tumors beyond MBC, including stomach and kidney cancer.

Four MBCs (30.8%) reported first-degree family history of BC/OC and six out of 13 (46.1%) had a first-degree family history of cancer at sites other than BC/OC.

Most male breast tumors were invasive ductal carcinomas (92.3%), at early stage (50.0%), severe grade (41.7%), estrogen and progesterone receptor positive and HER2 negative (76.9%, 84.6% and 92.3% respectively). Two out of 13 BC were triple negative BC (15.4%).

Moreover, the main information about lifestyle habits were available for 12 of the 13 (92.3%) MBC cases included in this study.

According to smoking habits, six cases out of 12 (50.0%) were non-smoker, one out of 12 (8.3%) was smoker and five out of 12 (41.7%) were classified as ex-smoker. According to alcohol consumption, seven out of 12 (58.3%) were alcohol consumers.

Table 4. Clinical-pathologic features of the MBC cases included in this study.

Sample ID	Age at diagnosis		Cancers in first degree relatives (age)	Pathologic characteristics of BC				Pathologic characteristics of other tumor(s)*
	MBC	Other primary tumors (type)		Tumor histotype	TNM stage*	Histologic grade*	ER/PR/HER2 status	
#1	63	86 (Stomach)	Ovary (62), Colorectal	IDC	2	3	+/+/+	Adenocarcinoma, NOS
#2	62	75 (Stomach)	-	IDC	1	3	-/-/-	Adenocarcinoma, NOS
#3	78 (contralateral)	76 (Kidney), 80 (Stomach)	-	IDC	2	1	-/+/-	Renal cell carcinoma
#4	70	74 (Stomach)	Stomach (2 relatives)	IDC	1	3	+/+/-	Intestinal type (T4, N2)
#5	52	67 (Prostate)	-	IDC	NA	NA	+/+/-	Adenocarcinoma, NOS (T2)
#6	65	69 (Prostate)	BC (51)	IDC	1	2	-/-/-	Adenocarcinoma, NOS (T3)
#7	73	73 (Prostate)	-	IDC	1	1	+/+/-	Adenocarcinoma, NOS
#8	79	81 (Prostate)	Leukemia (84), BC (89)	IDC	2	3	+/+/-	Adenocarcinoma, NOS
#9	91	91 (Colon)	Stomach, Colorectal, Lung	IDC	3	2	+/+/-	Mucinous adenocarcinoma (T3)
#10	55	66 (Basal cell carcinoma)	-	IDC	2	2	+/+/-	NA
#11	54	54 (Bladder)	BC (52), Melanoma	Other	1	1	+/+/-	Carcinoma, NOS (G3)
#12	70	67 (Lung)	Prostate (64), Liver (64)	IDC	1	3	+/+/-	Neoplasm, malignant
#13	62	56 (Tongue)	-	IDC	2	2	+/+/-	NA

*****Some data are not available. **Abbreviations:** MBCs: Male breast cancer cases; BC: Breast cancer; TNM: tumor, nodes, and metastases; ER: estrogen receptor; PR progesterone receptor; HER2: Human Epidermal Growth Factor (EGF) Receptor 2; IDC: invasive ductal carcinoma; NOS: not otherwise specified; NA: not available.

GERMLINE WHOLE-EXOME SEQUENCING IN MBC PATIENTS

Whole-exome sequencing data results

Sequencing data results of the 13 germline samples from the 13 MBC cases included in this study are reported in **Table 5**.

Overall, mean Q30 measure, that corresponds to the equivalent to the probability of an incorrect base call 1 in 1000 times, was 93.1%. As concern coverage analyses, the mean coverage was > 60x for 11 out of 13 (86.6%) germline MBC samples and mean uniformity of coverage was 96.5%.

Table 5. Sequencing data results of the 13 germline samples.

Sample ID	Q30 (%)	Mean Target Coverage Depth	Uniformity of Coverage (%)
#1	89.9%	152.7	96.8%
#2	93.5%	72.9	96.8%
#3	93.4%	65.7	96.7%
#4	93.4%	17.7	89.1%
#5	93.4%	68.5	97.5%
#6	93.0%	69.4	97.3%
#7	93.0%	42.6	97.2%
#8	93.6%	61.4	96.7%
#9	93.6%	101.2	97.8%
#10	93.0%	88.6	97.5%
#11	93.3%	68.6	97.4%
#12	93.6%	85.5	96.8%
#13	93.2%	64.0	97.5%
Mean	**93.1%**	**73.7**	**96.5%**

After the step of variant filtering, all PVs identified had been visualized and confirmed on IGV tool before proceeding with the analyses.

A total of eight germline PVs in five MBC cases (38.5%) were identified in *ATM, BRCA2, E2F4, ERCC3, FH, NTHL1, TGM4* and *ZFHX3* genes (**Table 6**).

Five out of eight (62.5%) PVs were frameshift variants, and three out of eight (37.5%) PVs were stop gained variants. Among these, the two PVs in *ATM* and *BRCA2* genes were previously identified in another study (Bucalo *et al*, 2023) and confirmed in this study by WES.

All germline PVs identified were also evaluated in the somatic samples of the same patient, as possible considered as loss of heterozygosity (LOH). All PVs identified in germline

samples were also detected in somatic samples, but no LOH was detected. An exception is represented by the *E2F4* p.Ser312_Ser316dup variant detected in MBC case #1. This variant was not detected in the male breast tumor of the MBC case #1, while it was detected in the stomach tumor of the same case.

Table 6. List of germline PVs identified through WES.

Sample ID	PH of tumor (age)	Gene	Variant type	Exon	Mutation		dbSNP ID
					Nucleotide change	Protein change	
#1	Breast (63); Stomach (86)	*BRCA2*	Frameshift variant	11	c. 6468_6469del	p. Gln2157fsTer18	-
		E2F4	Frameshift variant	7	c. 944_958dup	p. Ser312_Ser316dup	-
#5	Breast (52); Prostate (67)	*FH*	Frameshift variant	10	c. 1431_1433dup	p. Lys477dup	rs367543046
#7	Breast (73); Prostate (73)	*ATM*	Frameshift variant	10	c. 1402_1403del	p. Lys468fsTer18	rs587781347
		NTHL1	Stop gained	2	c. 244C>T	p. Gln82Ter	rs150766139
#8	Breast (79); Prostate (81)	*TGM4*	Stop gained	7	c. 806G>A	p. Trp269Ter	rs139860990
		ZFHX3	Frameshift variant	9	c. 5221_5223del	p. Gln1741del	-
#11	Breast (54); Bladder (54)	*ERCC3*	Stop gained	7	c. 1026C>A	p. Cys342Ter	rs752026166

Abbreviations: PH: personal history.

Clinical-pathologic characteristics of mutated cases

To investigate whether PVs in different genes could be associated with genetic susceptibility of different tumors, we analyzed the main clinical pathologic characteristics of mutated cases in comparison with mutated genes.

Case #1 was reported as carrier of PVs in *BRCA2* and *E2F4* genes. Both of PVs are frameshift variants. Case #1 developed MBC and stomach cancer at the age of 63 and 96, respectively. Interestingly, the breast tumor of case #1 was triple positive (**Table 6**).

Three of four (75%) MBC patients with prostate cancers carried at least one PVs.

In particular, case #5 was reported as carrier of a frameshift PV in *FH* gene. Case #5 developed MBC and prostate cancer at the age of 52 and 67, respectively. Case #7 was reported as carrier of a frameshift PV in *ATM* and a stop-gained PV in *NTHL1* genes. Case #7 developed MBC and prostate cancer, both at the age of 73. Case #8 was reported as carrier of a stop-gained PV in *TGM4* and a frameshift PV in *ZFHX3* genes. Case #8 developed MBC and prostate cancer, at the age of 79 and 81, respectively (**Table 6**).

Case #11 was reported as carrier of a stop-gained PV in *ERCC3* gene. Case #11 developed MBC and urinary bladder cancer, both at the age of 54.

SOMATIC WHOLE-EXOME SEQUENCING IN MBC PATIENTS

Whole-exome sequencing data results

Sequencing data results of the 22 tumor samples from the 13 MBC cases included in this study are reported in **Table 7.** Overall, mean Q30 measure was 89.6%. As concern coverage analyses, the mean coverage was > 60x for 16 out of 22 (72.7%) somatic samples, and mean uniformity of coverage was 88.9%. Notably, even if the mean coverage depth was suitable for the majority of tumor samples, the breast tumor sample of case #1 and the skin tumor sample of case #10 resulted with lower mean coverage, of 21.8x and 19.5x, respectively. However, the coverage of these two tumor samples was considered sufficient for the subsequent analysis.

Table 7. Sequencing data results of the 22 tumor samples included in this study.

Sample ID	Tumor site	Q30 (%)	Mean Target Coverage Depth	Uniformity of Coverage (%)
#1	Breast	89.0%	21.8	60.9%
#3	Breast	89.4%	96.4	94.5%
#8	Breast	90.3%	76.6	90.7%
#9	Breast	86.1%	29.2	90.2%
#10	Breast	86.3%	50.4	79.9%
#11	Breast	91.4%	116.9	92.7%
#12	Breast	91.4%	413.6	95.9%
#13	Breast	91.6%	240.8	95.6%
#1	Stomach	87.3%	48.6	88.5%
#2	Stomach	88.7%	71.1	89.9%
#3	Stomach	89.3%	31.1	72.7%
#3	Kidney	90.5%	71.3	86.4%
#4	Stomach	86.9%	68.6	91.5%
#5	Prostate	90.5%	103.3	89.9%
#6	Prostate	90.8%	78.3	90.4%
#7	Prostate	91.1%	162.8	94.2%
#8	Prostate	91.0%	64.6	89.1%
#9	Colon	91.6%	178.9	95.0%
#10	Skin	87.3%	19.5	86.4%
#11	Urinary Bladder	90.2%	103.6	92.8%
#12	Lung	90.2%	180.1	95.6%
#13	Tongue	90.4%	100.2	92.3%
Mean	**-**	**89.6%**	**105.8**	**88.9**

Somatic mutation profile of breast tumors

After comparing tumor and matched-normal samples to identify true somatic variants, a mean of 118,310 total variants, 45,755 PASS variants, and 23 cancer-specific driver somatic variants (range 10-44) were identified in the eight male breast tumors from the 13 MBCs **(Table 8)**.

Notably, breast tumors of MBC cases #11 and #13 showed an increased number of cancer-specific driver somatic variants compared to the mean value, 44 and 36 variants, respectively **(Table 8)**.

Table 8. Description of somatic variants identified in the eight male breast tumor samples.

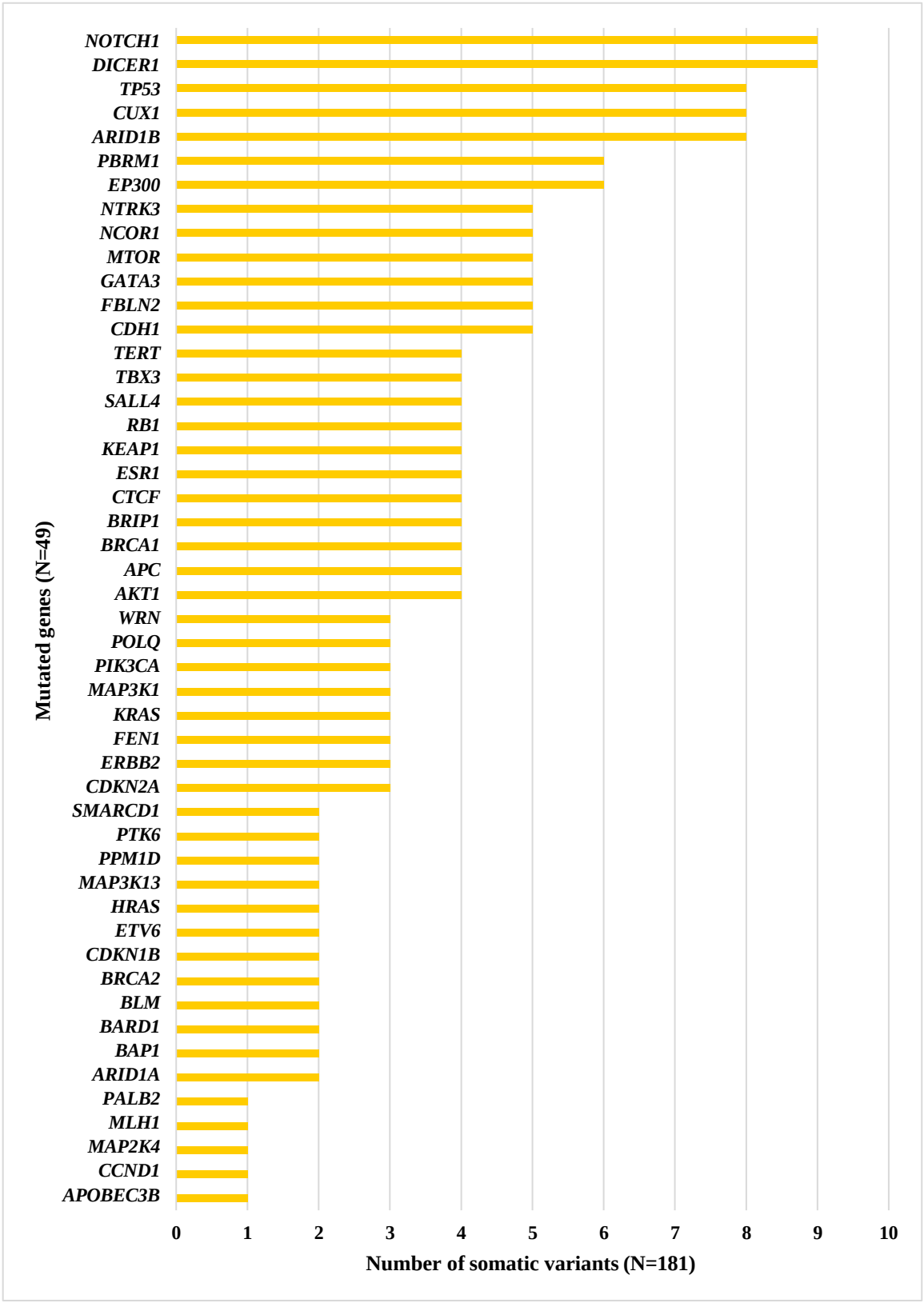
Sample ID	Tumor site	Total variants identified	PASS variants	Cancer-specific driver somatic variants
#1	Breast	73102	25643	10
#3	Breast	107380	44518	19
#8	Breast	132383	51567	19
#9	Breast	96096	27273	20
#10	Breast	116776	54612	18
#11	Breast	136931	53855	44
#12	Breast	122269	46249	15
#13	Breast	161546	62323	36
Mean	-	**118310**	**45755**	**23**

A total of 181 driver somatic variants were identified in 49 different genes. From one to nine somatic variants were identified in each gene **(Figure 6)**.

NOTCH1 and *DICER1* were the most frequently mutated genes, with nine out of 181 somatic variants detected (5.0%) in four (50.0%) and three (37.5%) breast tumors, each. Then, eight out of 181 (4.4%) somatic variants were identified in *ARID1B*, *CUX1* and *TP53* genes, in four (40.0%) breast tumors, each **(Figure 6)**.

Overall, 140 out of 181 (77.3%) somatic variants were missense, 27 (14.9%) stop-gained, five (2.8%) frameshift and nine (5.0%) splice-site variants.

Figure 6. Distribution of 181 driver somatic variants identified in the eight breast tumor samples, by gene.



Somatic mutation profile of other primary tumors

After comparing tumor and matched-normal samples to identify true somatic variants, a mean of 101,356 total variants, 40,236 PASS variants, and 24 cancer-specific driver somatic variants (range 4-59) were identified in the 22 second primary tumors from the 13 MBCs **(Table 9)**.

Notably, colon cancer sample of case #9, kidney cancer sample of case #3 and lung cancer sample of case #12 showed an increased number of cancer-specific driver somatic variants compared to the mean value, 59, 36 and 34 variants, respectively.

On the other hand, prostate cancer sample of case #7 and skin cancer sample of case #10 showed a decreased number of cancer-specific driver somatic variants compared to the mean value, eight and four variants, respectively.

Table 9. Description of somatic variants identified in the 22 second primary tumor samples.

Sample ID	Tumor site	Total variants identified	PASS variants	Cancer-specific driver somatic variants
#1	Stomach	47849	47555	24
#2	Stomach	104089	42762	26
#3	Stomach	78911	31179	22
#4	Stomach	111976	47433	19
#5	Prostate	85685	29064	10
#6	Prostate	116368	52436	22
#7	Prostate	87448	23998	8
#8	Prostate	111634	49041	15
#3	Kidney	137795	60242	36
#9	Colon	139522	53256	59
#10	Skin	78422	16247	4
#11	Urinary bladder	109052	37708	22
#12	Lung	104083	34749	33
#13	Tongue	106147	37635	29
Mean	-	**101356**	**40236**	**24**

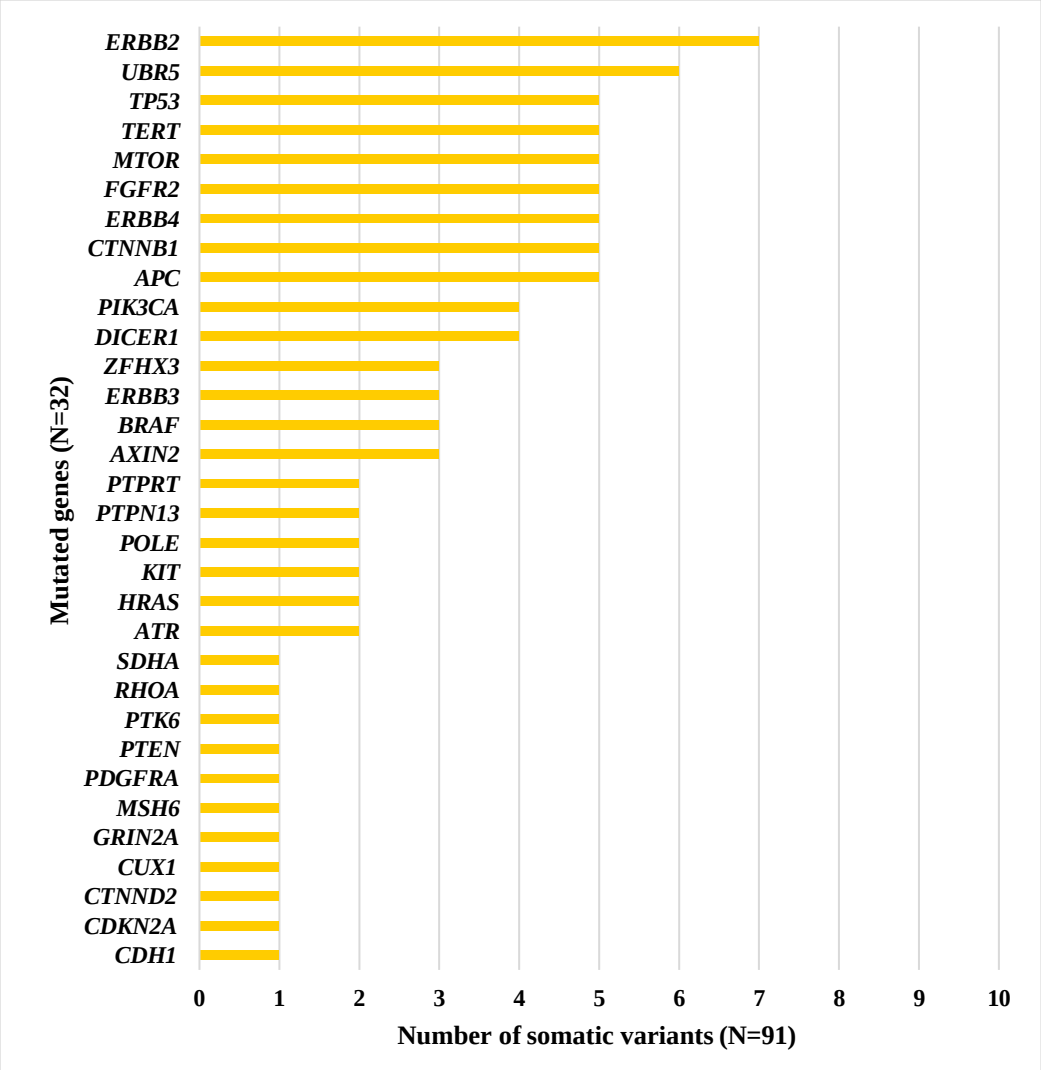
Stomach cancers

A total of 91 driver somatic variants were identified in 32 different genes in all four stomach tumor samples (cases #1, #2, #3 and #4). From one to seven somatic variants were identified in each gene **(Figure 7)**.

ERBB2 and *UBR5* were the most frequently mutated genes, with seven out of 91 (7.7%) and six out of 91 (6.6%) somatic variants detected in four (100.0%) and one (25.0%) stomach tumors, respectively. Then, five out of 91 (5.5%) somatic variants were identified in *APC*, *CTNNB1*, *ERBB4*, *FGFR2*, *MTOR*, *TERT* and *TP53* genes, each **(Figure 7)**.

Overall, 83 out of 91 somatic variants (91.2%) were missense, six (6.6%) stop-gained, one (1.1%) frameshift and one (1.1%) splice-site variants.

Figure 7. Distribution of 91 driver somatic variants identified in the four stomach tumor samples, by gene.



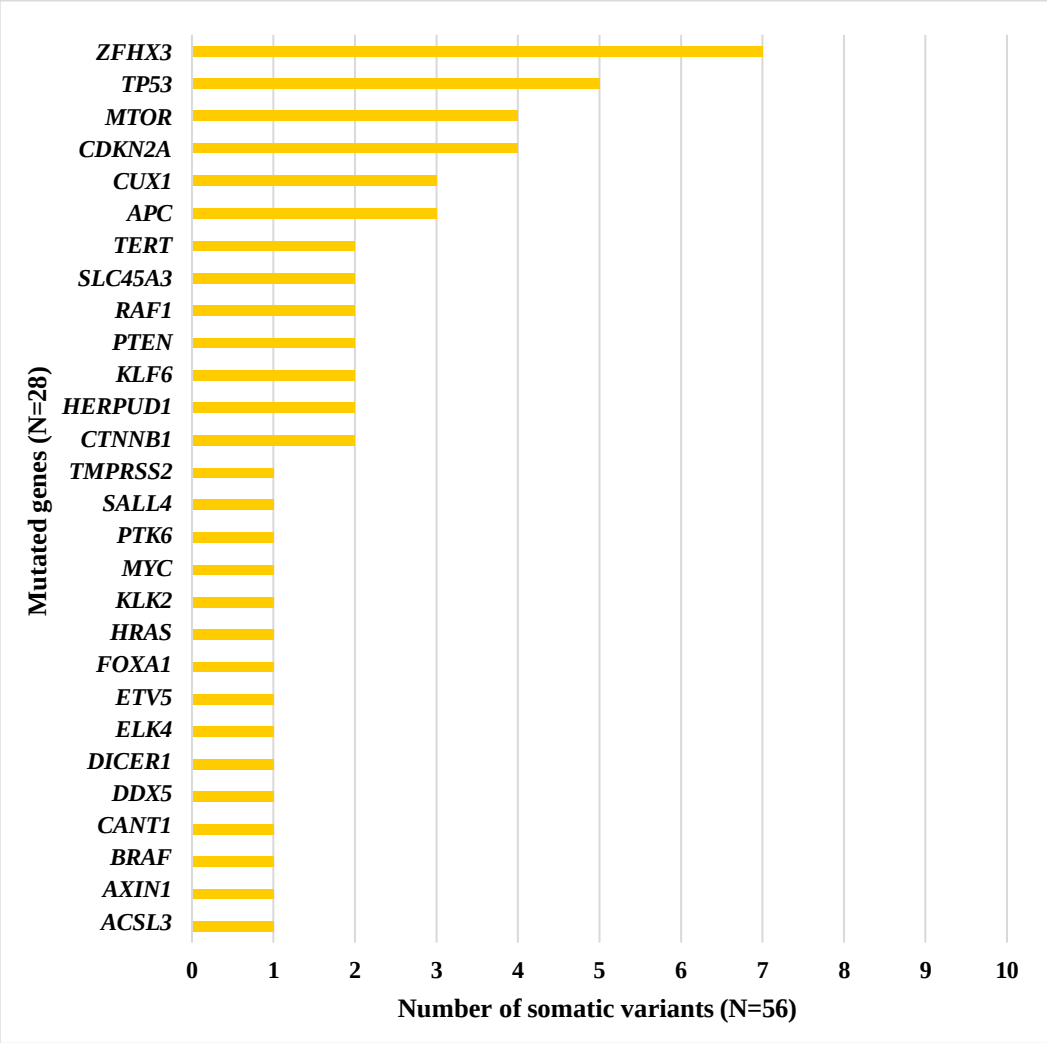
Prostate cancers

A total of 56 driver somatic variants were identified in 28 different genes in all four prostate samples (cases #5, #6, #7 and #8). From one to seven somatic variants were identified in each gene **(Figure 8)**.

ZFHX3 was the most frequently mutated gene, with seven out of 56 (12.5%) somatic variants detected in three (75.0%) prostate tumors. Then, five out of 56 (8.9%) somatic variants were identified in *TP53* gene, in two (50.0%) prostate tumors **(Figure 8)**.

Overall, 52 out of 56 somatic variants (92.9%) were missense, three (5.3%) stop-gained and one (1.8%) splice-site variants.

Figure 8. Distribution of 56 driver somatic variants identified in the four prostate tumor samples, by gene.

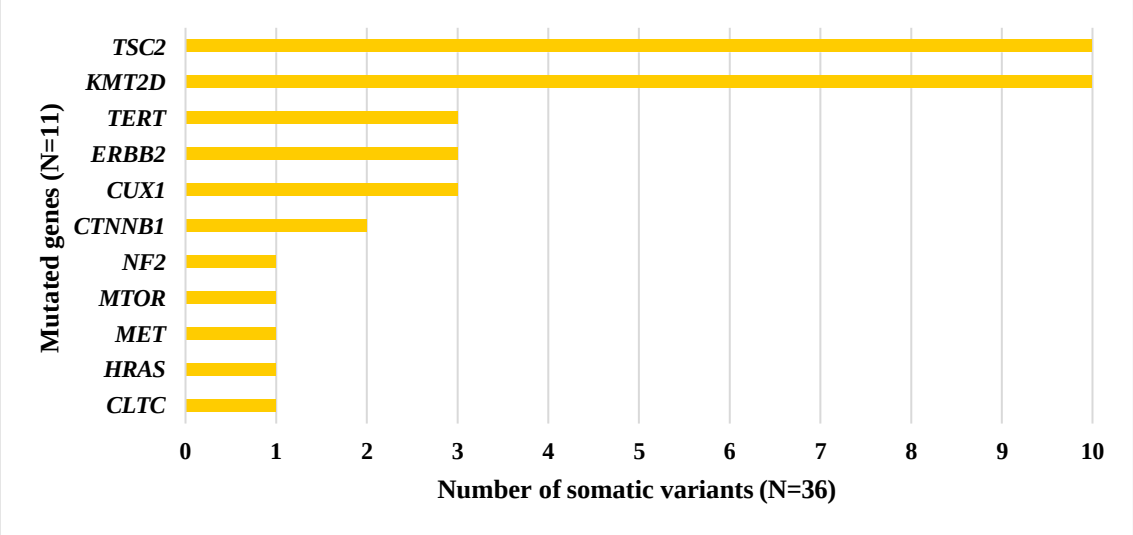


Kidney cancer

A total of 36 driver somatic variants were identified in 11 different genes in the kidney tumor sample (case #3). From one to 10 somatic variants were identified in each gene **(Figure 9)**. *KMT2D* and *TSC2* were the most frequently mutated genes, with 10 out 36 (27.8%) somatic variants detected in each gene. Then, three out of 36 (8.3%) somatic variants were identified in *CUX1*, *ERBB2* and *TERT* genes **(Figure 9)**.

Overall, 31 out of 36 somatic variants (86.1%) were missense, four (11.1%) stop-gained and one (2.8%) frameshift variants.

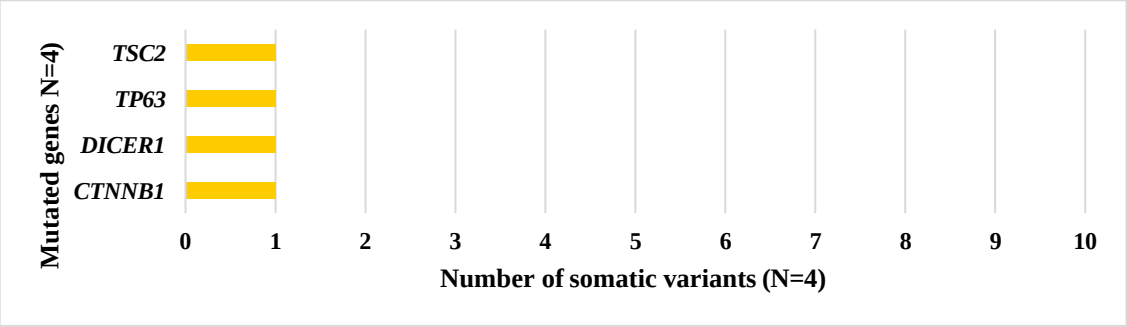
Figure 9. Distribution of 36 driver somatic variants identified in the kidney tumor sample, by gene.



Skin cancer

A total of four driver somatic variants were identified in *CTNNB1*, *DICER1*, *TP63* and *TSC2* genes in the skin tumor sample (case #10). Each gene carried only for one somatic variant **(Figure 10)**. Overall, two out of four somatic variants (50.0%) were missense, one (25.0%) stop-gained and one (25.0%) splice-site variant.

Figure 10. Distribution of 4 driver somatic variants identified in the skin tumor sample, by gene.



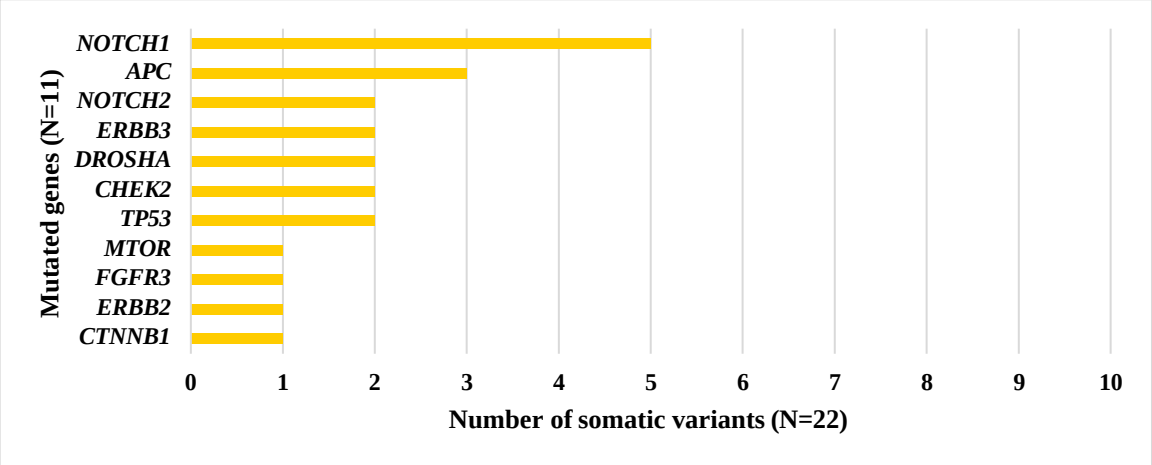
Urinary bladder cancer

A total of 22 driver somatic variants were identified in 11 different genes in the urinary bladder tumor sample (case #11). From one to five somatic variants were identified in each gene **(Figure 11)**.

NOTCH1 was the most frequently mutated gene, with five out of 22 (22.7%) somatic variants detected in each gene. Then, three out of 22 (13.6%) somatic variants were identified in *APC* gene **(Figure 11)**.

Overall, 19 out of 22 somatic variants (86.4%) were missense and four (13.6%) stop-gained variants.

Figure 11. Distribution of 22 driver somatic variants identified in the urinary bladder tumor sample, by gene.



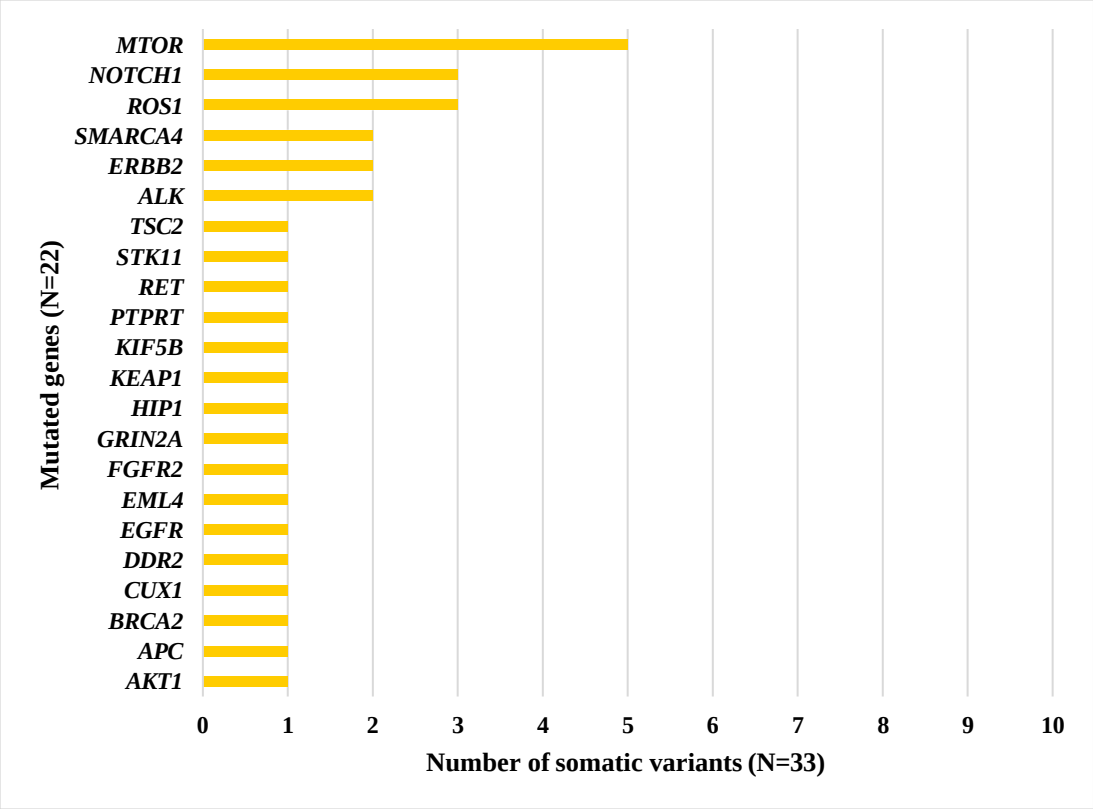
Lung cancer

A total of 33 driver somatic variants were identified in 22 different genes in the lung tumor sample (case #12). From one to five somatic variants were identified in each gene **(Figure 12)**.

MTOR was the most frequently mutated gene, with five out of 33 (15.1%) somatic variants detected in each gene. Then, three out 33 (9.1%) somatic variants were identified in *NOTCH1* and *ROS1* genes, each **(Figure 12)**.

Overall, 29 out of 33 somatic variants (87.8%) were missense, two (6.1%) stop-gained and splice-site variants, each.

Figure 12. Distribution of 33 driver somatic variants identified in the lung tumor sample, by gene.



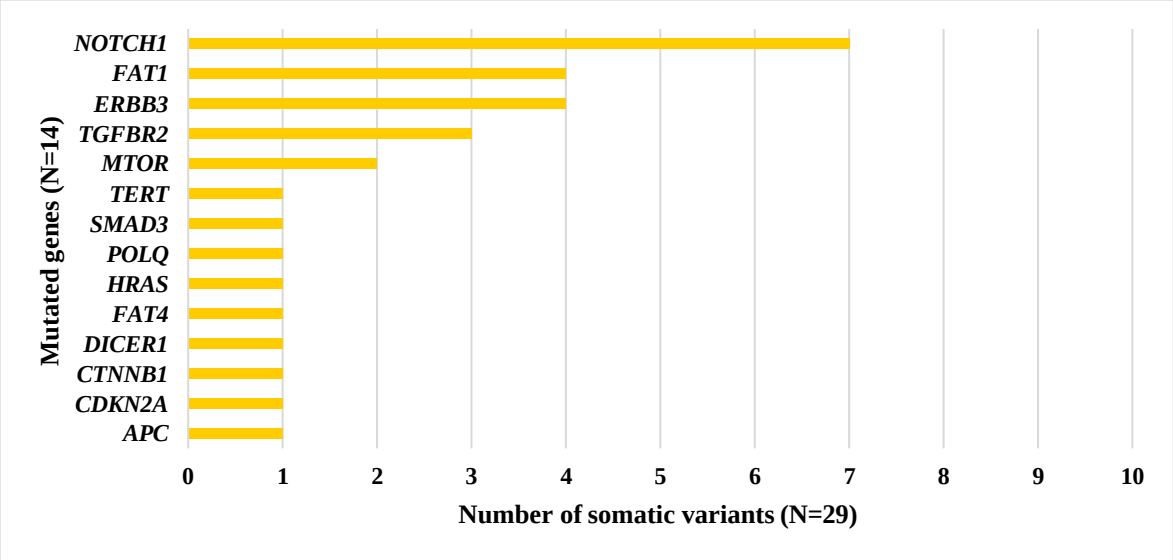
Tongue cancer

A total of 29 driver somatic variants were identified in 14 different genes in the tongue tumor sample (case #13). From one to seven somatic variants were identified in each gene **(Figure 13)**.

NOTCH1 was the most frequently mutated gene, with seven out of 29 (24.1%) somatic variants detected in each gene. Then, four out of 29 (13.8%) somatic variants were identified in *ERBB3* and *FAT1* genes, each **(Figure 13)**.

Overall, 26 out of 29 somatic variants (89.7%) were missense, two (6.9%) stop-gained and one (3.4%) frameshift variants.

Figure 13. Distribution of 29 driver somatic variants identified in the tongue tumor sample, by gene.



Colon cancer

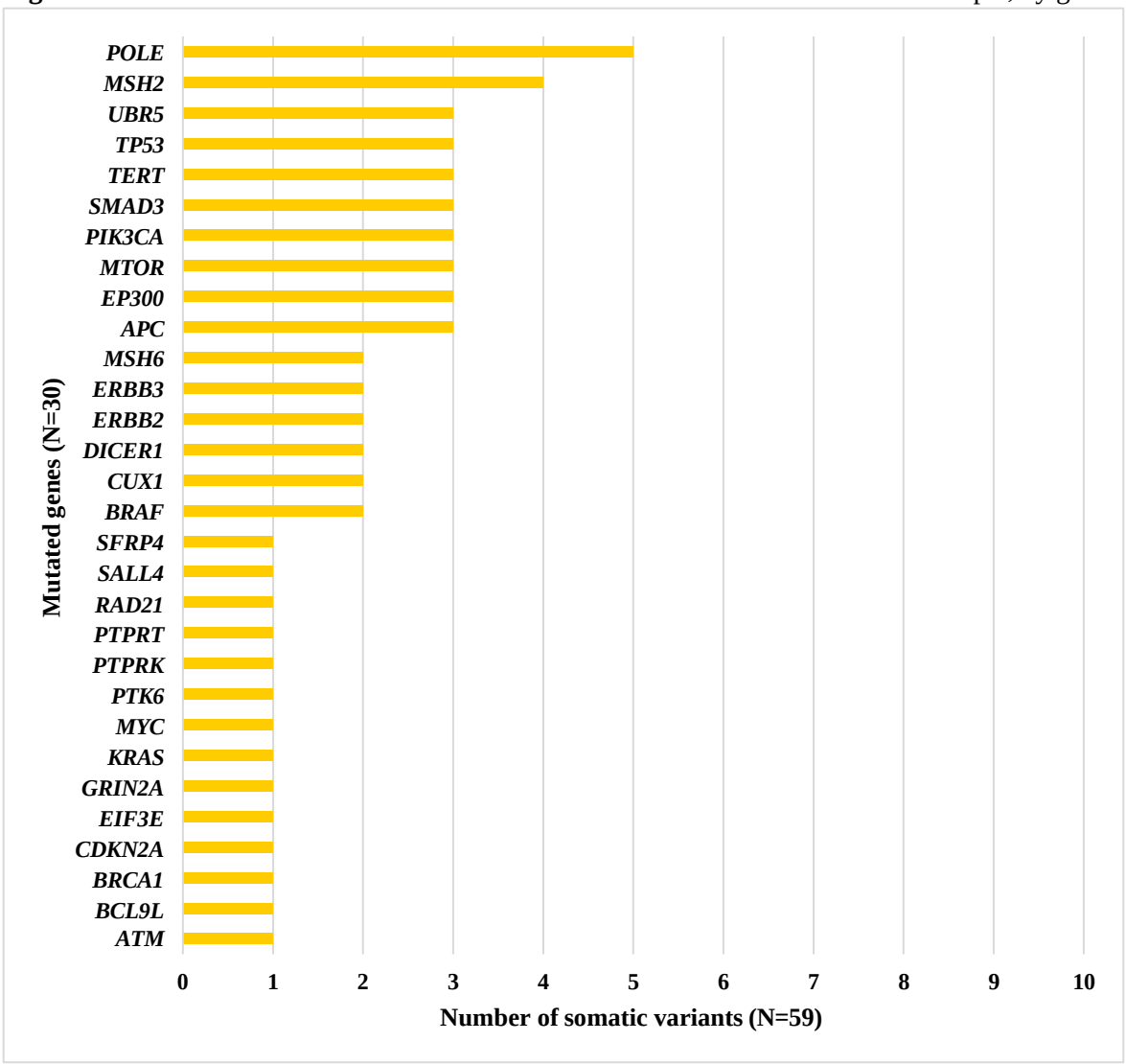
A total of 59 driver somatic variants were identified in 30 different genes in the colon tumor sample (case #9). From one to five somatic variants were identified in each gene **(Figure 14)**.

POLE was the most frequently mutated gene, with five out of 59 (8.7%) somatic variants detected in each gene.

Then, four out of 29 (13.8%) somatic variants were identified in *MSH2* gene **(Figure 14)**.

Overall, 45 out of 59 somatic variants (76.3%) were missense, 12 (20.3%) stop-gained and one (1.7%) frameshift and splice-site variant, each.

Figure 14. Distribution of 59 driver somatic variants identified in the colon tumor sample, by gene.



Identification of clinically actionable variants

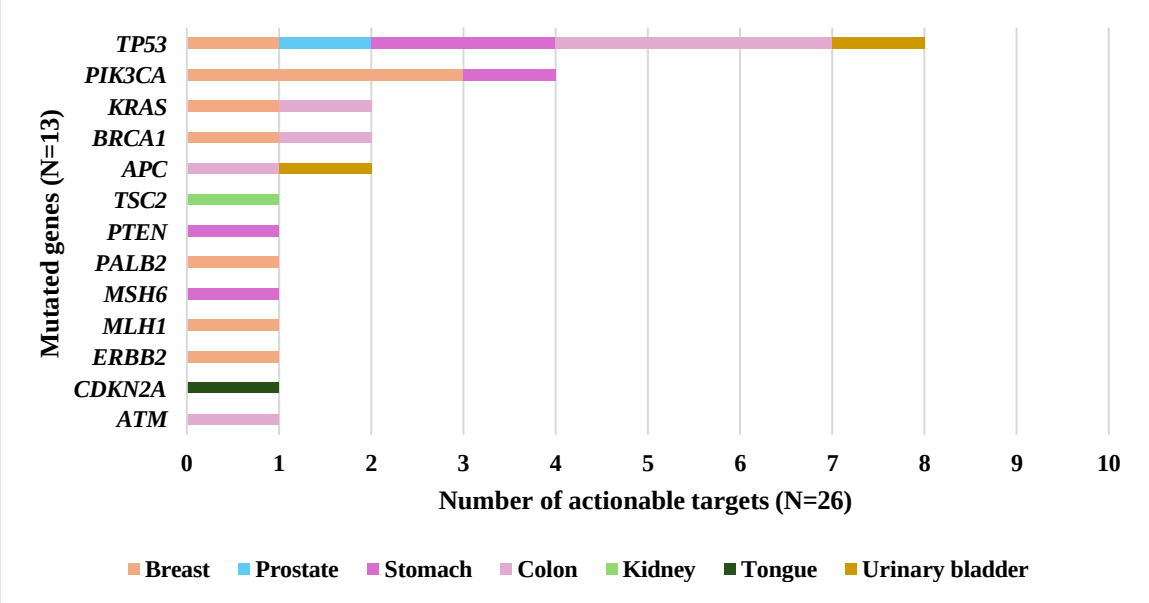
The COSMIC database was interrogated to evaluate whether the detected driver somatic variants were actionable targets in MBC, or other tumors. **Figure 15** shows the clinically actionable variants identified in all the 22 tumors (8 male breast and 14 multiple tumors) analyzed in this study, by gene.

Considering all 22 tumors analyzed, *TP53* emerged as the most representative gene, with eight out of 26 (30.8%) actionable molecular alterations detected, followed by *PIK3CA*, with four out of 26 (15.5%) alterations detected. Two out of 26 (7.8%) actionable molecular alterations were detected in *APC*, *BRCA1* and *KRAS* genes, whereas one out of 26 (3.9%) alteration was detected in *ATM*, *CDKN2A*, *ERBB2*, *MLH1*, *MSH6*, *PALB2*, *PTEN* and *TSC2* genes **(Figure 15)**.

Considering only the eight breast tumors, according to COSMIC database, nine clinically actionable alterations were detected in seven genes. *PIK3CA* emerged as the most representative gene, with three out of nine (33.3%) actionable molecular alterations detected, followed by *BRCA1*, *ERBB2*, *KRAS*, *MLH1*, *PALB2* and *TP53*, with one out of nine (11.1%) alteration detected, each **(Figure 15)**.

In general, from one to eight clinically actionable alterations were detected in each gene, in 12 out of 22 (54.5%) tumors, and in eight out of 13 MBCs (61.5%).

Figure 15. Clinically actionable alterations identified in the eight breast and in the 14 multiple tumors, by gene.



Intra-patient analysis of somatic mutation landscape

To identify possible driver somatic variants shared in the different tumors arising in the same patient, we compared the matched tumor-normal data of breast and the other primary tumors of the same case. Overall, a total of six driver somatic variants in different genes were identified to be shared in three out of the 13 MBCs (23.1%) (**Table 10**).

The reported somatic variants in *POLG* and in *SDHAF2* genes were shared in stomach and kidney tumors of case #3. The reported somatic variant in *FGFR1* gene was shared in breast and skin tumors of case #10, whether the reported somatic variants in *GATA3, SETD1B* and *TBX3* genes were shared in breast and lung tumors of case #12.

Five out of six variants (83.3%), identified in *FGFR1, GATA3, POLG, SETD1B* and *TBX3* genes were missense variants. One out of six variant (16.7%), identified in *SDHAF2* gene, was stop gained variant.

Among the shared variants between breast and lung tumors of case #12, the p.Met294Arg variant identified in *GATA3* gene was classified as variant with unknown clinical significance according to COSMIC classification.

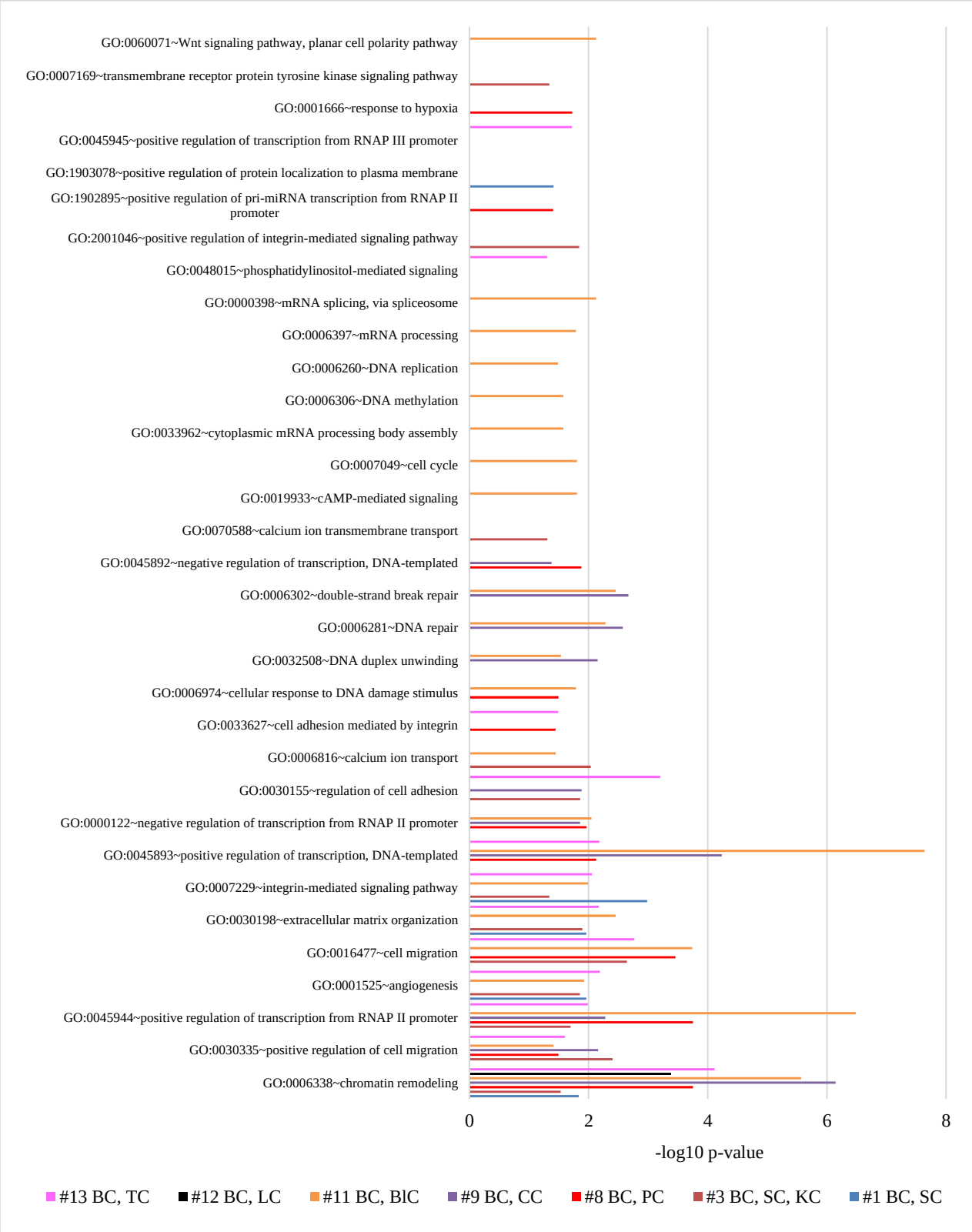
Table 10. Description of somatic variants shared among the same MBC case.

Sample ID	**Compared tumors**	**Gene**	**Variant type**	**Exon**	**Mutation**		**dbSNP ID**
					Nucleotide change	**Protein change**	
#3	Stomach, Kidney	*POLG*	Missense variant	16	c.2543G>A	p. Gly848Asp	-
		SDHAF2	Stop gained	2	c.181G>T	p. Glu61Ter	-
#10	Breast, Skin	*FGFR1*	Missense variant	8	c.958G>A	p. Asp320Asn	rs1563475734
#12	Breast, Lung	*GATA3*	Missense variant	4	c.881T>G	p. Met294Arg	-
		SETD1B	Missense variant	14	c.5242C>T	p. Arg1748Cys	-
		TBX3	Missense variant	2	c.626T>A	p. Leu209His	-

In addition to the analysis based on single nucleotide variants detection, the common pathways between the different tumors arising in the same patient were also investigated, in order to possibly add information on the shared mechanisms in carcinogenesis.
Based on GO classification, pathways statistically significant enriched in mutations were found in all matches intra-patient, with exception of MBC case #10.
In particular, pathway related to chromatin remodeling was found enriched in all matches (mean FDR p-value: 0.006), followed by pathways related to positive regulation of transcription from RNA polymerase II promoter and positive regulation of cell migration, enriched in five out of seven matches (mean FDR p-value 0.007 and 0.02, respectively) (**Figure 16**). Among all patients, the highest number of common pathways was found in the match between the breast and bladder cancer of case #11, with 22 common pathways, mainly represented by regulation of transcription pathways (**Figure 16**).

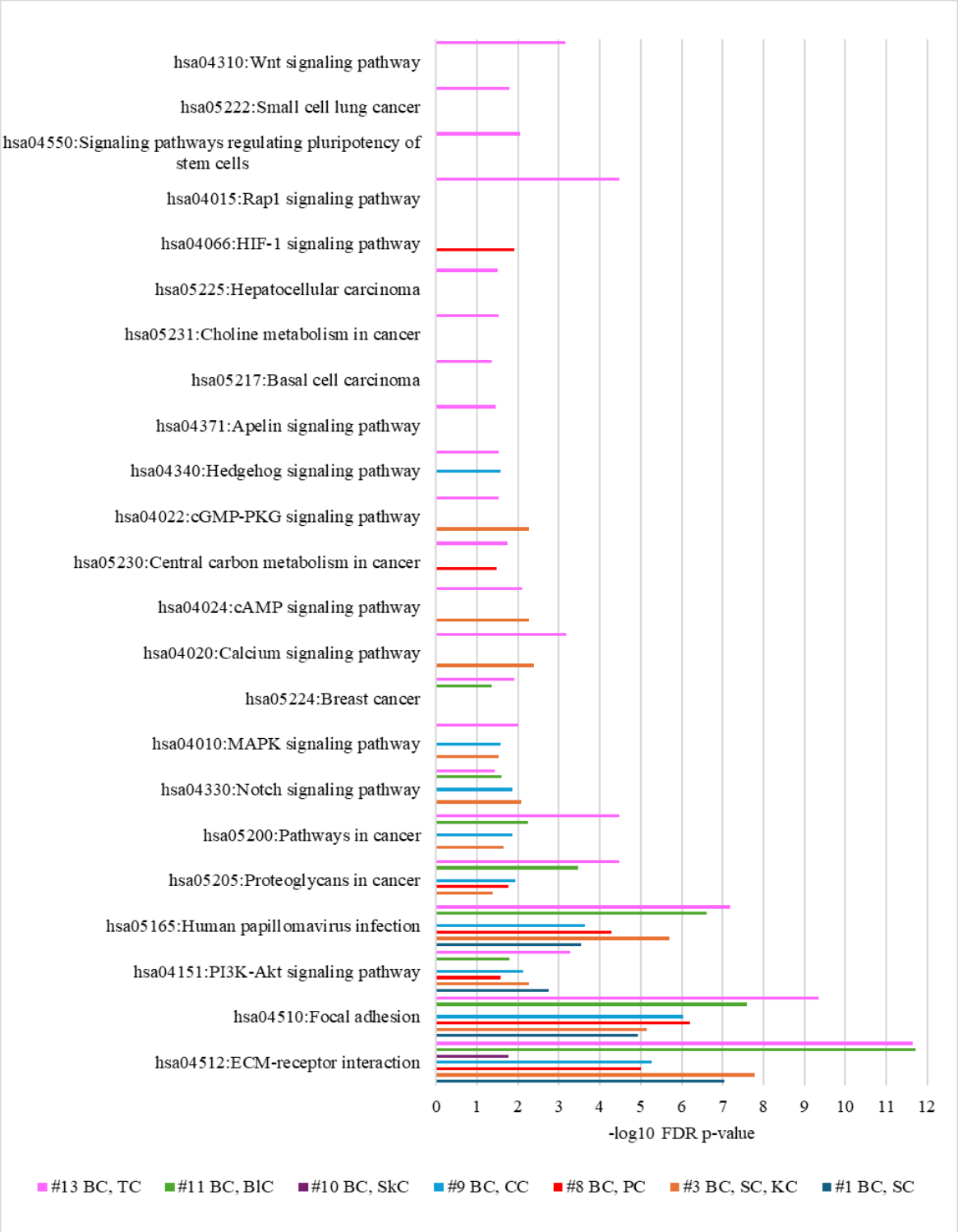
Based on KEGG classification, pathways statistically significant enriched in mutations were found in all matches intra-patient, with exception of MBC case #12.
In particular, pathway related to extracellular matrix-receptor (ECM-receptor) was found enriched in all matches (mean FDR p-value: 0.003), followed by pathways relate to focal adhesion, human papillomavirus (HPV) infection and PI3K-Akt signaling pathway, enriched in six out of seven matches (mean FDR p-value 3.43E-06, 9.57E-05 and 0.01, respectively) (**Figure 17**). Among all patients, the highest number of common pathways was found in the match between the breast and tongue cancer of case #13, with 22 common pathways, mainly represented by ECM-receptor interaction and focal adhesion (**Figure 17**).

Figure 16. Enrichment pathway analysis based on common intra-patient mutated genes according to GO classification.



Abbreviations: RNAP: RNA polymerase; BC: Breast cancer; TC: Tongue cancer, LC: Lung cancer; BlC: Bladder cancer; CC: Colon cancer; PC: Prostate cancer; SC: Stomach cancer, KC: Kidney cancer.

Figure 17. Enrichment pathway analysis based on common intra-patient mutated genes according to KEGG classification.



Abbreviations: BC: Breast cancer; TC: Tongue cancer, BlC: Bladder cancer; SkC: Skin cancer; CC: Colon cancer; PC: Prostate cancer; SC: Stomach cancer, KC: Kidney cancer.

DISCUSSION

MBC is considered a rare disease compared to the female counterpart, even if the annual incidence is continuing to arise, with consequent increasing attention (Ottini, 2014; Campos *et al,* 2021). Compared to FBC, MBC cases are diagnosed at a more advanced age and with a more severe clinical presentation, with the consequence of a poor prognosis (Ottini, 2014; Tedaldi *et al,* 2020).

Genetic susceptibility plays a key role in MBC development. One of the most important risk factors for MBC genetic susceptibility is a positive family history of BC (Korde *et al,* 2010; Khan & Tirona, 2021; Khare *et al,* 2024; Valentini *et al,* 2024). In fact, more than 20% of MBC patients have positive family history of BC and more than 20% develop a second non-breast tumor. This evidence suggests a relevant genetic component in MBC etiology (Rizzolo *et al,* 2019; Pellini *et al,* 2020; Angelats *et al,* 2021).

PVs in high-penetrance BC *BRCA2* and *BRCA1* genes accounts for about 13% and 2% of MBC cases, respectively (Ottini *et al,* 2012; Rizzolo *et al,* 2013; Rizzolo *et al,* 2019; Calip *et al,* 2021; Rolfes *et al,* 2022). In addition, multigene panel NGS studies conducted on MBC patients identified an additional range of 5-8% MBCs carrying PVs in different genes, mainly including *PALB2, ATM* and *CHEK2* genes (Pritzlaff *et al,* 2017; Neben *et al,* 2019; Rizzolo *et al,* 2019; Tedaldi *et al,* 2020; Bucalo *et al,* 2023). Thus, a large fraction of MBC cases still remains to be assigned to specific genetic risk factors.

Connected to this, from one our recent study, a fraction of high-risk MBC cases (*i.e.* MBC cases with BC family history and multiple primary malignancies) emerged as not mutated in known MBC susceptibility genes (Bucalo *et al,* 2023).

In this context, WES emerged as a crucial and powerful tool for exploring the extent to which rare mutations may explain the heritability of several types of cancer (Rotunno *et al,* 2020). To date, WES technology allowed the discovering of previously unknown cancer-related genes (Stadler *et al,* 2014). Alongside, some studies used WES also to identify somatic mutations on tumor samples in several types of cancer, also on multiple tumors arising in the same patient (Wang *et al,* 2020; Xue *et al,* 2020). In particular, matched germline-tumor WES in the same patient, is a useful tool to refine the interpretation of variants in cancer-predisposing genes; the advantages of this approach are amplified if patients with multiple primary tumors are considered.

With this in mind, the present study proposed a matched germline and tumor exome sequencing analysis with NovaSeq 6000 Illumina platform on 13 MBCs, previously tested negative for known MBC susceptibility genes. A total of 13 blood samples and 22 tumor samples were collected, for a total of 35 samples. The main goals of this study were to identify potential new genes associated with cancer genetic susceptibility, to investigate the somatic mutational landscape of MBC and the other multiple cancers arising in the same patient, and to characterize shared molecular pathways in carcinogenesis.
The purpose of this approach was to provide a comprehensive view of tumor driver changes, in order to expand our understanding of the biology of multi-cancer genomes and expand our knowledge on potential targets for drug development.

Regarding the main clinical-pathologic characteristics, the majority of our MBCs had invasive ductal carcinoma (92.3%), positive ER and PR hormonal receptors status (76.9% and 84.6%, respectively) and HER2 negative status (92.3%), in line with previously reported studies (Alsayed *et al*, 2019; Scarpitta *et al*, 2019; Ghani *et al*, 2020). As concern multiple tumors, each selected MBC case had at least one additional tumor among eight cancer types, mainly represented by stomach and prostate cancers. Among these, in three patients the male breast and the second primary tumor were diagnosed in the same year, in five patients with a difference of less than five years, and in five patients with a difference higher than five years between the two diagnoses. One recent study reported that synchronous multiple primary tumors (defined as primary cancers at different sites with a difference within six months for the time of diagnosis) had a gradual increase during the last years, even if molecular mechanisms involved are still unclear, with a consequent increased necessity of individualized treatments for synchronous multiple primary cancers (Kong *et al* 2022).

For the whole series analyzed, we also evaluated the lifestyle habits. Smoking and alcohol are not only suspected MBC risk factors, but they are also known causes of other types of cancer (Larsson *et al*, 2020).
According to a large recent review based on 139 cohort studies, alcohol consumption from light to heavy is significantly associated with the risk of colorectal cancer in both sexes, prostate cancer in males and breast cancer in females (Jun *et al*, 2023). Due to our results

combined with the literature, we are not able to exclude that lifestyle habits played a role in multiple tumors onset. However, as the present study is mainly focused on cancer genetic susceptibility, additional studies are needed to further understand the impact of environmental factors on multiple cancers developing.

Before proceeding with the subsequent analysis, WES quality was evaluated through measuring the uniformity of coverage, which resulted suitable for all samples.
Results showed that eight germline PVs in established or candidate cancer-associated genes, including *ATM, BRCA2, E2F4, ERCC3, FH, NTHL1, TGM4* and *ZFHX3*, were found in five out of 13 (38.5%) of the MBC cases analyzed.
All the germline PVs described were also confirmed in somatic samples, but no LOH was detected for any variant. BCs were reported to be highly heterogeneous, with the proportion of LOH ranging from 0.3% to >60%. Moreover, sequencing depth is one of the critical factors in determining the detection sensitivity of somatic events (Wang *et al,* 2004; Van Marcke *et al*, 2020). Considering that, we can hypothesize that LOH detection in our series could have been affected by the numerosity of MBC cases included in this study, and that, even if it resulted suitable for all samples, higher coverage would have been beneficial for more accurate LOH detection.
An exception is represented by the *E2F4* p.Ser312_Ser316dup variant detected in MBC case #1, which was detected in the stomach tumor of this case, but there is no evidence of this variant in the breast tumor of the same case. This incongruence could be explained by the low mean sequencing coverage of case #1 breast tumor, that could lead to the loss of some mutations. Apart from that, this data, not common in literature, could also be explain by lack of complete congruence between germline and somatic NGS testing; otherwise, this does not minimize the power or impact of the approach, but highlights the need to understand their possible limitations (Yap *et al,* 2023).

BRCA2 was mutated in case #1, which developed stomach cancer as second primary tumor. The role of germline PVs in *BRCA2* is well established in MBC genetic susceptibility (Valentini *et al,* 2024), and in the last years there is increasing evidence that gastric cancer may be part of the *BRCA2* cancer risk spectrum (Li *et al,* 2022).
Even *E2F4* was mutated in case #1. *E2F4* encodes a member of the E2F family of transcription factors. The *E2F4* PV found in MBC case #1 (c.944_958dup) was found in the

region of the polymorphic trinucleotide repeats of the gene, encoding a polyserine array, known to be unstable in gastrointestinal tumors (Schwemmle & Pfeifer, 2000). Even if *BRCA2* PV on its own could explain the genetic susceptibility of both cancers for case #1, also the detection of *E2F4* PV was interesting, since the co-occurrence of germline PVs in different genes could constitute an increasing genetic risk.

FH was mutated in case #5, which developed prostate cancer as second primary tumor. The *FH* gene encodes an enzyme called fumarase or fumarate hydratase. Fumarase participates in a series of reactions that characterize the Krebs cycle. Germline PVs in *FH* gene lead to a rare autosomal dominant syndrome called hereditary leiomyomatosis and kidney cancer, even if the role of this gene in MBC and prostate cancer needs to be more investigated (Gao *et al*, 2024).

ATM was mutated in case #7, which developed prostate cancer as second primary tumor. *ATM* is a well-established moderate-risk gene in FBC (Tedaldi *et al*, 2020; Hall *et al*, 2021; Dorling *et al*, 2021), and more recent studies indicate that *ATM* may be considered a moderate-risk gene in MBC (Bucalo *et al*, 2023). Germline *ATM* PVs have also been suggested to increase risks for developing other cancers, with some evidence also for prostate cancer (Pritchard *et al*, 2016; Hall *et al*, 2021), even if further studies are still needed.

Even *NTHL1* was mutated in case #7. *NTHL1* gene encodes a protein involved in the repair of DNA damage. Biallelic *NTHL1* PVs have been determined to cause a recessive multi-tumor syndrome, which is characterized especially colorectal cancer and FBC in women (Nurmi *et al*, 2023).

Moreover, data from an international multicenter study suggests that heterozygous germline PVs in *NTHL1* may be associated with low- to moderate- increased risk of FBC, while its role in MBC is still largely unknown (Li *et al*, 2021).

TGM4 was mutated in case #8, which developed prostate cancer as second primary tumor. Transglutaminase-4 is the protein encoded by *TGM4* gene. This protein is exclusively expressed in the prostate gland and has been suggested to be involved in certain medical conditions, such as infertility and possibly prostate cancer, while its role in BC needs to be elucidated (Ye *et al*, 2023).

Even *ZFHX3* was mutated in case #8. *ZFHX3* encodes a transcription factor with multiple homeodomains and zinc finger motifs, with role also as cell cycle inhibitor and tumor suppressor in several type of cancers. Frameshift mutations of *ZFHX3* gene were reported in metastatic or high-grade human prostate cancers (Hu *et al*, 2019). Moreover, exists the hypothesis that germline PVs in *ZFHX3* could be associated with hereditary-predisposing cancer syndrome (Rocca *et al*, 2023).

ERCC3 was mutated in case #11, which developed urinary bladder cancer as second primary tumor. *ERCC3* gene encodes a DNA helicase with a crucial role in RNA transcription and the nucleotide excision repair pathway.
Even if further studies are still needed to elucidate the possible role of this gene in MBC predisposition, in particular for the impact of these variants in the Italian population, it has been reported that variants in *ERCC3* gene are associated with an elevated risk of developing several types of cancer, including BC in women and bladder cancer (Vijai *et al*, 2016; Feki-Tounsi *et al*, 2017; Bonache *et al*, 2018; Chen *et al*, 2024).

Overall, at least one germline PV was detected in five out of the 13 MBCs analyzed. Among these five cases, three of them carried two PVs in two different genes (cases #1, #7 and #8). The co-occurrence of two PVs in the same case, even if in moderate- to low-penetrance genes (*i.e. NTHL1)* could modify and increase the impact of these variants on cancer genetic susceptibility.
Moreover, some of the mutated genes seem to be more associated with the multiple tumors than with MBC, as *ZFHX3* (case #8) for prostate cancer or *ERCC3* (case #11) for bladder cancer. This evidence highlights the importance of performing WES rather than targeted gene-panel sequencing on high-risk MBC patients, in order to have a more comprehensive on the genetic landscape, not necessarily strictly related on MBC genetic susceptibility. This approach may help this kind of patients and their families also at clinical level.

In our MBC series, the two (15.4% of the entire series) TNBC resulted negative for candidate germline PVs. These results seemed to be in contrast with the female counterpart, where TNBC is often associated with genetic susceptibility (Zhang *et al,* 2018; Yao *et al,* 2019). This evidence should suggest a little difference between female and male BC genetic susceptibility, even if further studies are needed to understand this hypothesis.

Matched tumor-normal pairs were performed for each tumor identified in the 13 MBC cases analyzed in this study, in order to identify true somatic variants.

Among the eight male breast tumor samples analyzed, a total of 181 driver somatic variants were identified in 49 different genes. *DICER1* and *NOTCH1* were the most frequently mutated genes, with 5.0% (9/181) somatic variants detected, each.

DICER1 gene encodes a protein involved in the production of microRNAs, playing a role in regulating the expression of other genes. As *DICER1* is involved in RNA interference, mutation in this gene have been associated with different multi-organ neoplastic and non-neoplastic conditions (Riascos *et al*, 2024). Germline mutations in *DICER1* gene are associated with DICER1 syndrome, a familial tumor predisposition, mainly characterized by pleuropulmonary blastoma and ovarian tumors, while somatic mutations were found in sporadic hepatocellular carcinomas associated with various etiologies (Foulkes *et al*, 2014; Caruso *et al*, 2017). One study reported that down-regulation of *DICER1* seems to be preferentially found in TNBC in females (Dedes *et al*, 2011).

NOTCH1 encodes a single-pass transmembrane receptor, member of the NOTCH family of proteins. A high rate of *NOTCH1* somatic mutations is observed in chronic lymphocytic leukemia, representing them a validated prognostic and potential predictive marker (Pozzo *et al*, 2022). In the context of multiple tumors, *NOTCH1* was found to be related to cancer cell metastasis (Hanahan & Weinberg, 2011).

In females, *NOTCH1* seemed to be enriched in the TNBC (Yuan *et al*, 2015). Moreover, one study classified *NOTCH1* as a FBC gene, since a frameshift mutation in its C-terminal regulatory region (in the same region in which mutation are known to activate *NOTCH1* in T-cell acute lymphoblastic leukemia) was identified (Jiao *et al*, 2012). *NOTCH1* somatic mutation are occasionally reported in MBC patients, even if is not possible at the moment to establish a correlation (Moelans *et al*, 2019).

As concern somatic mutations in the other primary tumors analyzed in this study, *NOTCH1* resulted the most mutated gene also in the bladder tumor sample (MBC case #11) and in the tongue tumor sample (MBC case #13), with 22.7% and 24.1% of the detected somatic variants, respectively.

A total of 22 driver somatic variants were identified in 11 different genes in the bladder tumor sample, and a total of 29 driver somatic variants were identified in 14 different genes in the tongue tumor sample.

In the last years, the dual oncogenic and tumor suppressive role of Notch signaling in bladder cancer has been established (Shi *et al*, 2024). One study reported that more than 40% of bladder cancers carry new inactivation mutations in components of Notch pathway (Rampias *et al*, 2014). Similarly, one other study revealed that, in bladder cancer, missense mutations in *NOTCH1* leads to functional loss of Notch pathway, enhancing the aggressiveness of bladder cancer (Maraver *et al*, 2015). However, further studies are necessary to better unveil the mechanism of Notch signaling in bladder cancer. As concern head and neck squamous cell carcinoma (the family of tongue cancer), genomic analysis including WES, found an important mutation rate in *NOTCH1*, reporting it as the gene with the highest mutation frequency just after *TP53* (Agrawal *et al*, 2011; Fukusumi & Califano, 2018).

Among the four stomach cancer samples analyzed, a total of 91 driver somatic variants were identified in 32 different genes. *ERBB2* was the most frequently mutated gene, with 7.7% (7/91) somatic variants detected. Interestingly, somatic variants in *ERBB2* gene were detected in all the four stomach tumors analyzed. *ERBB2* gene, also named *HER2* gene, encodes one tyrosine kinase receptor, member of the EGF receptor family. In literature *ERBB2* mutations were reported in gastric cancer patients, not necessarily associated with HER2 overexpression (Cai *et al*, 2019). In one study, the most common mutation was reported as located in the ERBB2 juxtamembrane domain (a part of the intracellular region) (Park *et al*, 2022). Among somatic mutations identified in our study, one out of seven (p.Glu698Lys, found in stomach cancer sample of MBC case #1) was in the juxtamembrane domain.

Among the four prostate cancer samples analyzed, a total of 56 driver somatic variants were identified in 28 different genes. *ZFHX3* was the most frequently mutated gene, with 12.5% (7/56) somatic variants detected. Interestingly, the MBC case #8, that carried the germline PV in *ZFHX3*, carried also one of the seven somatic variants found in *ZFHX3* (p.Arg2950His). One study that performed WES on 50 metastatic prostate cancer samples reported somatic mutation in six patients in different genes, among which was present also *ZFHX3* (Grasso *et al*, 2012). Nevertheless, further studies are needed to better elucidate the role of *ZFHX3* in prostate cancer.

A total of 36 driver somatic variants were identified in 11 different genes in the kidney tumor sample (case #3). *KMT2D* and *TSC2* were the most frequently mutated genes, with 27.8% (10/36) somatic variants detected, each. Notably, more than 50% of somatic mutations detected in kidney cancer sample of the case #3 were represented by these two genes. *KMT2D* encodes a Histone H3-Lysine 4 methyltransferase. This protein takes part of the large ASCOM protein complex, a transcriptional regulator of the beta-globin and estrogen receptor genes.

One study reported that genetic alterations of histone lysine methyltransferases are involved in generation and development of kidney cancer (Yan *et al*, 2019). Froimchuk *et al* reported that somatic mutations in *KMT2D* occurred in several types of cancer, including brain, lung, large intestine and endometrium, even if the reason *KMT2D* mutations are common in these cancers still needs to be further investigated (Froimchuk *et al*, 2017).

TSC2 gene is a tumor suppressor, negative regulator of the mTOR signaling. *TSC2* encodes for tuberin that, interacting with hamartin (encoded by the *TSC1* gene), forms a heterodimer that inhibits the activation of mTOR complex 1. In our kidney tumor sample, one somatic mutation in *MTOR* gene was also reported.

Chen *et al* reported somatic mutations in *TSC2* and *MTOR* genes (that lead to hyperactive mTOR complex 1 signaling) in sporadic renal carcinoma (Chen *et al*, 2019).

MTOR resulted the most mutated gene in the lung tumor sample of MBC case #12 (15.1% of the detected somatic variants). A total of 33 driver somatic variants were identified in 22 different genes in the lung tumor sample. In this sample, one somatic mutation in *TSC2* gene was also reported.

MTOR is altered in 3.33% of all cancers, with the prevalence of alterations registered in lung and colon adenocarcinomas (AACR Consortium, 2017). *MTOR* was reported as a therapeutic target for the treatment of non-small cell lung cancer, the type of lung cancer of case #12 (Somaiah & Simon, 2010; Ekman *et al*, 2012). In lung cancer, overexpression of the downstream of mTOR was associated with a poor prognosis (Zhang *et al*, 2019). At the moment, genes encoding components of PI3K–AKT–mTOR pathway resulted as frequently mutated in cancer, but few mutations have been characterized in *MTOR* gene. One study that identified 33 *MTOR* mutations leading to pathway hyperactivation in different cancer cell lines, including lung cancer, demonstrated that the hyperactivating *MTOR* mutations display heightened sensitivity to pharmacological treatments (Grabiner *et al*, 2014).

A total of 59 driver somatic variants were identified in 30 different genes in the colorectal tumor sample (case #9). *POLE* was the most frequently mutated gene, with 8.7% (5/59) somatic variants detected, each. Notably, this sample was the one with the highest number of somatic mutations detected. *POLE* encodes the catalytic subunit of DNA polymerase epsilon, involved in DNA-leading strand synthesis and base excision repair. Mutations in *POLE* gene have been associated with a particular subgroup of colorectal cancers, the microsatellite-stable (MSS) ultra-mutated colon cancer (Briggs & Tomlinson, 2013).
A paradigm of the relationship among *POLE*/*POLD1* alterations, MSI and tumor mutational burden (TMB) can be observed in a large study (Fabrizio *et al*, 2018) involving 6004 colorectal cancer patients, where only 5% showed MSI and high TMB. Furthermore, 2.9% of the MSS colorectal cancer patients has been shown to have a high TMB and greater presence of *POLE* variants (Fabrizio *et al*, 2018; Magrin *et al*, 2021). These finding are in line with our *POLE* mutated colon cancer case, that has been reported as MSS.

The COSMIC database was interrogated to evaluate whether the detected driver somatic variants were actionable targets in MBC or other tumors.
Considering all 22 tumors analyzed, *TP53* emerged as the most representative gene, followed by *PIK3CA*, with 30.8% (8/26) and 15.5% (4/26) actionable molecular alterations detected, each. If considering only the eight male breast tumors, *PIK3CA* emerged as the most representative gene, with 33.3% (3/9) actionable molecular alterations detected. Since *TP53* somatic mutations are one of the most frequent alterations in human cancers, we can hypothesize that the high presence of actionable alterations in *TP53* gene in our series of MBC cases is dragged by the other multiple tumors occurring in the patients analyzed (Olivier *et al*, 2010; Chen *et al*, 2022). This is supported by that, according to literature, recurrent *PIK3CA* actionable alterations are detected in MBCs, while *TP53* somatic mutations were significantly less frequent in MBC (Moelans *et al*, 2019). The rarity of *TP53* somatic variants in MBCs was also supported by one our recent study (Valentini *et al*, 2023).
Moreover, some actionable molecular alterations detected are likely to be tumor specific. One example is represented by the *TSC2* variant detected in kidney cancer sample of the case #3. In fact, *TSC2* is also one of the most mutated gene of this case.
Notably, the colorectal tumor of sample #9, apart from being the case with most driver somatic variants detected, was also the case in which most actionable variants were found, for a total of seven variants. Among actionable variants, *TP53* emerged as the most mutated

gene, with three out of the seven variants detected. One recent study using NGS in colorectal cancer patients, reported *TP53* as the most mutated gene, followed by *KRAS* and *APC*, both detected also in the colorectal cancer of case #9 (Lee CS *et al*, 2021).

To identify possible driver somatic variants shared in the different tumors arising in the same patient, we compared the matched tumor-normal data of MBC and the other primary tumors of the same case. Among the six shared driver somatic variants classified, the *GATA3* p.Met294Arg variant, identified in male breast and lung tumors of case #12, was classified as a Tier 3 variant (variant with unknown clinical significance) according to COSMIC classification.
GATA3 gene encodes the GATA binding protein 3, a transcription factor for a protein member of the GATA family. *GATA3* somatic mutations are an important factor involved in BC development; moreover, according to American Association for Cancer Research (AACR), *GATA3* somatic mutations are mostly registered in some specific types of cancer, including breast invasive ductal carcinoma and lung adenocarcinoma (Takaku *et al*, 2015; AACR Consortium, 2017).

As last part, enriched-pathway analysis intra-patient was performed using DAVID Database, together with GO and KEGG pathways. Pathways related to chromatin remodeling and to ECM-receptor were found enriched in all patients.
Since years ago, genetic alterations of chromatin remodeling factors have been linked to the development of cancer, considering that transcriptional control regulated by chromatin remodeling is essential for appropriate cell proliferation and differentiation (Davis & Brackmann, 2003). Chromatin remodeling uses several mechanisms to link the genome with its functional phenotype, and, with this in mind, the more recent molecular biology techniques are highlighting its role in disease development, particularly in cancer (Cheng & Solit, 2018). Therefore, a better understanding of chromatin remodeling is essential for developing new anticancer therapeutic strategies (Zhang & Li, 2022).
On the other hand, ECM is a major structural component of the tumor microenvironment, strictly connected with cancer development and progression (Popova & Jücker, 2022).
ECM-based prognostic gene signatures were constructed for different types of cancer, including bladder, breast, gastric and prostate cancers (Bergamaschi *et al*, 2008; Yang *et al*, 2018; Pang *et al*, 2019; Qing *et al*, 2020).

An exception among this type of analysis was represented by the match between male breast and lung tumors of case #12, where, based on KEGG classification, no statistically significant enriched pathway was found in this match. Notably, this was the only match intra-patient in which a common driver possibly actionable variant was found. These results found in case #12, highlighted the importance of performing different type of analysis in the same cancer case, at different level, for identifying biologically both important pathways and genes in human cancer, that sometimes could be present some differences.

This study presents a few limitations. Firstly, this is a pilot study on a small cohort of patients; however, this limitation had to be considered by taking into account the rarity of MBC, which significantly limits the MBC patients availability.
Secondly, the presence of pathogenic alterations other than gene variants (*i.e.* copy number variants and large rearrangements) was not evaluated in the current study. Further studies including a larger number of patients are needed to validate our molecular findings and address these issues.

CONCLUSIONS

In this study, a matched germline and somatic analysis was performed on 13 MBC cases with multiple primary tumors using WES.

Germline PVs in established or candidate cancer-associated genes were identified in 5 out of 13 (38.5%) MBC cases. No LOH was detected in the somatic samples for any of the identified germline PVs. The presence of PVs in genes not traditionally associated with breast cancer suggests a broader role in MBC susceptibility. Further research is needed to explore the clinical implications of these findings for MBC patients with multiple tumors and their families.

On average, approximately 20 cancer-specific driver somatic variants were identified in each tumor sample. *NOTCH1* emerged as one of the most frequently mutated genes in breast tumor samples, although additional studies are required to clarify its role in MBC pathogenesis.

Interestingly, the somatic analysis of non-breast tumors revealed a *POLE* mutation in a microsatellite stable (MSS) colon cancer sample showing the highest number of somatic mutations, consistent with the ultra-mutated phenotype observed in MSS *POLE*-mutated colon cancers.

Across all analyzed tumors, a total of 26 actionable alterations were detected, with *TP53* being the most frequently mutated gene, followed by *PIK3CA*. Specifically, *PIK3CA* was the most commonly altered gene in breast tumor samples. Notably, an actionable alteration in *TSC2* was detected in a kidney cancer sample.

The matched somatic analysis between breast and other primary tumors in the same patient identified shared variants, as well as shared pathways related to chromatin remodeling and extracellular matrix (ECM)-receptor interactions, deserving further investigations.

Overall, this study underscored the importance of applying WES to a unique subset of MBC patients with multiple primary tumors. Matched germline and somatic analyses have the potential to identify novel cancer susceptibility genes, actionable alterations, and key pathways that may be involved in the development of multiple cancers, providing valuable insights for both cancer research and personalized treatment strategies.

REFERENCES

1. AACR Project GENIE: powering precision medicine through an international consortium. The AACR Project GENIE Consortium. *Cancer Discovery*. 2017; 7(8):818-831.
2. Abdelwahab Yousef AJ. Male Breast Cancer: Epidemiology and Risk Factors. *Semin Oncol*. 2017; 44(4):267-272.
3. Abul-Husn NS, Soper ER, Odgis JA, *et al.* Exome sequencing reveals a high prevalence of BRCA1 and BRCA2 founder variants in a diverse population-based biobank. *Genome Med*. 2019; 12(1):2.
4. Adank MA, van Mil SE, Gille JJ, *et al*. PALB2 Analysis in BRCA2-like families. *Breast Cancer Res Treat*. 2011; 127:357–362.
5. Agrawal N, Frederick MJ, Pickering CR, *et al*. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. *Science.* 2011; 333(6046):1154-7.
6. AIRTUM. I numeri del cancro in Italia 2020.
7. Al Assaad M, Michaud O, Semaan A, *et al*. Whole-Genome Sequencing Analysis of Male Breast Cancer Unveils Novel Structural Events and Potential Therapeutic Targets. *Mod Pathol.* 2024; 37(4):100452.
8. Amir E, Freedman OC, Seruga B, *et al*. Assessing women at high risk of breast cancer: a review of risk assessment models. *J Natl Cancer Inst.* 2010; 102(10):680-91.
9. Ander Urruticoechea A, Purificacion Martinez P, Cristina Hernando C, *et al*. Ki67 as a prognostic factor in male breast cancer (male BC): Results from a large GEICAM Spanish cohort of male BC. *Journal of Clinical Oncology*. 2024; 42:16.
10. Anderson WF, Jatoi I, Tse J, *et al*. Male breast cancer: a population-based comparison with female breast cancer. *J Clin Oncol.* 2010; 28:232-9.
11. Angelats L, Estival A, Martinez-Cardús A, *et al.* Occult triple negative male breast cancer. The usefulness of molecular platforms. A case report. *Current Problems in Cancer: Case Reports.* 2021.
12. Antoniou AC, Casadei S, Heikkinen T, *et al*. Breast-cancer risk in families with mutations in PALB2. *N Engl J Med.* 2014; 371(6):497-506.

13. Bane AL, Beck JC, Bleiweiss I *et al*. BRCA2 mutation-associated breast cancers exhibit a distinguishing phenotype based on morphology and molecular profiles from tissue microarrays. *Am J Surg Pathol*. 2006; 31:121–128.
14. Barnes DR, Silvestri V, CIMBA. Breast and Prostate Cancer Risks for Male BRCA1 and BRCA2 Pathogenic Variant Carriers Using Polygenic Risk Scores. *J Natl Cancer Inst.* 2022; 114(1):109-122.
15. Bergamaschi A, Tagliabue E, Sørlie T, *et al*. Extracellular matrix signature identifies breast cancer subgroups with different clinical outcome. *J Pathol.* 2008; 214(3):357-67.
16. Bianchi A, Zelli V, D'Angelo A, *et al*. A method to comprehensively identify germline SNVs, INDELs and CNVs from whole exome sequencing data of BRCA1/2 negative breast cancer patients. *NAR Genom Bioinform.* 2024; 6(2).
17. Bianchi S, Palli D, Falchetti M, *et al*. ErbB-receptors expression and survival in breast carcinoma: a 15-year follow-up study. *J Cell Physiol.* 2006; 206:702-8.
18. Biserni GB, Di Oto E, Moskovszky LE, *et al*. Preferential expression of NY-BR-1 and GATA-3 in male breast cancer. *J Cancer Res Clin Oncol.* 2018; 144(2):199-204.
19. Blanco A, de la Hoya M, Balmana J, *et al.* Detection of a large rearrangement in PALB2 in Spanish breast cancer families with male breast cancer. *Breast Cancer Res Treat.* 2012; 132:307-315.
20. Bonache S, Esteban I, Moles-Fernández A, *et al*. Multigene panel testing beyond BRCA1/2 in breast/ovarian cancer Spanish families and clinical actionability of findings. *J Cancer Res Clin Oncol.* 2018; 144(12):2495-2513.
21. Boyd J, Rhei E, Federici MG, *et al*. Male breast cancer in the hereditary nonpolyposis colorectal cancer syndrome. *Breast Cancer Res Treat.* 1999; 53(1):87-91
22. Bradley K.L, Tyldesley S, Speers C.H, *et al*. Contemporary systemic therapy for male breast cancer. *Clin. Breast Cancer*. 2014; 14(1):31–39.
23. Breast Cancer Association Consortium (BCAC). Commonly studied single-nucleotide polymorphisms and breast cancer: results from the Breast Cancer Association Consortium. *J Natl Cancer Inst*. 2006; 98:1382–1396.

24. Briggs S, Tomlinson I. Germline and somatic polymerase ε and δ mutations define a new class of hypermutated colorectal and endometrial cancers. *J Pathol.* 2013; 230(2):148-53.
25. Brinton LA, Cook MB, McCormack V, *et al*. Anthropometric and hormonal risk factors for male breast cancer: Male Breast Cancer Pooling Project results. *J Natl Cancer Inst.* 2014; 106(3).
26. Brinton LA, Richesson DA, Gierach GL, *et al*. Prospective evaluation of risk factors for male breast cancer. *J Natl Cancer Inst*. 2008; 100(20):1477–1481.
27. Bucalo A, Conti G, Valentini V, *et al*. Male breast cancer risk associated with pathogenic variants in genes other than BRCA1/2: an Italian case-control study. *Eur J Cancer.* 2023; 188:183-191.
28. Bustreo S, Osella-Abate S, Cassoni P, *et al*. Optimal Ki67 cut-off for luminal breast cancer prognostic evaluation: a large case series study with a long-term follow-up. *Breast Cancer Res Treat.* 2016; 157(2):363-371.
29. Cai H, Jing C, Chang X, *et al*. Mutational landscape of gastric cancer and clinical application of genomic profiling based on target next-generation sequencing. *J Transl Med.* 2019; 17(1):189.
30. Calip GS, Kidd J, Bernhisel R, *et al.* Family history of breast cancer in men with non-BRCA male breast cancer: implications for cancer risk counseling. *Breast Cancer Res Treat.* 2021; 185(1):195-204.
31. Callari M, Cappelletti V, De Cecco L, *et al.* Gene expression analysis reveals a different transcriptomic landscape in female and male breast cancer. *Breast Cancer Res Treat.* 2011; 127:601-10.
32. Campos FAB, Rouleau E, Torrezan GT, *et al.* Genetic Landscape of Male Breast Cancer. *Cancers (Basel).* 2021; 13(14):3535.
33. Capalbo C, Buffone A, Vestri A *et al.* Does the search for large genomic rearrangements impact BRCA PRO carrier prediction? *J ClinOncol.* 2007; 25(18):2632–2634.
34. Cardoso F, Bartlett J, Slaets L, *et al.* Characterization of male breast cancer: first results of the EORTC10085/TBCRC/BIG/ NABCG International Male BC Program. *San Antonio Breast Cancer Symposium*. 2014, European Organization for Research and Treatment of Cancer: San Antonio, Texas.

35. Cardoso F, Bartlett JMS, Slaets L, *et al.* Characterization of male breast cancer: results of the EORTC 10085/TBCRC/BIG/NABCG International Male Breast Cancer Program. *Ann Oncol.* 2018; 29(2):405-417.
36. Caruso S, Calderaro J, Letouzé E, *et al*. Germline and somatic DICER1 mutations in familial and sporadic liver tumors. *J Hepatol.* 2017; 66(4):734-742.
37. Casadei S, Norquist BM, Walsh T, *et al.* Contribution of inherited mutations in the BRCA2-interacting protein PALB2 to familial breast cancer. *Cancer Res*. 2011; 71:2222-2229.
38. Castaneda CA, Castillo M, Bernabe LA, *et al.* A biomarker study in Peruvian males with breast cancer. *World J Clin Oncol.* 2021; 12(10):926–34.
39. Castéra L, Krieger S, Rousselin A, *et al.* Next-generation sequencing for the diagnosis of hereditary breast and ovarian cancer using genomic capture targeting multiple candidate genes. *Eur J Hum Genet*. 2014; 22(11):1305-13.
40. Catana A, Apostu AP, Antemie RG. Multi Gene Panel Testing for Hereditary Breast Cancer - Is It Ready to Be Used? *Med Pharm Rep*. 2019; 92(3):220-225.
41. Chapman PB, Hauschild A, Robert C *et al*. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med.* 2011; 364:2507–2516.
42. Chen X, Xiao H, Ning S, *et al*. ERCC3 Gene Associated with Breast Cancer: A Genetic and Bioinformatic Study. *The Breast Journal*. 2024.
43. Chen X, Zhang T, Su W, *et al*. Mutant p53 in cancer: from molecular mechanism to therapeutic modulation. *Cell Death Dis.* 2022; 13(11):974.
44. Chen YB, Mirsadraei L, Jayakumaran G, *et al*. Somatic Mutations of TSC2 or MTOR Characterize a Morphologically Distinct Subset of Sporadic Renal Cell Carcinoma With Eosinophilic and Vacuolated Cytoplasm. *Am J Surg Pathol.* 2019; 43(1):121-131.
45. Cheng ML, Solit DB. Opportunities and Challenges in Genomic Sequencing for Precision Cancer Care. *Ann Intern Med.* 2018; 168(3):221-222.
46. Co M, Lee A, Kwong A. Delayed presentation, diagnosis, and psychosocial aspects of male breast cancer. *Cancer Med.* 2020; 9(10):3305-3309.
47. Da Silva L, Lakhani SR. Pathology of hereditary breast cancer. *Mod Pathol*. 2010; 23(2):S46–S51.
48. Danielsson K, Mun LJ, Lordemann A, *et al.* Next-generation sequencing applied

to rare diseases genomics. *Expert Rev. Mol. Diagn.* 2014; 14,469-487.

49. Davis PK, Brackmann RK. Chromatin remodeling and cancer. *Cancer Biol Ther.* 2003; 2(1):22-9.

50. De Blok CJM, Wiepjes CM, Nota NM, *et al.* Breast cancer risk in transgender people receiving hormone treatment: nationwide cohort study in the Netherlands. *BMJ*. 2019; 365:l1652.

51. Deb S, Jene N, Fox SB. Genotypic and phenotypic analysis of familial male breast cancer shows under representation of the HER2 and basal subtypes in BRCA-associated carcinomas. *BMC Cancer*. 2012; 12:510.

52. Deb S, Lakhani SR, Ottini L, *et al.* The cancer genetics and pathology of male breast cancer. *Histopathology*. 2016; 68:110–118.

53. Dedes KJ, Natrajan R, Lambros MB, *et al*. Down-regulation of the miRNA master regulators Drosha and Dicer is associated with specific subgroups of breast cancer. *Eur J Cancer.* 2011; 47(1):138-50.

54. Ding YC, Steele L, Kuan CJ *et al*. Mutations in BRCA2 and PALB2 in male breast cancer cases from the United States. *Breast Cancer Res Treat*. 2011; 126:771–778.

55. Dong L, Wu N, Wang S *et al.* Detection of novel germline mutations in six breast cancer predisposition genes by targeted next-generation sequencing. *Hum Mutat.* 2018; 39(10):1442-1455.

56. Dorling L, Carvalho S, Allen J, *et al*. Breast cancer risks associated with missense variants in breast cancer susceptibility genes. *Genome Med.* 2022; 14(1):51.

57. Douillard JY, Oliner KS, Siena S *et al*. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. *N Engl J Med.* 2013; 369:1023–1034.

58. Easton DF, Pharoah PD, Antoniou AC, *et al*. Gene-panel sequencing and the prediction of breast-cancer risk. *N Engl J Med*. 2015; 372:2243–57.

59. Easton DF, Pooley KA, Dunning AM, *et al*. Genome-wide association study identifies novel breast cancer susceptibility loci. *Nature*. 2007; 447(7148):1087-93.

60. Ekman S, Wynes MW, Hirsch FR. The mTOR pathway in lung cancer and implications for therapy and biomarker analysis. *J Thorac Oncol*. 2012; 7(6):947-53.

61. Esposito A, Ablah E, Okut H, *et al*. Characteristics, treatment and outcomes of HER2 positive male breast cancer. *Am J Surg.* 2023; 225(3):489-493.
62. Fabrizio DA, George TJ Jr, Dunne RF, *et al*. Beyond microsatellite testing: assessment of tumor mutational burden identifies subsets of colorectal cancer who may respond to immune checkpoint inhibition. *J Gastrointest Oncol*. 2018; 9(4):610-617.
63. Fackenthal JD, Marsh DJ, Richardson AL, *et al*. Male breast cancer in Cowden syndrome patients with germline PTEN mutations. *J Med Genet*. 2001; 38(3):159-64.
64. Falchetti M, Lupi R, Rizzolo P, *et al.* BRCA1/BRCA2 rearrangements and CHEK2 common mutations are infrequent in Italian male breast cancer cases. *Breast Cancer Res Treat.* 2008; 110(1):161–167.
65. Fanale D, Amodeo V, Corsini LR, *et al*. Breast cancer genome-wide association studies: there is strength in numbers. *Oncogene.* 2012; 31(17):2121-8.
66. Feki-Tounsi M, Khlifi R, Louati I, *et al*. Polymorphisms in XRCC1, ERCC2, and ERCC3 DNA repair genes, CYP1A1 xenobiotic metabolism gene, and tobacco are associated with bladder cancer susceptibility in Tunisian population. *Environ Sci Pollut Res Int.* 2017; 24(28):22476-22484.
67. Felicio PS, Grasel RS, Campacci N, *et al.* Whole-exome sequencing of non BRCA1/BRCA2 mutation carrier cases at high-risk for hereditary breast/ovarian cancer. *Hum Mutat.* 2021; 42(3):290-299.
68. Feliubadaló L, Lopez-Doriga A, Castellsagué E *et al*. Next-generation sequencing meets genetic diagnostics: development of a comprehensive workflow for the analysis of BRCA1 and BRCA2 genes. *Eur J Hum Genet*. 2013; 21(8):864-70.
69. Fentiman IS. Male breast cancer is not congruent with the female disease. Critical Reviews in *Oncology/Hematology*. 2016; 101:119–124.
70. Fentiman IS, Fourquet A, Hortobagyi GN. Male breast cancer. *Lancet* 2006; 367:595–604.
71. Ferzoco RM, Ruddy KJ. The Epidemiology of Male Breast Cancer *Curr Oncol Rep*. 2016; 18(1):1.

72. Finkelman BS, Zhang H, Hicks DG, *et al*. The Evolution of Ki-67 and Breast Carcinoma: Past Observations, Present Directions, and Future Considerations. *Cancers (Basel).* 2023; 15(3):808.
73. Fostira F, Saloustros E, Apostolou P, *et al.* Germline deleterious mutations in genes other than BRCA2 are infrequent in male breast cancer. *Breast Cancer Res Treat.* 2018; 169(1):105-113.
74. Foulkes WD. Inherited susceptibility to common cancers. *N Engl J Med.* 2008; 359(20):2143-53.
75. Foulkes WD, Priest JR, Duchaine TF. DICER1: mutations, microRNAs and mechanisms. *Nat Rev Cancer.* 2014; 14(10):662-72.
76. Fox S, Speirs V, Shaaban AM. Male breast cancer: an update. *Virchows Arch.* 2021.
77. Frank TS, Deffenbaugh AM, Reid JE, *et al.* Clinical characteristics of individuals with germline mutations in BRCA1 and BRCA2: analysis of 10,000 individuals. *J Clin Oncol*. 2002; 20, 1480–1490.
78. Froimchuk E, Jang Y, Ge K. Histone H3 lysine 4 methyltransferase KMT2D. *Gene.* 2017; 627:337-342.
79. Fukusumi T, Califano JA. The NOTCH Pathway in Head and Neck Squamous Cell Carcinoma. *J. Dent. Res.* 2018; 97, 645–653.
80. Gampenrieder SP, Rinnerthaler G, Tinchon C, *et al*. Landscape of HER2-low metastatic breast cancer (MBC): Results from the Austrian AGMT_MBC-Registry. *Breast Cancer Res.* 2021; 23:112.
81. Gao F, Gu D, Zhang H, *et al*. Case report: response to immunotherapy and association with the fh gene in hereditary leiomyomatosis and renal cell cancer-associated renal cell cancer. *BMC Med Genomics.* 2024; 17(1):215.
82. García MJ, Fernández V, Osorio A, *et al.* Analysis of FANCB and FANCN/PALB2 fanconianemia genes in BRCA1/2-negative Spanish breast cancer families. *Breast Cancer Res Treat*. 2009; 113:545–551.
83. Ge Y, Sneige N, Eltorky MA, *et al*. Immunohistochemical characterization of subtypes of male breast carcinoma. *Breast Cancer Res.* 2009; 11:R28.

84. Ghoussaini M, Fletcher O, Michailidou K, *et al*. Genome-wide association analysis identifies three new breast cancer susceptibility loci. *Nat Genet*. 2012; 44(3):312-8.

85. Giordano SH, Buzdar AU, Hortobagyi GN. Breast cancer in men. *Ann Intern Med.* 2002; 137(8):678-87.

86. Giordano SH. A review of the diagnosis and management of male breast cancer. *Oncologist.* 2005; 10:471–479.

87. Goldhirsch A, Winer EP, Coates AS, *et al.* Panel members: personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer. *Ann Oncol.* 2013; 24:2206–2223.

88. Grabiner BC, Nardi V, Birsoy K, *et al*. A diverse array of cancer-associated MTOR mutations are hyperactivating and can predict rapamycin sensitivity. *Cancer Discov.* 2014; 4(5):554-63.

89. Grasso CS, Wu YM, Robinson DR, *et al*. The mutational landscape of lethal castration-resistant prostate cancer. *Nature.* 2012; 487(7406):239-43.

90. Gucalp A, Traina TA, Eisner JR, *et al.* Male Breast Cancer: A Disease Distinct from Female Breast Cancer. *Breast Cancer Res.* 2019; 173, 37–48.

91. Hall MJ, Bernhisel R, Hughes E, *et al.* Germline Pathogenic Variants in the Ataxia Telangiectasia Mutated (ATM) Gene are Associated with High and Moderate Risks for Multiple Cancers. *Cancer Prev Res (Phila)*. 2021; 14(4):433-440.

92. Hallamies S, Pelttari LM, Poikonen-Saksela P, *et al*. CHEK2 c.1100delC mutation is associated with an increased risk for male breast cancer in Finnish patient population. *BMC Cancer.* 2017; 17(1):620.

93. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011; 144(5):646-74.

94. Hansen J. Elevated risk for male breast cancer after occupational exposure to gasoline and vehicular combustion products. *Am J Indust Med.* 2000; 37(4):349-52.

95. Hansen TO, Jønson L, Albrechtsen A, *et al*. Large BRCA1 and BRCA2 genomic rearrangements in Danish high-risk breast-ovarian cancer families. *Breast Cancer Res Treat.* 2009; 115:315–323.

96. Hartman P, Beckman K, Silverstein K, *et al*. Next generation sequencing for clinical diagnostics: Five year experience of an academic laboratory. *Molecular Genetics and Metabolism Reports*. 2019; 19,100464.

97. Hao S, Huang M, Tian W, *et al*. Whole Exome Sequencing in the Male Breast Cancer with Prolactinoma: A Case Report and Literature Review. *J Breast Cancer.* 2020; 23(6):656-664.

98. Honrado E, Osorio A, Palacios J, *et al*. Pathology and gene expression of hereditary breast tumors associated with BRCA1, BRCA2 and CHEK2 gene mutations. *Oncogene.* 2006; 25:5837–5845.

99. Hong JH, Ha KS, Jung YH, *et al.* Clinical Features of Male Breast Cancer: Experiences from Seven Institutions Over 20 Years. *Cancer Res Treat.* 2016; 48(4):1389-1398.

100. Howlader N, Noone AM, Krapcho M, *et al*. SEER Cancer Statistics Review, 1975-2011, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2011/, based on November 2013 SEER data submission, posted to the SEER web site, April 2014.

101. Howlader N, Noone AM, Krapcho M, *et al*. SEER cancer statistics review. *National Cancer Institute: Bethesda, MD.* 2016; 1975–2013.

102. Hu Q, Zhang B, Chen R, *et al*. ZFHX3 is indispensable for ERβ to inhibit cell proliferation via MYC downregulation in prostate cancer cells. *Oncogenesis.* 2019; 8(4):28.

103. Humphries MP, Sundara Rajan S, Honarpisheh H, *et al*. Characterisation of male breast cancer: a descriptive biomarker study from a large patient series. *Sci Rep.* 2017; 7:45293.

104. Imyanitov EN, Kuligina ES, Sokolenko AP, *et al*. Hereditary cancer syndromes. *World J Clin Oncol.* 2023; 14(2):40-68.

105. Iorfida M, Bagnardi V, Rotmens N, *et al*. Outcome of male breast cancer: a matched single-institution series. *Clin. Breast Cancer*. 2014; 14(5):371–377.

106. Ishii T, Nakano E, Watanabe T, *et al.* Epidemiology and practice patterns for male breast cancer compared with female breast cancer in Japan. *Cancer Med.* 2020; 9(16):6069-6075.

107. Jiao X, Wood LD, Lindman M, *et al*. Somatic mutations in the Notch, NF-KB, PIK3CA, and Hedgehog pathways in human breast cancers. *Genes Chromosomes Cancer.* 2012; 51(5):480-9.

108. Johansson I, Killander F, Linderholm B, *et al.* Molecular profiling of male breast cancer - lost in translation? *Int J Biochem Cell Biol*. 2014; 53:526-35.

109. Johansson I, Nilsson C, Berglund P, *et al.* Gene expression profiling of primary male breast cancers reveals two unique subgroups and identifies N-acetyltransferase-1 (NAT1) as a novel prognostic biomarker. *Breast Cancer Res.* 2012; 14(1):R31.

110. Jones S, Hruban RH, Kamiyama M, *et al.* Exomic sequencing identifies PALB2 as a pancreatic cancer susceptibility gene. *Science, 324.* 2009; 217.

111. Jun S, Park H, Kim UJ, *et al*. Cancer risk based on alcohol consumption levels: a comprehensive systematic review and meta-analysis. *Epidemiol Health.* 2023; 45:e2023092.

112. Jung SY, Han W, Lee JW, *et al*. Ki-67 Expression Gives Additional Prognostic Information on St. Gallen 2007 and Adjuvant! Online Risk Categories in Early Breast Cancer. *Ann. Surg. Oncol.* 2009; 16:1112–1121.

113. Karamanakos P, Mitsiades CS, Lembessis P, *et al*. Male breast adenocarcinoma in a prostate cancer patient following prolonged anti-androgen monotherapy. *Anticancer Res.* 2004; 24:1077-81.

114. Karapetis CS, Khambata-Ford S, Jonker DJ *et al*. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med.* 2008; 359:1757-1765.

115. Karhu R, Laurila E, Kallioniemi A, *et al*. Large genomic BRCA2 rearrangements and male breast cancer. *Cancer Detect Prev.* 2006; 30:530–534.

116. Kaufmann M, Pusztai L, Biedenkopf Expert Panel Members. Use of standard markers and incorporation of molecular markers into breast cancer therapy: consensus recommendations from an International Expert Panel. *Cancer.* 2011; 117(8):1575–82.

117. Khan MH, Allerton R, Pettit L. Hormone Therapy for Breast Cancer in Men. *Clin Breast Cancer.* 2015; 15:245-50.

118. Khan NAJ, Tirona M. An updated review of epidemiology, risk factors, and management of male breast cancer. *Med Oncol.* 2021; 38(4):39.

119. Khare VS, Huda F, Misra S, *et al*. Male Breast Cancer: An Updated Review of Patient Characteristics, Genetics, and Outcome. *Int J Breast Cancer.* 2024; 2024:9003572.

120. Kiiski JI, Pelttari LM, Khan S, *et al*. Exome sequencing identifies FANCM as a susceptibility gene for triple-negative breast cancer. *Proc Natl Acad Sci U S A*. 2014; 111(42):15172-7.

121. Kleibl Z, Kristensen VN. Women at high risk of breast cancer: Molecular characteristics, clinical presentation and management. *Breast.* 2016; 28:136-44.

122. Kong Y, Li J, Lin H, *et al*. Landscapes of synchronous multiple primary cancers detected by next-generation sequencing. *FEBS Open Bio.* 2022; 12(11):1996-2005.

123. Korde LA, Zujewski JA, Kamin L, *et al.* Multidisciplinary meeting on male breast cancer: summary and research recommendations. *J Clin Oncol*. 2010; 28, 2114–22.

124. Kornegoor R, Verschuur-Maes AHJ, Buerger H, *et al*. Molecular subtyping of male breast cancer by immunohistochemistry. *Mod. Pathol*. 2012; 25:398–404.

125. Ku CS, Naidoo N, Pawitan Y. Revisiting Mendelian disorders through exome sequencing. *Hum Genet.* 2011; 129:351–370.

126. Lapunzina P, López RO, Rodríguez-Laguna L, *et al.* Impact of NGS in the medical sciences: genetic syndromes with an increased risk of developing cancer as an example of the use of new technologies. *Genet Mol Biol*. 2014; 37(1 Suppl):241-9.

127. Larsson SC, Carter P, Kar S, *et al*. Smoking, alcohol consumption, and cancer: A mendelian randomisation study in UK Biobank and international genetic consortia participants. *PLoS Med.* 2020; 17(7):e1003178.

128. Lecarpentier J, Silvestri V, Kuchenbaecker KB, *et al.* Prediction of Breast and Prostate Cancer Risks in Male BRCA1 and BRCA2 Mutation Carriers Using Polygenic Risk Scores. *J Clin Oncol.* 2017; 35(20):2240-2250.

129. Lek M, Karczewski KJ, Minikel EV, *et al*. Analysis of protein-coding genetic variation in 60,706 humans. *Nature.* 2016; 536:285–91.

130. Lee CS, Song IH, Lee A, *et al* Enhancing the landscape of colorectal cancer using targeted deep sequencing. *Sci Rep.* 2021; 11(1):8154.

131. Lee JS, Zhao H. On Estimation of Allele Frequencies via Next-Generation DNA Resequencing with Barcoding. *Stat Biosci.* 2013; 5(1):26-53.

132. Leone JP, Leone J, Zwenger AO, *et al*. Prognostic significance of tumor subtypes in male breast cancer: a population-based study. *Breast Cancer Res Treat*. 2015; 152(3):601–9.

133. Li N, Zethoven M, McInerny S, *et al*. Evaluation of the association of heterozygous germline variants in NTHL1 with breast cancer predisposition: an international multi-center study of 47,180 subjects. *NPJ Breast Cancer.* 2021; 7(1):52.

134. Li S, Silvestri V, Leslie G, *et al*. Cancer Risks Associated with *BRCA1* and *BRCA2* Pathogenic Variants. *J Clin Oncol.* 2022; 40(14):1529-1541.

135. Liede A, Karlan BY, Narod SA. Cancer risks for male carriers of germline mutations in BRCA1 or BRCA2: a review of the literature. *J Clin Oncol.* 2004; 22(4):735–742.

136. Liu Y, Yin W, Yan T, *et al*. The clinical significance of Ki-67 as a marker of prognostic value and chemosensitivity prediction in hormone-receptor-positive breast cancer: A meta-analysis of the published literature. *Curr. Med. Res. Opin.* 2013; 29:1453–1461.

137. Losurdo A, Rota S, Gullo G, *et al*. Controversies in clinicopathological characteristics and treatment strategies of male breast cancer: A review of the literature. *Crit Rev Oncol Hematol.* 2017; 113:283-291.

138. Lu C, Xie M, Wendl MC, *et al*. Patterns and functional implications of rare germline variants across 12 cancer types. *Nat Commun.* 2015; 6:10086.

139. Łukasiewicz S, Czeczelewski M, Forma A, *et al*. Breast Cancer-Epidemiology, Risk Factors, Classification, Prognostic Markers, and Current Treatment Strategies-An Updated Review. *Cancers (Basel).* 2021; 13(17):4287.

140. Ly D, Forman D, Ferlay J, *et al.* An international comparison of male and female breast cancer incidence rates. *Int J Cancer.* 2013; 132:1918-1926.

141. MacArthur DG, Balasubramanian S, Frankish A, *et al*. A systematic survey of loss-of-function variants in human protein-coding genes. *Science.* 2012; 335(6070):823-8.

142. Magliacane G, Grassini G, Bartocci P *et al*. Rapid targeted somatic mutation analysis of solid tumors in routine clinical diagnostics. *Oncotarget.* 2015.

143. Magrin L, Fanale D, Brando C, *et al*. POLE, POLD1, and NTHL1: the last but not the least hereditary cancer-predisposing genes. *Oncogene.* 2021; 40(40):5893-5901.

144. Maguire S, Perraki E, Tomczyk K, *et al.* Common Susceptibility Loci for Male Breast Cancer. *J Natl Cancer Inst*. 2021; 113(4):453-461.

145. Maraver A, Fernandez-Marcos PJ, Cash TP, *et al*. NOTCH pathway inactivation promotes bladder cancer progression. *J Clin Invest.* 2015; 125(2):824-30.

146. Marshall CR, Chowdhury S, Taft RJ, *et al*. Best practices for the analytical validation of clinical whole-genome sequencing intended for the diagnosis of germline disease. *NPJ Genomic Medicine*. 2020; 5,47.

147. Masala G, Ambrogetti D, Assedi M, *et al*. Dietary and lifestyle determinants of mammographic breast density. A longitudinal study in a Mediterranean population. *Int J Cancer.* 2006; 118(7):1782-9.

148. Masci G, Caruso M, Caruso F, *et al*. Clinicopathological and immunohistochemical characteristics in male breast cancer: a retrospective case series. *Oncologist.* 2015; 20(6):586–92.

149. Matthijs G, Souche E, Alders M, *et al*. Guidelines for diagnostic next-generation sequencing. *European Journal of Human Genetics*. 2016; 24(10), 1515.

150. Mavaddat N, Barrowdale D, Andrulis IL, *et al*. Pathology of breast and ovarian cancers among BRCA1 and BRCA2 mutation carriers: results from the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA). Cancer Epidemiol Biomarkers Prev. 2012; 21:134–147.

151. Mavaddat N, Michailidou K, Dennis J, *et al*. Polygenic Risk Scores for Prediction of Breast Cancer and Breast Cancer Subtypes. *Am J Hum Genet.* 2019; 104(1):21-34.

152. McCarthy JJ, McLeod HL, Ginsburg GS. Genomic medicine: a decade of successes, challenges, and opportunities. *Sci Transl Med.* 2013; 5(189):189sr4.

153. Meijers-Heijboer H, van den Ouweland A, Klijn J, *et al.* Low-penetrance susceptibility to breast cancer due to CHEK2 (*) 1100delC in noncarriers of BRCA1 or BRCA2 mutations. *Nat Genet*. 2002; 31(1):55-9.

154. Metzker ML. Sequencing technologies - the next generation. *Nat Rev Genet.* 2010; 11(1):31- 46.

155. Miao H, Verkooijen HM, Chia KS, *et al.* Incidence and outcome of male breast cancer: an international population-based study. *J Clin Oncol.* 2011; 29(33):4381-6.

156. Misyura M, Zhang T, Sukhai MA, *et al*. Comparison of Next-Generation Sequencing Panels and Platforms for Detection and Verification of Somatic Tumor Variants for Clinical Diagnostics. *J Mol Diagn.* 2016; 18(6):842-850.

157. Moelans CB, de Ligt J, van der Groep P, *et al.* The molecular genetic make-up of male breast cancer. *Endocr Relat Cancer.* 2019; 26(10):779–94.

158. Mouelle M, Meka E, Mathelin C, *et al.* Management of men with high genetic risk of breast cancer. Is there a place for screening or risk-reducing surgery? Case report and review. *Current Problems in Cancer: Case Reports.* 2023.

159. Narasimhan V, Hunt KA, Mason D, *et al*. Health and population effects of rare gene knockouts in adult humans with related parents. *Science.* 2016; 8624:1–8.

160. Neben CL, Zimmer AD, Stedden W, *et al.* Multi-Gene Panel Testing of 23,179 Individuals for Hereditary Cancer Risk Identifies 585 Pathogenic Variant Carriers Missed by Current Genetic Testing Guidelines. *J Mol Diagn*. 2019; 21, 646–57.

161. Ng SB, Turner EH, Robertson PD, *et al*. Targeted capture and massively parallel sequencing of 12 human exomes. *Nature, 461*. 2009; 272-276.

162. Nurmi AK, Pelttari LM, Kiiski JI, *et al*. NTHL1 is a recessive cancer susceptibility gene. *Sci Rep.* 2023; 13(1):21127.

163. Olivier M, Hollstein M, Hainaut P. TP53 mutations in human cancers: origins, consequences, and clinical use. *Cold Spring Harb Perspect Biol*. 2010; 2(1):a001008.

164. Orr N, Cooke R, Jones M, *et al*. Genetic variants at chromosomes 2q35, 5p12, 6q25.1, 10q26.13, and 16q12.1 influence the risk of breast cancer in men. *PLoS Genet.* 2011; 7:e1002290.

165. Orr N, Lemnrau A, Cooke R, *et al*. Genome-wide association study identifies a common variant in RAD51B associated with male breast cancer risk. *Nat Genet.* 2012; 44(11):1182–1184.

166. Ortega-Lozano AJ, Gómez-Caudillo L, Briones-Herrera A, *et al*. Characterization of Mitochondrial Proteome and Function in Luminal A and Basal-like Breast Cancer Subtypes Reveals Alteration in Mitochondrial Dynamics and Bioenergetics Relevant to Their Diagnosis. *Biomolecules.* 2022; 12(3):379.

167. Ottini, L. Male breast cancer: A rare disease that might uncover underlying pathways of breast cancer. *Nat. Rev. Cancer.* 2014; 14(10):643.

168. Ottini L, Masala G, D'Amico C, *et al*. BRCA1 and BRCA2 mutation status and tumor characteristics in male breast cancer: a population-based study in Italy. *Cancer Res.* 2003; 63(2):342-7.

169. Ottini L, Palli D, Rizzo S, *et al*. Male breast cancer. *Crit Rev Oncol Hematol.* 2010; 73(2):141-55.

170. Ottini L, Rizzolo P, Zanna I, *et al.* BRCA1/BRCA2 mutation status and clinical-pathologic features of 108 male breast cancer cases from Tuscany: a population-based study in central Italy. *Breast Cancer Res Treat.* 2009; 116(3):577-86.

171. Ottini L, Silvestri V, Rizzolo P, *et al*. Clinical and pathologic characteristics of BRCA-positive and BRCA-negative male breast cancer patients: results from a collaborative multicenter study in Italy. *Breast Cancer Res Treat.* 2012; 134(1):411-8.

172. Ottini L, Silvestri V, Saieva C, *et al*. Association of low-penetrance alleles with male breast cancer risk and clinicopathological characteristics: results from a multicenter study in Italy. *Breast Cancer Res Treat.* 2013; 138(3):861-8.

173. Padron-Monedero A, Koru-Sengul T, Tannenbaum SL, *et al*. Smoking and survival in male breast cancer patients. *Breast Cancer Res Treat.* 2015; 153(3):679-87.

174. Palli D, Masala G, Mariani-Costantini R, *et al*. A gene-environment interaction between occupation and BRCA1/BRCA2 mutations in male breast cancer? *Eur J Cancer.* 2004; 40(16),2474-9.

175. Pang X, Xie R, Zhang Z, *et al*. Identification of SPP1 as an Extracellular Matrix Signature for Metastatic Castration-Resistant Prostate Cancer. *Front Oncol.* 2019; 9:924.

176. Park S, Ahn S, Kim DG, *et al*. High Frequency of Juxtamembrane Domain *ERBB2* Mutation in Gastric Cancer. *Cancer Genomics Proteomics.* 2022; 19(1):105-112.

177. Park WK, Nam SJ, Kim SW, *et al*. The Impact of HER2-Low Expression on Oncologic Outcomes in Hormone Receptor-Positive Breast Cancer. *Cancers.* 2023; 15:5361.

178. Paulo P, Maia S, Pinto C *et al.* Targeted next generation sequencing identifies functionally deleterious germline mutations in novel genes in early-onset/familial prostate cancer. *PLoS Genet*. 2018; 14(4).

179. Peiffer DS, Zhao F, Chen N, *et al*. Clinicopathologic Characteristics and Prognosis of ERBB2-Low Breast Cancer Among Patients in the National Cancer Database. *JAMA Oncol.* 2023; 9:500–510.

180. Pellini F, Granuzzo E, Urbani S, *et al*. Male Breast Cancer: Surgical and Genetic Features and a Multidisciplinary Management Strategy. *Breast Care (Basel).* 2020; 15(1):14-20.

181. Perou CM, Sorlie T, Eisen MB. Molecular portraits of human breast tumours. *Nature.* 2000; 406(6797):747–752.

182. Pharoah PD, Antoniou A, Bobrow M, *et al.* Polygenic susceptibility to breast cancer and implications for prevention. *Nat Genet.* 2002; 31:33–36.

183. Piscuoglio S, Ng CK, Murray MP, *et al*. The Genomic Landscape of Male Breast Cancers. *Clin Cancer Res.* 2016; 22:4045-56.

184. Popova NV, Jücker M. The Functional Role of Extracellular Matrix Proteins in Cancer. *Cancers (Basel).* 2022; 14(1):238.

185. Pozzo F, Bittolo T, Tissino E, *et al*. Multiple Mechanisms of NOTCH1 Activation in Chronic Lymphocytic Leukemia: NOTCH1 Mutations and Beyond. *Cancers.* 2022; 14(12):2997.

186. Pritchard CC, Mateo J, Walsh MF, *et al*. Inherited DNA-Repair Gene Mutations in Men with Metastatic Prostate Cancer. *N Engl J Med.* 2016; 375(5):443-53.

187. Pritzlaff M, Summerour P, McFarland R, *et al.* Male breast cancer in a multi-gene panel testing cohort: insights and unexpected results. *Breast Cancer Res Treat.* 2017; 161(3):575-586.

188. Qing L, Gu P, Liu M, *et al*. Extracellular Matrix-Related Six-lncRNA Signature as a Novel Prognostic Biomarker for Bladder Cancer. *Onco Targets Ther*. 2020; 13:12521-12538.

189. Rabbani B, Tekin M, Mahdieh N. The promise of whole-exome sequencing in medical genetics. *J. Hum. Genet, 59*. 2014; 5-15.

190. Rahman N, Seal S, Thompson D, *et al.* PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nat Genet.* 2007; 39:165-167.

191. Rampias T, Vgenopoulou P, Avgeris M, *et al*. A new tumor suppressor role for the Notch pathway in bladder cancer. *Nat Med.* 2014; 20(10):1199-205.

192. Reddington R, Galer M, Hagedorn A, *et al*. Incidence of Male Breast Cancer in Scotland over a Twenty-Five-Year Period (1992–2017). *Eur J Surg Oncol.* 2020; 46(8):1546-1550.

193. Rey JM, Ducros V, Pujol P *et al.* Improving Mutation Screening in Patients with Colorectal Cancer Predisposition Using Next-Generation Sequencing. *J Mol Diagn.* 2017; 19(4):589-601.

194. Riascos MC, Huynh A, Faquin WC, *et al*. Expanding Our Knowledge of DICER1 Gene Alterations and Their Role in Thyroid Diseases. *Cancers*. 2024; 16(2):347.

195. Rizzolo P, Silvestri V, Falchetti M, *et al*. Breast Cancer: Not Only a "Woman's" Disease. *Current Women's Health Reviews.* 2012; 8:55-64.

196. Rizzolo P, Silvestri V, Tommasi S, *et al.* Male breast cancer: genetics, epigenetics, and ethical aspects. *Ann Oncol.* 2013; 24:Suppl8:viii75-viii82.

197. Rizzolo P, Zelli V, Silvestri V, *et al*. Insight into genetic susceptibility to male breast cancer by multigene panel testing: Results from a multicenter study in Italy. *Int J Cancer*. 2019; 145(2):390-400.

198. Rocca C, Rocca G, Zampieri P, *et al*. Somatic and Germline Variants Affect Prognosis and Susceptibility in Prostate Cancer. *Anticancer Res.* 2023; 43(7):2941-2949.

199. Rolfes M, Borde J, Möllenhoff K, *et al*. Prevalence of Cancer Predisposition Germline Variants in Male Breast Cancer Patients: Results of the German Consortium for Hereditary Breast and Ovarian Cancer. *Cancers (Basel).* 2022; 14(13):3292.

200. Rosenblatt KA, Thomas DB, McTiernan A, *et al*. Breast cancer in men: aspects of familial aggregation. *J Natl Cancer Inst.* 1991; 83(12):849–854.

201. Rotunno M, Barajas R, Clyne M, *et al*. A Systematic Literature Review of Whole Exome and Genome Sequencing Population Studies of Genetic Susceptibility to Cancer. *Cancer Epidemiol Biomarkers Prev.* 2020; 29(8):1519-1534.

202. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A.* 1977; 74(12):5463-7.

203. Sarmiento S, McColl M, Musavi L, *et al*. Male Breast Cancer: A Closer Look at Patient and Tumor Characteristics and Factors That Affect Survival Using the National Cancer Database. *Breast Cancer Res Treat.* 2020; 180, 471–9.

204. Scarpitta R, Zanna I, Aretini P, *et al.* Germline investigation in male breast cancer of DNA repair genes by next-generation sequencing. *Breast Cancer Res Treat.* 2019; 178(3), 557-564.

205. Schrader KA, Stratton KL, Murali R, *et al*. Genome Sequencing of Multiple Primary Tumors Reveals a Novel PALB2 Variant. *J Clin Oncol.* 2016; 34(8):e61-7.

206. Schwemmle S, Pfeifer GP. Genomic structure and mutation screening of the E2F4 gene in human tumors. *Int J Cancer.* 2000; 86(5):672-7.

207. Shaaban AM, Ball GR, Brannan RA, *et al*. A comparative biomarker study of 514 matched cases of male and female breast cancer reveals gender specific biological differences. *Breast Cancer Res Treat.* 2012; 133(3):949-58.

208. Shi Q, Xue C, Zeng Y, *et al*. Notch signaling pathway in cancer: from mechanistic insights to targeted therapies. *Signal Transduct Target Ther.* 2024; 9(1):128.

209. Siegel R L, Miller KD, Fuchs HE, *et al*. Cancer Statistics, 2021. *CA A Cancer J Clin.* 2021; 71(1):7–33.

210. Silvestri V, Barrowdale D, Mulligan AM, *et al*. Male breast cancer in BRCA1 and BRCA2 mutation carriers: pathology data from the Consortium of Investigators of Modifiers of BRCA1/2. *Breast Cancer Res.* 2016; 18(1):15.

211. Silvestri V, Leslie G, CIMBA Group, *et al*. Characterization of the Cancer Spectrum in Men with Germline BRCA1 and BRCA2 Pathogenic Variants: Results From the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA). *JAMA Oncol.* 2020; 6(8):1218-1230.

212. Silvestri V, Rizzolo P, Falchetti M, *et al.* Mutation analysis of BRIP1 in male breast cancer cases: a population-based study in Central Italy. *Breast Cancer Res Treat.* 2011a; 126(2):539-543.

213. Silvestri V, Rizzolo P, Falchetti M, *et al.* Mutation screening of RAD51C in male breast cancer patients. *Breast Cancer Res.* 2011b; 13(1):404.

214. Silvestri V, Rizzolo P, Scarnò M, *et al.* Novel and known genetic variants for male breast cancer risk at 8q24.21, 9p21.3, 11q13.3 and 14q24.1: results from a multicenter study in Italy. *Eur J Cancer*. 2015; 51(16):2289-95.

215. Silvestri V, Rizzolo P, Zanna I, *et al*. PALB2 Mutations in male breast cancer: a population-based study in Central Italy. *Breast Cancer Res Treat.* 2010; 122:299–301.

216. Silvestri V, Rizzolo P, Zelli V, *et al*. A possible role of FANCM mutations in male breast cancer susceptibility: Results from a multicenter study in Italy. *Breast.* 2018; 38,92-97.

217. Silvestri V, Valentini V, Bucalo A, *et al*. HER2-Low Expression in Male Breast Cancer: Results from a Multicenter Series in Italy. *Cancers (Basel).* 2024; 16(3):548.

218. Silvestri V, Zelli V, Valentini V, *et al.* Whole-exome sequencing and targeted gene sequencing provide insights into the role of PALB2 as a male breast cancer susceptibility gene. *Cancer*. 2017; 123(2):210-218.

219. Sluiter MD, van Rensburg EJ. Large genomic rearrangements of the BRCA1 and BRCA2 genes: review of the literature and report of a novel BRCA1 mutation. *Breast Cancer Res Treat.* 2011; 125:325–349.

220. Snape K, Ruark E, Tarpey P, *et al*. Predisposition gene identification in common cancers by exome sequencing: insights from familial breast cancer. *Breast Cancer Res Treat.* 2012; 134(1):429-33.

221. Sokolenko AP, Suspitsin EN, Kuligina ESh, *et al*. Identification of novel hereditary cancer genes by whole exome sequencing. *Cancer Lett.* 2015; 369(2):274-88.

222. Somaiah N, Simon GR. Molecular targeted agents and biologic therapies for non-small cell lung cancer. *J Thorac Oncol.*; 5(12 Suppl 6):S434-54.

223. Speirs V and Shaaban AM. The Rising Incidence of Male Breast Cancer. *Breast Cancer Res Treat.* 2009; 115, 429–30.

224. Stadler ZK, Schrader KA, Vijai J, *et al*. Cancer genomics and inherited risk. *J. Clin. Oncol, 32.* 2014; 687-698.

225. Sun JW, Li XR, Gao HY *et al*. Electromagnetic field exposure and male breast cancer risk: a meta-analysis of 18 studies. *Asian Pac J Cancer Prev.* 2013; 14, 523-528.

226. Susswein LR, Marshall ML, Nusbaum R *et al*. Pathogenic and likely pathogenic variant prevalence among the first 10,000 patients referred for next-generation cancer panel testing. *Genet Med.* 2016; 18(8), 823–832.

227. Syrjäkoski K, Kuukasjärvi T, Auvinen A, *et al.* CHEK2 1100delc is not a risk factor for male cancer population. *Int J Cancer.* 2004; 108:475–476.

228. Takaku M, Grimm SA, Wade PA. GATA3 in Breast Cancer: Tumor Suppressor or Oncogene? *Gene Expr*. 2015; 16(4):163-8.

229. Tedaldi G, Tebaldi M, Zampiga V, *et al*. Male Breast Cancer: Results of the Application of Multigene Panel Testing to an Italian Cohort of Patients. *Diagnostics (Basel).* 2020; 10(5):269.

230. Thomas E. Original research: men's awareness and knowledge of male breast cancer. *Am J Nurs.* 2010; 110(10):32–7.

231. Thompson ER, Gorringe KL, Rowley SM, *et al.* Prevalence of PALB2 mutations in Australian familial breast cancer cases and controls. *Breast Cancer Res*. 2015; 17:111.

232. Tournier I, Paillerets BB, Sobol H, *et al*. Significant contribution of germline BRCA2 rearrangements in male breast cancer families. *Cancer Res.* 2004; 64:8143–8147.

233. Trujillano D, Weiss ME, Schneider J *et al*. Next-generation sequencing of the BRCA1 and BRCA2 genes for the genetic diagnostics of hereditary breast and/or ovarian cancer. *J Mol Diagn*. 2015;17(2):162-70.

234. Tung N, Battelli C, Allen B, *et al*. Frequency of mutations in individuals with breast cancer referred for BRCA1 and BRCA2 testing using next-generation sequencing with a 25-gene panel. *Cancer*. 2015; 121:25–33.

235. Turnbull C, Ahmed S, Morrison J, *et al.* Genome-wide association study identifies five new breast cancer susceptibility loci. *Nat Genet.* 2010; 42:504–507.

236. Valentini V, Silvestri V, Bucalo A, *et al*. Molecular profiling of male breast cancer by multigene panel testing: Implications for precision oncology. *Front Oncol.* 2023; 12,1092201.

237. Valentini V, Bucalo A, Conti G, *et al.* Gender-Specific Genetic Predisposition to Breast Cancer: BRCA Genes and Beyond. *Cancers.* 2024; 16, 579.

238. Van Marcke C, Helaers R, De Leener A, *et al*. Tumor sequencing is useful to refine the analysis of germline variants in unexplained high-risk breast cancer families. *Breast Cancer Res.* 2020; 22(1):36.

239. van Vugt MATM, Parkes EE. When breaks get hot: inflammatory signaling in BRCA1/2-mutant cancers. Trends. *Cancer*. 2022; 8(3):174-189.

240. Vandenberga LN, Schaeberleb CM, Rubinb BS, *et al*. The male mammary gland: a target for the xenoestrogen bisphenol A. *Reprod Toxicol.* 2013; 37:15–23.

241. Varga Z, Li Q, Jochum W, *et al*. Ki-67 assessment in early breast cancer: SAKK28/12 validation study on the IBCSG VIII and IBCSG IX cohort. *Sci. Rep.* 2019; 9:13534.

242. Vargas AC, Reis-Filho JS, Lakhani SR. Phenotype-genotype correlation in familial breast cancer. *J Mammary Gland Biol Neoplasia.* 2011; 16:27–40.

243. Vestergaard LK, Oliveira DNP, Høgdall CK, *et al*. Next generation sequencing technology in the clinic and its challenges. *Cancers.* 2021; 13(8):1751.

244. Vijai J, Topka S, Villano D, *et al*. A Recurrent ERCC3 Truncating Mutation Confers Moderate Risk for Breast Cancer. *Cancer Discov.* 2016; 6(11):1267-1275.

245. Vietri MT, Caliendo G, Casamassimi A, *et al.* A novel PALB2 truncating mutation in an Italian family with male breast cancer. *Oncol Rep.* 2015; 33:1243-1247.

246. Villeneuve S, Cyr D, Lynge E *et al*. Occupation and occupational exposure to endocrine disrupting chemicals in male breast cancer: a case control study in Europe. *Occupat Environ Med.* 2010; 7, 837-844.

247. Wang ZC, Lin M, Wei LJ, *et al*. Loss of heterozygosity and its correlation with expression profiles in subclasses of invasive breast cancers. *Cancer Res.* 2004; 64(1):64-71.

248. Wang F, Shu X, Meszoely I, *et al*. Overall Mortality After Diagnosis of Breast Cancer in Men vs Women. *JAMA Oncol.* 2019; 5(11):1589-1596.

249. Wang A, Li Z, Wang M, *et al*. Molecular characteristics of synchronous multiple gastric cancer. *Theranostics.* 2020; 10(12):5489-5500.

250. Walsh T, Lee MK, Casadei S *et al.* Detection of inherited mutations for breast and ovarian cancer using genomic capture and massively parallel sequencing. *Proc Natl Acad Sci USA.* 2010; 107, 12629-12633.

251. Walsh T, Casadei S, Lee MK, *et al.* Mutations in 12 genes for inherited ovarian, fallopian tube, and peritoneal carcinoma identified by massively parallel sequencing. *Proc Natl Acad Sci USA.* 2011; 108, 18032-18037.

252. Wasielewski M, den Bakker MA, van denOuweland A, *et al.* CHEK21100delC and male breast cancer in the Netherlands. *Breast CancerRes Treat* 2009; 116:397–400.

253. Weiss JR, Moysich KB, Swede H. Epidemiology of male breast cancer. *Cancer Epidemiol Biomarkers Prev.* 2005; 14(1), 20-6.

254. Won HS, Ahn J, Kim Y,. Clinical significance of HER2-low expression in early breast cancer: A nationwide study from the Korean Breast Cancer Society. *Breast Cancer Res.* 2022; 24:22.

255. Xue L, Li W, Fan X, *et al*. Identification of second primary tumors from lung metastases in patients with esophageal squamous cell carcinoma using whole-exome sequencing. *Theranostics.* 2020; 10(23):10606-10618.

256. Yan L, Zhang Y, Ding B, *et al*. Genetic alteration of histone lysine methyltransferases and their significance in renal cell carcinoma. *PeerJ.* 2019; 7:e6396.

257. Yang X, Leslie G, Doroszuk A, *et al.* Cancer risks associated with germline PALB2 pathogenic variants: an international study of 524 families. *J Clin Oncol.* 2020; 38, 674–685.

258. Yang Z, Zhang C, Qi W, *et al*. Tenascin-C as a prognostic determinant of colorectal cancer through induction of epithelial-to-mesenchymal transition and proliferation. *Exp Mol Pathol.* 2018; 105(2):216-222.

259. Yao Y, Chu Y, Xu B, *et al*. Risk factors for distant metastasis of patients with primary triple-negative breast cancer. *Biosci Rep.* 2019; 39(6):BSR20190288.

260. Yap TA, Stadler ZK, Stout LA, *et al*. Aligning Germline Cancer Predisposition With Tumor-Based Next-Generation Sequencing for Modern Oncology Diagnosis, Interception, and Therapeutic Development. *Am Soc Clin Oncol Educ Book.* 2023; 43:e390738.

261. Yuan X, Zhang M, Wu H, *et al*. Expression of Notch1 Correlates with Breast Cancer Progression and Prognosis. *PLoS One.* 2015; 10(6):e0131689.

262. Ye L, Sanders AJ, Jiang WG. Transglutaminase-4 (Prostate Transglutaminase), a Potential Biological Factor and Clinical Indicator for the Diagnosis and Prognosis of Prostate Cancer. *Anticancer Res.* 2023; 43(1):291-296.

263. Yeo ZX, Wong JC, Rozen SG *et al*. Evaluation and optimisation of indel detection workflows for ion torrent sequencing of the BRCA1 and BRCA2 genes. *BMC Genomics.* 2014; 15:516.

264. Yu XF, Feng WL, Miao LL, *et al*. The prognostic significance of molecular subtype for male breast cancer: a 10-year retrospective study. *Breast.* 2013; 22:824–827.

265. Zhang FL, Cao JL, Xie HY, *et al*. Cancer-Associated MORC2-Mutant M276I Regulates an hnRNPM-Mediated CD44 Splicing Switch to Promote Invasion and Metastasis in Triple-Negative Breast Cancer. *Cancer Res.* 2018; 78(20):5780-5792.

266. Zhang FL, Li DQ. Targeting Chromatin-Remodeling Factors in Cancer Cells: Promising Molecules in Cancer Therapy. *International Journal of Molecular Sciences*. 2022; 23(21):12815.

267. Zhang X. Exome sequencing greatly expedites the progressive research of Mendelian diseases. *Front Med, 8.* 2014; 42-57.

268. Zhang X, Cheng D, Liu Y, *et al*. Gephyrin suppresses lung squamous cell carcinoma development by reducing mTOR pathway activation. *Cancer Manag Res.* 2019; 11:5333-5341.

269. Zheng G, Leone JP. Male Breast Cancer: An Updated Review of Epidemiology, Clinicopathology, and Treatment. *J Oncol.* 2022.