Impact on breast cancer susceptibility and clinicopathological traits of common genetic polymorphisms in *TP53*, *MDM2* and *ATM* genes in Sardinian women

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Abstract. Common variants of genes involved in DNA damage correction [tumor protein p53 (*TP53)*, murine double 2 homolog oncoprotein (*MDM2)* and ataxia-telengiectasia mutated (*ATM*)] may serve a role in cancer predisposition. The purpose of the present study was to investigate the association of five variants in these genes with breast cancer risk and clinicopathological traits in a cohort of 261 women from northern Sardinia. Polymorphic variants in *TP53* (rs17878362, rs1042522 and rs1625895), *MDM2* (rs2279744) and *ATM* (rs1799757) were determined by PCR and TaqMan single nucleotide polymorphism assay in patients with breast cancer (n=136) and healthy controls (n=125). Association with clinicopathological (e.g., age at diagnosis, lymph node involvement, clinical stage) and lifestyle factors (e.g., smoking status, alcohol intake, contraceptive use) was also evaluated. *TP53* rs17878362 and rs1625895 polymorphisms were associated with decreased risk of BC diagnosis in patients older than 50 years (codominant and recessive models) and post-menopause (recessive model). Furthermore, there was a significant association between lymph node status (positive vs. negative) and *ATM* rs1799757-delT in dominant and additive models and between *MDM2* rs2279744-allele and use of oral contraceptives. This analysis suggested that *TP53* rs17878362 and rs1625895 may affect age of onset of breast cancer and *ATM* rs1799757 and *MDM2* rs2279744 may be associated with lymph node status and prolonged use of oral contraceptives, respectively.

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Introduction

Breast cancer (BC) is a key public health issue worldwide. It is the most frequently diagnosed malignancy in women and has surpassed lung cancer as the leading cause of global cancer incidence, representing 11.7% of all cancer cases diagnosed in 2020 (1). Global BC mortality shows a continuing downward trend due to widespread mammography screening, leading to early diagnosis, and the advent of personalized medicine (2,3).

The etiology of BC is multifactorial and highly complex. Numerous epidemiological studies have indicated that this disease is caused by a combination of multiple genetic and environmental, making the analysis of causative factors complex (4-9).Gene expression profiling studies have established the heterogeneous molecular nature of BC, which is considered as a collection of distinct 'intrinsic' subtypes (including *Luminal A*, *Luminal B*, *ERBB2+* and *Basal-like*) characterized by variable biological and clinical behavior and response to treatment (10,11).

Furthermore, linkage studies have identified germline mutations in high-penetrance (capable of causing the disease by itself) BC susceptibility genes, including *BRCA1*, *BRCA2*, tumor protein p53 (*TP53)*, partner and localizer of BRCA2, ataxia-telengiectasia mutated (*ATM)* and checkpoint kinase (*CHEK)2*, which are responsible for 5-10% of BC in the general population (12,13).

However, 90-95% of BC cases are sporadic forms in which general risk factors and single nucleotide polymorphisms (SNPs) in key genes involved in BC serve a role (14). Genome-wide association studies have identified numerous low-penetrance alleles with variable frequency in different

ethnic groups that are present in a high percentage of individuals and contribute to breast carcinogenesis (15-17) SNPs are implicated in genetic predisposition or resistance to a particular disease. Numerous SNPs have been associated with protein expression or changes in gene function via amino acid or indirect epigenetic changes (18-20).

Somatic mutations of the tumor suppressor gene, which encodes *TP53*, are the most frequent genetic alterations associated with several types of human cancer, such as colorectum, pancreas, ovarian, lung carcinomas (21-23). In humans, this gene encodes a 53 kDa nuclear phosphoprotein comprising 393 amino acids that form five highly conserved regions and four functional domains (24) with tetrameric transcription factor function that are involved in regulation of cell cycle progression, maintenance of genomic integrity, autophagy, inhibition of angiogenesis, differentiation, senescence and apoptotic cell death (25-27).

SNPs associated with BC have been identified in *TP53*, murine double 2 homolog oncoprotein (*MDM2*) and *ATM*. SNPs in *TP53*, *MDM2* and *ATM* contribute to individual susceptibility to cancer risk via alterations in gene expression regulation and exhibit wide geographical and ethnic variation (28-32). Furthermore, they have been the subject of functional case-control studies on BC to assess their role as genetic determinants on cancer risk, progression, treatment outcome and survival in patients with cancer (33-35).

The most well-characterized intronic SNP in *TP53* is PIN 16bp (rs17878362), a 16-base pair insertion at nucleotide 11,951 within intron 3 (36). The r.13494 G to A nucleotide transversion (rs1625895) is a rare polymorphism located in intron 6, which is one of the hotspots for *TP53* mutation (37). *TP53* R72P (rs1042522) is the most widely investigated SNP in cancer genetic epidemiology (38-40). It is a non-synonymous change of arginine (R72 form CGC) to proline (P72 form CCC) in exon 4 codon 72. This SNP is located in a proline-rich region of the putative SH3 binding domain; the two isoforms, R and P, differ in their biochemical and biological properties. The R72 variant exhibits markedly superior potential to induce apoptosis due to increased mitochondrial localization (41). On the other hand, the P72 variant has greater binding affinity with the transcriptional machinery to activate transcription, inducing higher levels of arrest in G_1 phase and facilitating repair of damaged DNA (42-44) *TP53* is a tumor suppressor gene that, under normal conditions, exerts its protective role via activation of a range of anti-proliferative responses triggered by DNA damage, hypoxia, oxidative stress and oncogene activation (21,45,46). Somatic mutations in *TP53* gene occur in ~31% of all cancers and 23% of BC cases; it is the second most frequently mutated gene after *PI3KCA* protooncogene (47,48).

MDM2 oncogene consists of 12 exons encoding a protein composed of 491 amino acids containing a P53 binding domain in the N-terminal and a RING domain responsible for E3 ligase activity at the C-terminal (49). In addition, mutations and polymorphic variants that alter the key role of MDM2 in control of P53 function manifest as cancer-associated phenotypes (50). Although multiple SNPs have been described in *MDM2*, the most characterized is the 309 T>G (rs2279744) variant at nucleotide 309 downstream of exon 1 in the P2 promoter (28,29,38,51-54).

ATM was mapped to chromosome 11q22-23 and contains 66 exons which encode a large protein (350 kDa) of 3,056 amino acids (55). ATM protein is a member of the PI3K-related protein kinase family. It has serine-threonine kinase activity that phosphorylates numerous substrates including proteins encoded by BC susceptibility genes such as *TP53*, *BRCA1* and *CHEK2* (56-58). ATM protein serves multiple roles in human cell biology: Its function is essential for recognition and repair of double-strand breaks in DNA, oxidative stress, control of cell cycle checkpoints, transcriptional regulation and apoptosis control (30). The polymorphism IVS24-9 delT (rs1799757) alters the acceptor splice site of intron 24 and increases BC risk by inducing genetic instability and normal response to DNA damage (59,60).

Several studies have been published on the potential association between polymorphic variants, especially variants of *TP53* (and, to a lesser extent, *MDM2* and *ATM*) and BC risk in different populations, showing controversial results, with *TP53* associated with BC risk and with no effect on BC risk (53,61-65).

The present case-control study aimed to analyze the effect of common genetic variants in *TP53*, *MDM2* and *ATM* and general risk factors for BC in a cohort from the north of Sardinia. Given its peculiar history of genetically isolated population, the Sardinian population represents European genetic variability while also including variants that are particularly frequent due to genetic drift or natural selection, and thus is an ideal population for genetic studies (66). The present study evaluated the role on BC susceptibility conferred by *TP53* rs17878362, rs1042522 and rs1625895, *MDM2* rs2279744 and *ATM* rs1799757 by allele frequency analysis and haplotype association and assessed their association with clinicopathological and lifestyle traits. The present study also verified the frequency of cumulative effects between three *TP53* polymorphic variants and SNPs of *MDM2* and *ATM*. A further aim of the study was to evaluate the role of causal risk factors that contribute to BC development in a cohort of patients and healthy women from northern Sardinia.

Materials and methods

Sampling plan. The case-control study, conducted between May 2017 and January 2020, involved a total of 261 unrelated women (aged 26-86): 136 patients with BC registered at the Medical Oncology Unit in Sassari University Hospital, Sassari, Italy, and a control group of 125 healthy women, who had never previously been affected by any tumor, registered at the Unit of Occupational Medicine of the University of Sassari, Sassari, Italy. Written informed consent was obtained from each participant and the protocol was reviewed and approved by the Azienda Sanitaria Locale Sassari Bioethics Committee (approval no. 2468/CE, 14/03/2017). The study was conducted in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki).

To recruit only individual representative of the Sardinian population, the participants were selected on the basis of the place of birth of their grandparents (all born in Sardinia). All participants completed a questionnaire to collect information on etiological factors underlying the onset of BC. In answering

the questions, the participants were asked to consider their entire, or almost entire, life span.

The following data of patients were taken from medical records: age of disease onset, tumor histology, stage according to TNM classification, hormonal receptor, HER2 and Ki67 expression, distant metastasis and molecular subtype.

Polymorphism genotyping. *EDTA* treated blood samples (5 ml) were obtained from all participants upon enrolment and stored at -20˚C until further use. Genomic DNA was extracted from 200 μl peripheral blood by QIAmp DNA Blood Mini kit (Qiagen GmbH) according to the manufacturer's instructions. *TP53* Ins16bp was determined by PCR analysis in a final volume of 20 μl reaction mixture containing 100 ng DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1.25 units Taq Gold DNA polymerase (Applied Biosystems; Thermo Fisher Scientific, Inc.) and 0.6 μM sense primer 5'-CCATGGGACTGACTTTTCTGC-3' and antisense primer 5'-GGGGACTGTAGATGGGTGAA-3'. PCR conditions were as follows: initial denaturation at 95˚C for 5 min, followed by 5 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec, 30 cycles of 95˚C for 30 sec, 57˚C for 30 sec and 72˚C for 30 sec and final extension at 72˚C for 5 min, as previously described (67). The PCR products were electrophoresed on a 3% Metaphor agarose gel, stained with ethidium bromide and visualized by UV trans-illumination. Genotypes of *TP53* Arg72Pro (cat. no. C_2403545_10), TP53 r.13494G>A (cat. no. C_8727782_20), MDM2 309T>G (cat. no. C_15968533_20) and ATM IVS24-9 (cat. no. C_33307825_10) were detected using TaqMan SNP assay kits (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. Briefly, 12.5 ng genomic DNA in 5 μl was added to a 25 μl reaction well with 20 μl reaction mix containing forward and reverse primers and two allele-specific fluorescent labelled probes, one wild-type (VIC) and one variant allele specific (FAM). PCR and allele detection were performed using the ABI Prism 7300 Sequence Detection System.

Statistical analysis. Minor allele frequency of polymorphisms in the Sardinian population was verified using public Sardinia Pheweb (pheweb.irgb.cnr.it/). SNP deviations from Hardy-Weinberg Equilibrium (HWE) in the control population were assessed independently for each SNP using 1-degree-of-freedom χ^2 test, with SNPassoc v. 2.0-11 (68). Student's paired t-test and Fisher's exact test implemented in the R base package v. 4.2.1 (https://www.r-project.org/) were used to evaluate the differences in descriptive variables at diagnosis, such as age or body mass index (BMI), between the cases and controls. For each SNP, genotypic and allelic association were tested with regression analysis under multiple inheritance models (additive, dominant, codominant, over-dominant and recessive), using a custom R script and SNPassoc package (association function), to compare patients with controls. The R package fsmb v. 0.7.3 was used to calculate odds ratio (OR) and 95% confidence interval (CI) according to the Woolf method (69): when 0 count was observed, Gart-adjusted logit interval was calculated. Before association, potential confounders (such as age and BMI) were tested by including them as covariates in the association models; accordingly, age and BMI were added as covariates in the analysis. Haplotype analysis was restricted to polymorphisms located on the same chromosome and genetic region (for example, variants encompassing a 1 Mb region). Measures of linkage disequilibrium (LD) between each pair of SNPs (D' and r2 statistic) and haplotype reconstruction were obtained in the 1000 genomes European population (https://www.internationalgenome.org/) (70) using the LDpair web tool (ldlink.nci.nih.gov/?tab=ldpair). The most common haplotype was selected as the reference. OR and 95% CI were calculated to estimate the degree of association between haplotype and risk of BC. A threshold of $P \leq 0.05$ was used to assess statistical significance for each genetic association. Bonferroni's correction was applied to adjust for multiple testing with a significance threshold of $P \leq 0.01$ (0.05/number of inheritance models tested for each genetic variant) for differences in descriptive variables at diagnosis between cases and controls. To evaluate the interaction between lifestyle factors and SNPs with BC risk, a logistic regression model was constructed.

Results

Comparison of questionnaire information between BC patients and controls. The primary hormonal/reproductive, lifestyle/environmental and familial cancer risk factors of cases and controls from the questionnaire analysis are summarized in Table I. The mean age of cases and controls was 56.80 and 50.71 years, respectively ($P=1.122x10^{-5}$). There were significant differences in BMI (OR: 3.150, 95% CI: 1.666-5.957, $P=5x10^{-4}$), alcohol intake (OR: 1.955, 95% CI: 1.024-3.733, $P=2.85x10^{-2}$) and competitive sport during adolescence (OR: 2.648, 95% CI: 1.577-4.444, $P=3x10^{-4}$) between BC cases and controls. Regarding the role of genetic factors, first-degree relatives (mother, sisters, brothers and cousins and aunts only of first-degree) of cases more frequently experienced onset of BC at an age <45 years (OR: 3.573, 95% CI: 2.002-6.379, $P=1.77x10^{-5}$). Furthermore, there was more frequent occurrence in number of familial cases of BC ≥3 and cancers of any organ ≥4 (OR: 4.139, 95% CI: 1.818-9.426, $P=7x10^{-4}$ and OR: 1.848, 95% CI: 1.113-3.067, $P=2.4x10^{-2}$ respectively), as well as more frequent occurrence of benign pre-tumor lesions (OR: 2.438, 95% CI: 1.207-4.924, $P=1.7x10^{-2}$) between cases compared with controls. There were no statistically significant differences between cases and controls regarding all hormonal/reproductive (only age of menarche and nulliparity exhibited a small role; $P=8x10^{-2}$ and $P=9x10^{-2}$ respectively) and other lifestyle/environmental risk factors, although secondhand smoke exposure ($P=7x10^{-2}$) appeared to have a small effect. Questionnaire analysis data showed a relevant impact on BC risk of both lifestyle and genetic factors in patients compared to controls.

Clinicopathological characteristics of patients with BC. Clinical and pathological data of all patients are summarized in Table II. In brief, the analysis of BC cases in the north Sardinia cohort revealed that most cases were diagnosed in woman aged >40 years (83.82%) and who were pre-menopausal (56.68%). The most common histological type was invasive ductal carcinoma (85.29%), and more frequently the tumors showed early clinical stage (0-II, 66.18%); 72.79% of tumors expressed estrogen receptor (ER), 67.65% expressed

Table I. Hormonal/reproductive, lifestyle/environmental and familial cancer risk factors in patients and controls.

Risk factor	Cases (n.136) n, %	Controls (n.125) n, %	OR (CI 95%)	P (chi sq)
Age at menarche <13 y	70, 51.5	50, 40.0	1.591 (0.974-2.600)	0.08306
Nulliparity	38, 27.9	48, 38.4	0.622 (0.370-1.046)	0.09611
Age at first bird ≥35 y	12, 8.8	17, 13.6	0.615 (0.281-1.345)	0.3033
No breastfeeding	58, 42.6	58, 46.4	0.859 (0.527-1.401)	0.6278
CO ≥10 years	53, 39.0	49, 39.2	0.990 (0.602-1.629)	1
[a]Age at menopause >51 y	21, 15.4	19, 15.2	1.019 (0.519-2.000)	1
BMI >27	43, 31.6	16, 12.8	3.150 (1.666-5.957)	0.0004963
Drinking	31, 22.8	17, 13.6	1.876 (0.980-3.592)	0.07919
[b]Smoke ≥10 p/y	37, 27.2	34, 27.2	1.000 (0.580-1.726)	1
Passive smoke	78, 57.4	57, 45.6	1.604 (0.984-2.617)	0.07603
No sport	91, 66.9	74, 59.2	1.394 (0.841-2.309)	0.2452
No sport in adolescence	100, 73.5	64, 51.2	2.648 (1.577-4.444)	0.000317
Chemicals exposure	27, 19.9	17, 13.6	1.574 (0.811-3.053)	0.237
[c]Benign breast lesions	30, 22.1	13, 10.4	2.438 (1.207-4.924)	0.01781
Bilateral BC in family	23, 16.9	16, 12.8	1.387 (0.695-2.765)	0.449
BC <45 y in family	57, 41.9	21, 16.8	3.573 (2.002-6.379)	1.77e-05
Ovary cancer in family	19, 14.0	10, 8.0	1.868 (0.833-4.189)	0.1815
N. BC ≥3 in family	30, 22.1	8, 6.4	4.139 (1.818-9.426)	0.0006557
N. all tum ≥4 in family	62, 45.6	39, 31.2	1.848 (1.113-3.067)	0.02401

[a]Only natural menopause was considered (55 BC patients and 60 healthy controls). [b]Smoking status at the time of recruitment or in the past. Pack/years (p/y) is calculated as follows: (Years of smoking x number of cigarettes/day)/20. [c]Parameters considered in first-degree relatives (parent, sibling, or child). OR, odds ratio; y, years; OC, oral contraceptives; BMI, body mass index; BC, breast cancer.

progesterone receptor (PgR) and 30.88% were HER2+. Finally, the molecular subtypes of our BC cohort were: 58 Luminal A (42.65%), 21 Luminal B-like Her-2 neg (15.44%), 21 Luminal B-like Her-2 pos (15.44%), 21 Her-2 overexpressing (15.44%) and 15 Triple negative (11.03%).

Genetic variants and BC risk association. *TP53* (rs17878362) wild-type allele resulted in 153 bp fragment; 169 bp fragment was the variant allele (Fig. S1). None of the SNPs were discarded there was no deviation from HWE in the control population (*TP53* rs17878362 HWEp=0.795, rs1042522 HWEp=0.826, and rs1625895 HWEp=1, *ATM* rs1799757, HWEp=0.306 and *MDM2* rs2279744 HWEp=0.848). The distribution of allele frequencies of these SNPs in controls and BC cases in the Sardinian population is shown in Table III. In the distribution of alleles analyzed under multiple genetic inheritance models (codominant, dominant, recessive, over-dominant and additive), no statistically significant effect on the risk of BC was found in the two cohorts (association analysis adjusted for age and BMI). Haplotype analysis combining *TP53* genotypes and those of *MDM2* and *ATM* polymorphisms did not reveal significant differences associated with BC (data not shown). The SNPs of *TP53*, *ATM* and *MDM2* considered in the present study have not shown effects on BC susceptibility.

Association between genotypes and clinical features and lifestyle. The present study evaluated the potential association between SNP genotypes in codominant, dominant, recessive, over-dominant and log-additive inheritance models and clinicopathological parameters such as familiar BC cases and other primary tumors, age at diagnosis, lymph node involvement, clinical stage, ER, Ki67 and HER2 status and menopause status at diagnosis. *TP53* rs17878362 and rs1625895 polymorphisms were associated with a decreased risk of BC diagnosis at an age >50 years in codominant (ins/ins vs. del/del OR 0.01, 95% CI: 0.00-0.30, P=4.2x10^{-2}; A/A vs. G/G OR 0.01, 95% CI: 0.00-0.28, P=4.8x10^{-2}) and recessive models (ins/ins vs. del/ins OR: 0.01, 95% CI: 0.00-0.28, P=1.1x10^{-2}; A/A vs. A/G OR 0.01, 95% CI: 0.00-0.28, P=1.4x10^{-2}, Table IV). SNPs were associated with a decreased risk of BC diagnosis in post-menopausal cases in the recessive inheritance model (ins/ins vs. del/ins OR 0.03, 95% CI: 0.00-0.68, P=2.4x10^{-2}; A/A vs. A/G OR 0.03, 95% CI: 0.00-0.69, P=4.0x10^{-2}; association analysis adjusted for age and BMI; Table IV). Furthermore, there was an association between lymph node (positive vs. negative) status and allele delT in *ATM* rs1799757 dominant and additive models (T/delT-delT/delT vs. T/T OR 0.43, 95% CI: 0.19-0.98, P=4.0x10^{-2} and OR 0.43, 95% CI: 0.19-0.94, P=3.0x10^{-2}, respectively, Table IV). The study also evaluated the potential association between distribution of SNP genotypes and lifestyle traits such as smoking status, alcohol intake and oral contraceptive (OC) use. There was an interaction between use of OC for <10 and ≥10 years and the rs2279744 polymorphism of *MDM2* in BC cases (83/53) and controls (76/49). GG genotype with OC intake for >10 years was associated with an increase in BC risk (OR: 3.43, 95% CI: 0.92-12.78, P=4.8x10^{-2}, association analysis adjusted for age

Table II. Clinicopathological features of breast cancer cases.

Variable	Cases (%), n=136
Age at diagnosis, years (Mean=52.10)	
≤40	22.00 (16.18)
>40	114.00 (83.82)
Menopause status at diagnosis	
Pre-	73.00 (56.68)
Post-	63 (46.32)
Histological subtype	
Ductal	116.00 (85.29)
Lobular	10.00 (7.35)
Other	10.00 (7.35)
ER expression	
Positive	99.00 (72.79)
Negative	37.00 (27.21)
PgR expression	
Positive	92.00 (67.65)
Negative	44.00 (32.35)
HER2	
Positive	42.00 (30.88)
Negative	89.00 (65.44)
Missing	5.00 (3.68)
Ki67	
≤30%	104.00 (76.47)
>30%	31.00 (22.79)
Missing	1.00 (0.73)
Distant metastasis	
M0	112.00 (82.35)
M1	22.00 (16.18)
Missing	2.00 (1.47)
Clinical stage	
Early (0, I, II)	90.00 (66.18)
Advanced (III, IV)	39.00 (28.68)
Missing	7.00 (5.15)
Molecular subtype	
Luminal A	58.00 (42.65)
Luminal B-like HER2-negative	21.00 (15.44)
Luminal B-like HER2-positive	21.00 (15.44)
HER2-overexpressing	21.00 (15.44)
Triple negative	15.00 (11.03)

ER, estrogen receptor; PgR, progesterone receptor. Missing=number of patients for which this particular value is not available.

and BMI; Table V). Of the 21 homozygotes of the G allele, 5 were ER-negative and 16 ER-positive with a frequency of the G allele equal to 0.31 and 0.38 respectively (data not shown). Furthermore, association analysis suggested an association between *TP53* rs1042522 Pro allele/*MDM2* rs2279744 T allele combination ($P=5.6x10^{-2}$) and *TP53* rs1042522 recessive Arg/Arg-Pro/Arg recessive model and premenopausal status ($P=5.3x10^{-2}$; Table SI and SII association analysis adjusted for age and BMI). The present study showed that the investigated SNPs affect some clinical characteristics and are associated with prolonged OC intake in BC patients.

Discussion

BC etiology is extremely complex, it might be partially explained by individual genetic susceptibility, as well as by numerous extrinsic factors linked to lifestyle, which modify the normal biology of the mammary glandular epithelium during the woman's life. The present study investigated the association of 5 variants in *TP53*, *MDM2* and *ATM* (rs17878362, rs1042522, rs1625895, rs2279744, rs1799757) with BC susceptibility, clinicopathological and lifestyle traits in a cohort of Sardinian women. *TP53* rs17878362 and rs1625895 polymorphisms were associated with decreased risk of BC diagnosis both in patients aged >50 years and those who were post-menopausal. Moreover, there was a significant association between lymph node status and *ATM* rs1799757-delT and *MDM2* rs2279744-allele and OC use.

When analyzing BC risk in the two groups using multiple genetic models, there was no statistically significant difference for polymorphisms *TP53* rs17878362, rs1042522 and rs1625895. Published data report controversial results regarding the role of these SNPs in BC predisposition, showing increase or decrease of BC susceptibility risk, when other factors are not taken into consideration. This may be due to factors such as differences in geographical location and ethnicity, methodological approach and composition of the analyzed cohort. In this regard, large collaborative multi-center studies should be undertaken with the same methodology with particular attention paid to the ancestral genetic origins of the population under consideration (71-86).

Regarding the rs17878362 Ins16bp and rs1625895 13494G>A *TP53* polymorphisms, the presence of Ins-allele and AA-allele, respectively, was associated with a decreased probability of BC diagnosis in those aged >50 years (codominant model and recessive model) and with pre-menopausal status (recessive model). Certain evidence supports the hypothesis that non-coding genetic variations may be important in regulating P53 activity by initiating, for example, aberrant splicing of pre-messenger RNA and production of mRNA that is translated into a defective protein (87-89). Both rs17878362 Ins16bp and rs1625895 13494G> A are intronic polymorphisms with a potential biological association with certain types of cancer, such as lung, colorectal and ovarian (67,78,83,90,91). As for the first, the variant A2 allele (16p duplication) causes an alteration in mRNA processing (90). Based on the present results, it might be hypothesized that the risk factors promoting BC combined with Ins and A alleles of the rs17878362 and rs1625895 polymorphisms respectively, affect the age of BC onset.

Here, the *MDM2* 309T> G polymorphism did not reveal any role in BC risk; however, there was a significant association between the GG variant and BC risk increase in those using OC for ≥10 years. In 2004, Bond *et al* (28) demonstrated an increase in the binding affinity of the consensus sequence of *MDM2* promoter with the transcription factor Sp1 in conditions of homozygosity of the G allele of SNP309T>G, producing an 8-fold increase in *MDM2* mRNA and a 4-fold

Table III. Genotype and allele frequencies of *TP53*, *MDM2* and *ATM* variants in BC cases and controls (adjusted for age and BMI).

A, *TP53* (rs17878362). Ref. allele, NoIns; alt. allele, Ins16

Model	Genotype	Cases (%), n=136.00	Controls (%), n=125.00	OR (95% CI)	P-value
Allele	Del	220.00 (80.88)	196.00 (78.40)	1.00	
	Ins	52.00 (19.12)	54.00 (21.60)	1.16 (0.74-1.83)	0.51
Codominant	Del/del	88.00 (64.70)	76.00 (60.80)	1.00	
	Del/ins	44.00 (32.35)	44.00 (35.20)	0.85 (0.49-1.47)	
	Ins/ins	4.00 (2.94)	5.00 (4.00)	0.55 (0.13-2.40)	0.65
Dominant	Del/del	88.00 (64.70)	76.00 (60.80)	1.00	
	Del/ins + ins/ins	48.00 (35.29)	49.00 (39.20)	0.82 (0.48-1.39)	0.45
Recessive	Del/del + del/ins	132.00 (97.06)	120.00 (96.00)	1.00	
	Ins/ins	4.00 (2.94)	5.00 (4.00)	0.59 (0.14-2.50)	0.47
Over-dominant	Del/del + ins/ins	92.00 (67.65)	81.00 (64.80)	1.00	
	Del/ins	44.00 (32.35)	44.00 (35.20)	0.87 (0.50-1.51)	0.62
Log-additive	0,1,2	136.00 (52.10)	125.00 (47.90)	0.81 (0.51-1.29)	0.38

B, *TP53* (rs1042522). Ref. allele, C; alt. allele, G

Model	Genotype	Cases (%), n=136.00	Controls (%), n=125.00	OR (95% CI)	P-value
Allele	G (Arg)	202.00 (74.26)	179.00 (71.60)	1.00	
	C (Pro)	70.00 (25.73)	71.00 (28.40)	1.14 (0.76-1.72)	0.55
Codominant	G/G	75.00 (55.15)	63.00 (50.40)	1.00	
	G/C	52.00 (38.23)	53.00 (42.40)	0.76 (0.44-1.30)	
	C/C	9.00 (6.62)	9.00 (7.20)	0.60 (0.21-1.72)	0.46
Dominant	G/G	75.00 (55.15)	63.00 (54.40)	1.00	
	G/C + C/C	61.00 (44.85)	62.00 (49.60)	0.74 (0.44-1.23)	0.24
Recessive	G/G + G/C	127.00 (93.38)	116.00 (92.80)	1.00	
	C/C	9.00 (6.62)	9.00 (7.20)	0.68 (0.24-1.89)	0.45
Over-dominant	G/G + C/C	84.00 (61.76)	72.00 (57.60)	1.00	
	G/C	52.00 (38.23)	53.00 (42.40)	0.81 (0.48-1.36)	0.42
Log-additive	(0,1,2)	136.00 (52.10)	125.00 (47.90)	0.77 (0.50-1.17)	0.21

C, *TP53* (rs1625895). Ref. allele, A; alt. allele, G

Model	Genotype	Cases (%), n=136.00	Controls (%), n=125.00	OR (95% CI)	P-value
Allele	G	221.00 (81.25)	200.00 (80.00)	1.00	
	A	51.00 (18.75)	50.00 (20.00)	1.08 (0.68-1.71)	0.74
Codominant	G/G	88.00 (64.71)	80.00 (64.00)	1.00	
	G/A	45.00 (33.09)	40.00 (32.00)	0.92 (0.53-1.61)	
	A/A	3.00 (2.20)	5.00 (4.00)	0.37 (0.07-1.82),	0.45
Dominant,	G/G	88.00 (64.71)	80.00 (64.00)	1.00	
	G/A + A/A	48.00 (35.29)	45.00 (36.00)	0.86 (0.50-1.47),	0.57
Recessive,	G/G + G/A	133.00 (97.79)	120.00 (96.00)	1.00	
	A/A	3.00 (2.20)	5.00 (4.00)	0.38 (0.08-1.85),	0.22
Over-dominant	G/G + A/A	91.00 (66.91)	85.00 (68.00)	1.00	
	G/A	45.00 (33.09)	40.00 (32.00)	0.96 (0.55-1.67),	0.89
log-Additive	(0,1,2)	136.00 (52.10)	125.00 (47.90)	0.81 (0.50-1.30),	0.38

Table III. Continued.

D, *MDM2* (rs2279744). Ref. allele, T; alt. allele, G

Model	Genotype	Cases (%), n=136.00	Controls (%), n=125.00	OR (95% CI)	P-value
Allele	T	176.00 (64.71)	157.00 (62.80)	1.00	
	G	96.00 (35.29)	93.00 (37.20)	1.08 (0.75-1.58)	0.71
Codominant	T/T	61.00 (44.85)	50.00 (40.00)	1.00	
	T/G	54.00 (39.71)	57.00 (45.60)	0.81 (0.46-1.42)	
	G/G	21.00 (15.44)	18.00 (14.40)	0.98 (0.45-2.11),	0.75
Dominant,	T/T	61.00 (44.85)	50.00 (40.00)	1.00	
	TG + G/G	75.00 (55.15)	75.00 (60.00)	0.85 (0.51-1.43),	0.55
Recessive,	TT + T/G	115.00 (84.56)	107.00 (85.60)	1.00	
	G/G	21.00 (15.44)	18.00 (14.40)	1.09 (0.53-2.22),	0.82
Over-dominant	T/T + G/G	82.00 (60.29)	68.00 (54.40)	1.00	
	T/G	54.00 (39.71)	57.00 (45.60)	0.82 (0.49-1.37),	0.45
log-Additive	(0,1,2)	136.00 (52.10)	125.00 (47.90)	0.94 (0.66-1.36),	0.76

E, *ATM* (rs1799757). Ref. allele, T; alt. allele, delT

Model	Genotype	Cases (%), n=136.00	Controls (%), n=125.00	OR (95% CI)	P-value
Allele	T	235.00 (86.40)	213.00 (85.20)	1.00	
	delT	37.00 (13.60)	37.00 (14.80)	1.10 (0.65-1.86)	0.71
Codominant	T/T	100.00 (73.53)	89.00 (71.20)	1.00	
	T/delT	35.00 (25.73)	35.00 (28.00)	1.00 (0.56-1.79)	
	delT/delT	1.00 (0.73)	1.00 (0.80)	0.37 (0.02-6.29),	0.80
Dominant,	T/T	100.00 (75.53)	89.00 (71.20)	1.00	
	T/delT + delT/delT	36.00 (26.47)	36.00 (28.80)	0.97 (0.54-1.73),	0.92
Recessive,	T/T + T/delT	135.00 (99.26)	124.00 (99.20)	1.00	
	delT/delT	1.00 (0.73)	1.00 (0.80)	0.37 (0.02-6.27),	0.50
Over-dominant	T/T + delT/delT	101.00 (74.26)	90.00 (72.00)	1.00	
	T/delT	35.00 (25.73)	35.00 (28.00)	1.01 (0.56-1.80),	0.98
log-Additive	(0,1,2)	136.00 (52.10)	125.00 (47.90)	0.94 (0.54-1.63),	0.82

Del, deletion; Ins, insertion; BMI, body mass index.

increase in Mdm2 protein, resulting in the attenuation of P53 pathway both *in vitro* and *in vivo*. As this polymorphism is localized in a promoter region regulated by a hormonal signaling pathway and the G allele of SNP309T> G increases the affinity of co-transcriptional activator for nuclear hormone receptor (such as Sp1), there is accelerated hormone-dependent tumor formation (92). To the best of our knowledge, the present study is the first to report an association between the *MDM2* rs2279744 GG genotype and a history of OC use for ≥10 years, these results are supported by data previously described.

ATM IVS24-9 polymorphism did not increase susceptibility to BC in the present case-control study. However, heterozygous T/-T and homozygous -T/-T genotypes in dominant and log-additive models were significantly associated with negative status of lymph node involvement at diagnosis. To the best of our knowledge, however, functional and case-control studies are scarce (59,93-96). The IVS24-9 polymorphism is a splice acceptor site that increases BC risk by favoring genetic instability and normal response to DNA damage (97); additional studies are needed to define the putative role of lymph node negativity. *ATM* has numerous polymorphic sites and some may confer risk of BC and other tumors (30,95,96), although these conclusions are limited and conflicting and must therefore be confirmed by further functional and case-control studies.

Regarding analysis of lifestyle and demographic factors, there was only a small association between the age of menarche onset and nulliparity; however this was not significant. Research conducted in different populations and with a higher number of samples indicate increased relative risk associated with longer fertile period (early menarche and late menopause), nullity or low parity, first full-term pregnancy after 30-35 years, failure

Table IV. Association of *TP53* polymorphisms and age and menopausal status and *ATM* polymorphism and lymph nodes status at diagnosis (adjusted by age and BMI).

Variable	SNP	Models and allele	n	OR (95% CI)	P-value
Age at diagnosis, years ≤50/>50 (n=67/69)	*TP53* rs17878362,	Codominant Del/del	45/43	1.00	
		Del/ins	19/25	1.05 (0.38-2.89)	
		Ins/ins	3/1	0.01 (0.00-0.30)	$4.2x10^{-2}$
		Recessive Del/del-del/ins	64/68	1.00	
		Ins/ins	3/1	0.01 (0.00-0.28)	$1.1x10^{-2}$
	TP53 rs1625895	Codominant G/G	5/43	1.00	
		A/G	20/25	0.96 (0.35-2.64)	
		A/A	2/1	0.01 (0.00-0.28)	$4.8x10^{-2}$
		Recessive G/G-A/G	65/68	1.00	
		A/A	2/1	0.01 (0.00-0.28)	$1.4x10^{-2}$
Menopausal status at diagnosis Pre-/post- (n=73/63)	*TP53* rs17878362,	Recessive Del/del-del/ins	70/62	1.00	
		Ins/ins	3/1	0.03 (0.00-0.68)	$3.4x10^{-2}$
		Recessive G/G-A/G	71/62	1.00	
		A/A	2/1	0.03 (0.00-0.69)	$4.0x10^{-2}$
Lymph nodes at diagnosis Negative/positive (n=67/64),	*ATM* rs1799757	Dominant T/T	44/52	1.00	
		T/delT-delT/delT	23/12	0.43 (0.19-0.98)	$4.0x10^{-2}$
		Log additive (0,1,2)		0.43 (0.19-0.94)	$3.0x10^{-2}$

Del, deletion; Ins, insertion; BMI, body mass index.

Table V. Association of polymorphism rs2279744 of *MDM2* and duration of oral contraceptive use in cases and controls (adjusted by age and BMI).

Oral contraceptive use, years	Allele	Control (n=125)	Cases (n=136)	OR (95% CI)	P-value
<10	T/T	30	37	1.00	
	T/G	32	36	1.04 (0.51-2.12)	
	G/G	14	10	0.60 (0.22-1.60)	
≥10	T/T	20	24	1.57 (0.69-3.57)	
	T/G	25	18	0.80 (0.36-1.80)	
	G/G	4	11	3.43 (0.92-12.78)	$4.8x10^{-2}$

to breastfeed and OC use (4-7,98-100). However, other epidemiological investigations provide conflicting data, attributing little value to these factors and considering them important only for surveillance (8,101-103). Here, there was a strong association between high BMI, frequent (daily/often) alcohol intake and absence of physical activity during adolescence and increased risk of BC. A sedentary lifestyle combined with being overweight/obese has adverse health effects and leads to an increased BC risk, particularly in postmenopausal women (104-106). This is due to altered cellular sensitivity to insulin, inflammation and cytokine production, overexpression of leptinin adipose tissue, bioavailability of sex hormones and activation/variation of epigenetic mechanisms (107-109). In obese women, the adipose tissue represents an important source of endogenous estrogens due to conversion of androgenic precursors (110). Secondly, the adipose microenvironment is similar to the tumor microenvironment in cellular composition, low-grade chronic inflammation and high ratio of reactive species oxygen to antioxidants (111).

Physical activity and BC have been linked in numerous studies (112,113). This is reported to be associated with decreased BC risk in post-menopausal women and mortality (114,115). Practicing intense physical activity in adolescence decreases BC risk by delaying the age of menarche onset and decreasing the amount of bioavailable circulating hormones (116,117). Acting on these modifiable risk factors, through regular physical activity and control of body weight, could contribute to risk reduction by modifying metabolic and hormonal status.

The assessment of genetic risk share indicates that BC cases occurring at a relatively early age in first-degree relatives, high number of familial BC (≥3) and primary tumors

in other locations (≥4) and benign breast lesions were risk factors for BC. Similar results have been reported in other studies (118-121).

In conclusion, genetic analysis of *TP53* rs17878362, rs1042522, and rs1625895, *MDM2* rs2279744 and *ATM* rs1799757 suggested that no polymorphic allele, alone or in combination within haplotypes, was associated with BC risk in Sardinian women. However, *TP53* Ins16bp and 13494G>A SNPs showed a significant association with age of BC onset and menopausal status in BC patients. However, the most significant result was *MDM2* 309T>G polymorphism. To the best of our knowledge, the present study is the first to suggest an increased risk among GG-carrier patients who have taken OC for >10 years. The present results contribute to the characterization of the genetic BC susceptibility profile. However, caution is required when drawing conclusions and further studies are needed to elucidate the role of these polymorphisms in predisposition and as predictors of treatment outcome and prognostic markers in BC.

The descriptive analysis of questionnaires confirmed the key role of lifestyle/environmental and genetic/familial causal factors in increasing the relative risk of BC. At present, knowledge of breast carcinogenesis remains incomplete and although causative modifiable and immutable factors are known, it is not possible to identify subgroups of women who will develop BC, except those showing a high familial genetic risk.

Future large-scale studies should simultaneously consider intrinsic and extrinsic risk determinants, their interaction and their association with the individual genetic predisposition.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MRM conceived and coordinated the study and drafted the manuscript. PC, MF, MRM, GP, MI, AA and MS designed the methodology, collected, analyzed and visualized data. GP, AC, MRDM, GD, CA and CC performed experiments. MRM, AS, MB, AP, CP, VS and MB were involved in sample selection, collection and analysis of results. MRDM, MF, MI, MS, AA and AP edited the manuscript. MF and MRM confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by Azienda Sanitaria Locale (ASL) Sassari Bioethical Committee and written informed consent was obtained from each participant, according to Italian Legislation (approval no. 2468/CE 14/03/2017). All data are treated according to the EU General Data Protection Regulation 2016/679.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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