Prevalence and Spectrum of BRCA Germline Variants in Central Italian High Risk or Familial Breast/Ovarian Cancer Patients: A Monocentric Study

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Received: 17 July 2020; Accepted: 6 August 2020; Published: 12 August 2020

Abstract: Hereditary breast and ovarian cancers are mainly linked to variants in BRCA1/2 genes. Recently, data has shown that identification of BRCA variants has an immediate impact not only in cancer prevention but also in targeted therapeutic approaches. This prospective observational study characterized the overall germline BRCA variant and variant of uncertain significance (VUS) frequency and spectrum in individuals affected by breast (BC) or ovarian cancer (OC) and in healthy individuals at risk by sequencing the entire BRCA genes. Of the 363 probands analyzed, 50 (13.8%) were BRCA1/2 mutated, 28 (7.7%) at BRCA1 and 23 (6.3%) at BRCA2 gene. The variant c.5266dupC p.(Gln1756Profs) was the most frequent alteration, representing 21.4% of the BRCA1 variants and 12.0% of all variants identified. The variant c.6313delA p.(Ile2105Tyrfs) of BRCA2 was the most frequent alteration observed in 6 patients. Interestingly, two new variants were identified in BRCA2. In addition, 25 different VUS were identified; two were reported for the first time in BRCA1 and two in BRCA2. The number of triple-negative BCs was significantly higher in patients with the pathogenic BRCA1/2-variant (36.4%) than in BRCA1/2 VUS (16.0%) and BRCA1/2 wild-type patients (10.7%) (p < 0.001). Our study reveals that the overall frequency of BRCA germline variants in the selected high-risk Italian population is about 13.8%. We believe that our results could have significant implications for preventive strategies for unaffected BRCA-carriers and effective targeted treatments such as PARP inhibitors for patients with BC or OC.

Keywords: BRCA1/2 variant carrier; breast cancer; VUS; genetic testing; risk evaluation

1. Introduction

Breast cancer (BC) is the most common cancer and leading cause of cancer-related mortality among women worldwide. In Europe, approximately 500,000 women are diagnosed with BC annually, and in 2018, BC cases were responsible for a third of all cancer related deaths (about 130,000) [1]. Most women with breast or ovarian cancer (OC) have a sporadic rather than an inherited cancer.
However, the majority of hereditary breast and ovarian cancers (HBOC) are due to highly penetrant germline BRCA variants, which are inherited in an autosomal-dominant fashion: breast cancer susceptibility gene 1 (BRCA1) or breast cancer susceptibility gene 2 (BRCA2). In these patients, there are frequently several generations of women affected with BC (often premenopausal) and, in some families, OC as well. The prevalence of BRCA variants varies based on a number of factors, including type of cancer and age at diagnosis. For individuals whose ethnicity is associated with higher variant frequency, particularly Ashkenazi Jews, any personal or family history of BC is sufficient to warrant consideration of BRCA testing. Aside from Ashkenazi Jews, founder variants have also been reported worldwide in populations from the Netherlands, Sweden, Hungary, Iceland, Italy, France, South Africa, Pakistan, Asia, and among French Canadians, Hispanics, and African Americans [2–5].

In a recent study, the incidences of BC and OC were reported to be 72% or 44% in BRCA1 carriers and 69% or 17% in BRCA2 carriers, respectively [6,7]. Other BRCA-associated malignancies such as prostate, male breast and pancreatic cancer may also be observed. Less commonly, BC is due to other hereditary syndromes, such as Li-Fraumeni and Cowden, which are associated with variants in the TP53 and PTEN genes, respectively [8]. BC is the most prevalent cancer type and the first cause of death among women in Italy [9]. International guidelines, in cases of known variants in the family, early-onset or triple-negative cancers and multiple relatives with cancer, suggest referral for genetic counseling [10,11]. In recent years, poly(ADP-ribose) polymerase (PARP) inhibitors have been developed that target BRCA pathogenic variants in various cancer types including breast and ovarian cancers [12]. Thus, the detection of BRCA variants has a relevant impact both in cancer prevention and in targeted treatment. Typically, variant screening has been performed among affected women, selected on the basis of young age at diagnosis or family cancer history. The aim of this study is to determine the overall germline BRCA variant frequency and spectrum in healthy Italian individuals at risk or affected by BC or OC by molecular genetic analysis of regions of BRCA1 and BRCA2 genes.

2. Materials and Methods

2.1. Patients and Samples

Individuals referring to genetic counseling at the Medical Oncology Division of the S. Maria della Misericordia Hospital (Perugia-Italy) in the years 2010–2016 at risk or with a history of BC or OC were included in the study. This cohort of 363 women/men was selected according to the Italian Medical Oncology Association (AIOM) guidelines [13] based on age at BC/OC onset, number of cancer cases in I- and II degree relatives, and pathological characteristics of BC. Several genetic risk assessment methods are available to estimate the probability of BRCA variant in individuals in order to select them for molecular diagnosis [14]. Genetic testing was performed on all individuals >18 years old selected according to the AIOM guidelines and these criteria do not differ from other jurisdictions in Italy.

- Knowledge of pathogenetic mutation in the family
- Males affected by breast cancer
- Women with breast and ovarian cancer
- Women affected by breast cancer <36 years old
- Women affected by triple negative breast cancer <60 years old
- Women with bilateral breast cancer <50 years old
- Women with breast cancer <50 years old AND first degree familiarity of:
  1. Breast cancer <50 years old
  2. No-mucinous, no-border line ovarian cancer (all ages)
  3. Bilateral breast cancer
  4. Male breast cancer
We chose, however, to utilize BRCAPRO software that is based on Bayes’ theorem; this requires data on all first, second and third degree relatives of the family proband and incorporates as prior probabilities incidence rates in the US population, allele variant frequencies and penetrances estimated from studies in families with several BC or OC cases [15–17]. For unaffected individuals we utilized the Cuzick–Tyrer model that, developed for the International Breast Intervention Study (IBIS-1), incorporates the assessment of additional hereditary factors, body mass index, menopausal status and hormone replacement therapy use [18]. We considered it suitable for genetic testing of BRCA variant individuals with an estimated life-time risk of disease ≥10%. The study was conducted in accordance with Good Clinical Practice and the ethical principles of the Declaration of Helsinki and approved by the S. Maria della Misericordia Ethics Committee (CE, protocol 2207/2010). We obtained written informed consent from all participants. Clinical data such as age at diagnosis, hystotype, grading, stage, tumor invasiveness, and receptor status were gathered.

St Gallen guidelines were used to classify BC subtype, based on receptor status [19]. Data about a second BC and/or OC or other malignancies and the family cancer history in I and II degree relatives were also collected.

2.2. BRCA1/2 Analysis

Ten milliliters of whole blood mixed with EDTA were collected from each patient. Genomic DNA was extracted from blood using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) and quantified using the Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, M0, Italy). All 23 coding exons of BRCA1 (exons 2 to 24) and 26 coding exons of BRCA2 (exons 2 to 27) were amplified in 33 and 46 amplicons, respectively. The primers were designed to cover all coding exons and adjacent 20 base pair introns. The amplified DNA fragments were sequenced using the BigDyeTerminator v.3.1 cycle sequencing kit (Thermo Fisher Scientific) on a 3500 Genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing chromatograms were analyzed for variant detection using Seqscape software v.2.7 (Applied Biosystems, Foster City, CA, USA). In all cases, samples harboring variants were re-amplified and re-sequenced using the same experimental conditions. All sequences were compared with the BRCA1 (NM_007294.3) and BRCA2 (NM_000059.3) reference sequences for variant detection. To identify gross deletions/insertions not detectable by sequencing on the BRCA1/2 genes, we performed the Multiplex Ligation-dependent Probe Amplification (MLPA) using the SALSA P002 BRCA1 and SALSA P045 BRCA2 MLPA probe mix assays (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer’s instructions. Coffalyser V9.4 software (MRC-Holland, Amsterdam, The Netherlands) was used to analyze MLPA results.

2.3. Variant Classification

According to the IARC recommendations [20], we classified genetic variants identified into five classes. To annotate BRCA1/2 variants we used: databases such as Breast Cancer Information Core (BIC) [21], BRCA Share (formerly Universal Variant Database) [22], Leiden Open Variation Database (LOVD) [23], ClinVar-NCBI Database, and American College of Medical Genetics (ACMG) guidelines [24].

Variants not found in these databases were classified on the basis of their characteristics. All variants with conflicting interpretation results by ClinVar-NCBI Database were considered as VUSs. The classification of variants initially considered as VUS was subjected to regular updates, by reviewing the literature and publicly available databases to the best of our knowledge, and modified accordingly. Frameshift and nonsense VUS leading to a premature stop codon were considered likely-pathogenic-class4 and classified in accordance with the ACMG guidelines. All variants were reported according to Human Genome Variation Society nomenclature [25] according to ENIGMA.
(Evidence-based Network for the Interpretation of Germline Mutant Alleles) consortium rules for variant classification to obtain the most recent information on variant reclassifications.

2.4. Data Collection and Statistical Analysis

Data were collected using a management system that is integrated with the Umbria Cancer Registry application system [26].

Descriptive statistics of patients’ characteristics and sequencing results were presented as median and range for continuous data and as natural frequencies and percentages for categorical data. Pearson Chi-square test or an appropriate Fisher Exact test were used to compare tabular proportions. All data analyses were performed using R software version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria).

2.5. Immunohistochemistry Analysis of Breast Tumor Samples

Tumor immunohistochemical (IHC) analysis was performed for estrogen receptor (ER) (clone 1D5 diluted 1:15), progesterone receptor (PgR) (clone 1A6 diluted 1:15), and Ki-67 (clone MIB1 diluted 1:15) using the automated platform Bond III (Leica Biosystem, MI, Italy). IHC analysis for evaluation of human epidermal growth factor receptor 2 (HER2) status was performed using the HercepTest™ kit (Dako, Glostrup, Denmark) with an automated system (Autostainer Link 48, Dako) according to the manufacturer’s instructions. HER2 status was defined as negative (HercepTest scores of 0 or 1 +), doubtful (2 + score), and positive (3 ± score). To confirm HER2 status when IHC results were doubtful, we used Fluorescence in-situ hybridization test using a HER2 FISH PharmDx™ kit (Dako Glostrup, Denmark), and gene amplification was recorded when the HER2/centromeric probe for chromosome 17 signal ratio was ≥2.0.

3. Results

3.1. Patient Characteristics

This prospective observational study included 363 Central Italian individuals: 263 (72.4%) with BC (median age 46 years), 16 (4.4%) with other tumors, and 84 (23.1%) with no tumor. Of the 263 BC patients, 217 (82.5%) had a first BC, 44 (16.7%) a second BC and 2 (0.8%) had subsequent three BC. Among the 10 patients with OC, 3 had initial OC and 7 had a second OC after BC. The BRCA2 pathogenic variants were significantly prevalent in patients with initial BC (p = 0.006, Fisher Exact test) while BRCA1 pathogenic variants were significantly present in patients with OC (p < 0.001, Fisher Exact test). BC and OC patient tumor characteristics are summarized in Table 1. The majority of individuals genotyped with no a priori data on familial variant 269/363 (74.1%) were tested because of personal history of cancer while 94/363 (25.9%) were referred for oncogenetic counselling and genotyping because of a family history suggestive of inherited predisposition to cancer.
Table 1. Population Characteristics.

|                   | BRCA1  | BRCA2  | BRCA1/2 |
|-------------------|--------|--------|---------|
|                   | Pathogenic/Likely | VUS | Pathogenic Variants | Pathogenic/Likely | VUS | Pathogenic Variants | VUS | Pathogenic Variants |
| Overall Central Italian individuals (N, %) | 363 (100.0) | 28 (7.7) | 9 (2.5) | 326 (89.9) | 23 (6.3) | 21 (5.8) | 319 (87.9) | 50 ** (13.8) | 28 (7.7) | 285 (78.5) |
| Age at diagnosis, years | Median Range (Min-Max) | 47 (19–84) | 49 (22–69) | 54 (37–74) | 47 (19–84) | 48 (27–72) | 50 (19–84) | 47 (19–84) | 51 (27–74) | 47 (19–84) |
| p-value * | 0.165 | 0.444 | 0.09 |
| Tumor Type | | | |
| Breast cancer (BC) | First BC | 217 (59.9) | 15 (53.6) | 5 (15.6) | 197 (56.4) | 11 (47.8) | 13 (61.9) | 193 (60.5) | 25 (50.0) | 14 (57.1) | 176 (61.8) |
| Second BC | 4 (12.1) | 4 (14.3) | 2 (6.7) | 11 (2.7) | 3 (13.0) | 7 (33.3) | 34 (10.7) | 7 (9.8) |
| Third BC | 2 (0.5) | 0 (0.0) | 0 (0.0) | 2 (0.6) | 1 (4.4) | 0 (0.0) | 1 (0.3) | 0 (0.0) | 1 (0.4) |
| Other tumors | 16 (4.4) | 2 (7.1) | 1 (11.1) | 13 (4.0) | 2 (8.7) | 2 (4.8) | 13 (4.8) | 4 (2.6) |
| No tumors | 84 (23.1) | 7 (25) | 0 (0.0) | 76 (23.3) | 26 (8.7) | 7 (26.0) | 78 (26.0) | 28 (9.8) |
| p-value * | 0.898 | 0.006 | 0.006 |
| Ovarian cancer (OC) | First OC | 3 (0.8) | 2 (7.1) | 0 (0.0) | 1 (0.3) | 0 (0.0) | 3 (0.0) | 2 (0.0) | 0 (0.0) | 3 (0.3) |
| Both BC and OC | 5 (17.9) | 5 (17.9) | 0 (0.0) | 2 (0.6) | 0 (0.0) | 0 (0.0) | 4 (1.9) | 6 (1.9) |
| No | 269 (96.4) | 21 (75.0) | 9 (0.0) | 323 (99.1) | 23 (100.0) | 20 (95.2) | 310 (97.2) | 43 (96.0) | 27 (99.3) |
| p-value * | <0.001 | 0.779 | <0.001 |

Abbreviations: BC, breast cancer; OC, ovarian cancer; VUS, variant of uncertain significance. * Pearson Chi-square test or the Fisher Exact test, as appropriate. ** One patients possess the pathogenic variants of both BRCA1 and BRCA2 genes simultaneously (ID 606). *** the individuals were all Caucasians.
3.2. BRCA Variants and Patient Characteristics

A total of 363 oncogenetic genotyping results were performed in the present study, 351 in females (97.7%) and 12 (3.3%) in males. Overall, 50/363 (13.8%) genotyping individuals carried one pathogenic/likely pathogenic variant in either BRCA gene, including 28 (7.7%) pathogenic/likely pathogenic BRCA1 variants and 23 (6.3%) pathogenic/likely pathogenic BRCA2 variants (Table 2A). One patient had two variants in both BRCA1 and BRCA2 genes (sample ID 606, Table 2A). Thirteen of 50 (26.0%) variants found were carried in people with no history of cancer and 38/50 variants (76.0%) were detected in patients affected by BC. Of the BC BRCA-mutated patients, 21 (56.7%) were affected by a variant of BRCA1 and 17 (45.3%) by a BRCA2 variant. Of 13 women or men without personal history of cancer, 7 (53.8%) were affected by variants of BRCA1 and 6 (46.2%) by variants of BRCA2. On the whole, the majority of BRCA pathogenic variants were reported to be in exon 11 for both genes: 10 (43.5%) variants in exon 11 of BRCA1 and 13 (56.5%) of BRCA2 gene, respectively. All detected pathogenic/likely pathogenic variants with the exception of three in splice sites of BRCA2 gene and three variants missense of BRCA1 gene, the cause being either termination or a frameshift in BRCA proteins. Five BRCA-variant carriers (17.9%) were affected from both BC and OC. Of seven patients presented with bilateral BC (14.6%), three BRCA1 and four BRCA2 pathogenic variants were found.

3.3. Cohort Spectrum and Variant Detection Rate

Table 2A lists the pathogenic/likely-pathogenic variants detected in the BRCA1 and BRCA2 genes, and Table 2B shows the BRCA1 and BRCA2 VUS variants as well as their frequencies. We found 14 different pathogenic/likely-pathogenic variants in BRCA1 gene and 16 in BRCA2 gene. Overall, of the 30 pathogenic/likely-pathogenic variants, 2 (6.6%) were novel variants in exon 17 of BRCA2 (c.7828_7834delGTGGATC p.(Val2610fs); c.7852_7862delATTTGGGTTTA, p.(Ile2618fs)) not previously reported in BIC, LOVD, ClinVar-NCBI Database, BRCA-Share or any published literature. Besides the detrimental variant detected, 9 and 16 VUS were identified in the BRCA1 and BRCA2 genes, respectively. Of these 25 BRCA1/2 VUS, 2 are reported here for the first time in BRCA1 (c.4986 + 47A > G (IVS16 + 47A > G) in exon 16; c.5407-72delAAAA (IVS22-72delAAAA) in exon 23) and 2 in BRCA2 (c.4504C > A p.(Gln1502Lys) in exon 11; c.7618-11delATTTT (IVS15-11delATTTT) in exon 16). The most frequent VUS variant detected in exon 11 of BRCA2 c.5972C > T p.(Ala1991Val) was observed in five patients. Seven women presented at the same time a VUS and a pathogenic variant, three patients with VUS resulted affected by both OC and BC and six patients had bilateral BC.

3.4. Recurrent Pathogenic/Likely-Pathogenic BRCA1/2 Variants

Of the 30 distinct pathogenic/likely-pathogenic BRCA variants in our patient cohort, 23 were observed only once; 5 in BRCA1 and 2 in BRCA2 variants were detected in at least two or more. These seven variants were detected in 23.3% of all patients with pathogenic BRCA variant. The most frequent pathogenic variant detected in BRCA1 c.5266dupC p.(Gln1756Profs) exon 20 and BRCA2 c.6313delA p.(Ile2105Tyrfs) exon 11, was observed in six patients, respectively (Table 2A).
Table 2. (A) List of BRCA1 and BRCA2 pathogenic/likely pathogenic variants detected in 50 Central Italian individuals. (B) List of BRCA1 and BRCA2 Variants of Uncertain Significance (VUS) variants detected in 33 Central Italian individuals *.

**Table 2 (A) List of BRCA1 and BRCA2 Pathogenic/Likely-Pathogenic Variants Detected on 50 Central Italian Individuals**

| Sample ID | Gene | Exon/Intron | HGVS cDNA (BRCA1 NM_007294.3) (BRCA2 NM_000059.3) | HGVS Protein | Variant Type | IARC Classification | ClinVar | BRCA Share-BIC-LOVD | N. |
|-----------|------|-------------|---------------------------------------------------|---------------|--------------|--------------------|---------|---------------------|----|
| 66,101    | BRCA1| 2           | c.68_69delAG                                      | p.(Glu23Valfs) Frameshift deletion | Class-5       | Pathogenic         | Pathogenic | 2                   |
| 315       | BRCA1| 3           | c.116G > A                                        | p.(Cys39Tyr) Missense | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 909       | BRCA1| 5           | c.181T > G                                        | p.(Cys61Gly) Missense | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 403       | BRCA1| 11          | c.1999C > T                                       | p.(Gln662Ter) Nonsense | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 833       | BRCA1| 11          | c.3228_3229delAG                                  | p.(Gly1077Alafs) Frameshift deletion | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 265,287,471,524 | BRCA1| 11          | c.2406_2409delGAGT                               | p.(Gln804Valfs) Frameshift deletion | Class-5       | Pathogenic         | Pathogenic | 4                   |
| 475,606,1341 | BRCA1| 11          | c.3326-3329delAAA                                 | p.(Lys1109Serfs) Frameshift deletion | Class-5       | Pathogenic         | Pathogenic | 3                   |
| 223       | BRCA1| 11          | c.3599_3600delAG                                  | p.(Gln1200Argfs) Frameshift deletion | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 443       | BRCA1| 12          | c.4117G > T                                       | p.(Glu1373Ter) Nonsense | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 161       | BRCA1| 16          | c.4964_4982del19                                  | p.(Ser1651Tyrfs) Frameshift deletion | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 270,300,358,1011 | BRCA1| 17          | c.5062_5064delGTT                               | p.(Val1688del) Inframe deletion | Class-5       | Pathogenic         | Pathogenic | 4                   |
| 50        | BRCA1| 18          | c.5096G > A                                       | p.(Arg1699Gln) Missense | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 47,150,746,938,943,609 | BRCA1| 20          | c.5266dupC                                       | p.(Gln1756Profs) Frameshift insertion | Class-5       | Pathogenic         | Pathogenic | 6                   |
| 932       | BRCA1| 23          | c.5445G > A                                       | p.(Trp1818Ter) Nonsense | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 616       | BRCA2| 2           | c.67 + 1G > A                                    | - Splicing       | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 606       | BRCA2| 8           | c.632 − 2A > G                                   | - Splicing       | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 289       | BRCA2| 8           | c.658_659delGT                                   | p.(Val2201Leufs) Frameshift deletion | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 426       | BRCA2| 11          | c.3919delG                                       | p.(Glu1307Lysfs) Frameshift deletion | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 352       | BRCA2| 11          | c.4284delT                                       | p.(Gln1429Serfs) Frameshift deletion | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 959       | BRCA2| 11          | c.5645C > A                                       | p.(Ser1882Ter) Nonsense | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 865,946,1004 | BRCA2| 11          | c.5722_5723delCT                                 | p.(Leu1908Argfs) Frameshift deletion | Class-5       | Pathogenic         | Pathogenic | 3                   |
| 424       | BRCA2| 11          | c.6039delA                                       | p.(Val2014Tyrfs) Frameshift deletion | Class-5       | Pathogenic         | Pathogenic | 1                   |
| 48,78,291,564,614,615 | BRCA2| 11          | c.6313delA                                       | p.(Ile2105Tyrfs) Frameshift deletion | Class-5       | Pathogenic         | Pathogenic | 6                   |
| Sample ID | Gene | Exon/Intron | HGVS cDNA (BRCA1 NM_007294.3) (BRCA2 NM_000059.3) | HGVS Protein | Variant Type | IARC Classification | ClinVar | BRCA Share-BIC-LOVD N. |
|-----------|------|-------------|--------------------------------------------------|---------------|--------------|---------------------|---------|------------------------|
| 618       | BRCA2| 17          | c.7828_7834delGTGCCATC                           | p.(Val2612fs) | Frameshift deletion | Class-4 | -                     | - | 1 |
| 367       | BRCA2| 17          | c.7852_7862delATTGGGTTTA                         | p.(Ile2618fs) | Frameshift deletion | Class-4 | -                     | - | 1 |
| 260       | BRCA2| 18          | c.8174G>A                                      | p.(Trp2725Ter) | Nonsense        | Class-5 | Pathogenic Pathogenic  | 1 |
| 393       | BRCA2| 19          | c.8487 + 1G > A                                | -             | Splicing        | Class-5 | Pathogenic UV/Pathogenic | 1 |
| 295       | BRCA2| 20          | c.8537_8538delAG                               | p.(Glu2846Glyfs) | Frameshift deletion | Class-5 | Pathogenic Pathogenic  | 1 |
| 640       | BRCA2| 22          | c.8878C>T                                     | p.(Gln2960Ter) | Nonsense        | Class-5 | Pathogenic Pathogenic  | 1 |
| 571       | BRCA2| 22          | c.8930delA                                    | p.(Tyr2977Phefs) | Frameshift deletion | Class-5 | Pathogenic Pathogenic  | 1 |

| Sample ID | Gene | Exon/Intron | HGVS cDNA (BRCA1 NM_007294.3) (BRCA2 NM_000059.3) | HGVS Protein | Variant Type | IARC Classification | ClinVar | BRCA Share-BIC-LOVD N. |
|-----------|------|-------------|--------------------------------------------------|---------------|--------------|---------------------|---------|------------------------|
| 879       | BRCA1| 2           | c.-77delTGT (IVS0-77delTGT)                       | -             | Intron        | Class-3 | -                     | - | 1 |
| 733       | BRCA1| 7           | c.335A>G                                      | p.(Asn112Ser) | missense      | Class-3 | -                     | VUS | 1 |
| 632       | BRCA1| 11          | c.734A>T                                     | p.(Asp245Val) | missense      | Class-3 | VUS                   | VUS | 1 |
| 635       | BRCA1| 11          | c.3711A>G                                   | p.(Ile1237Met) | missense      | Class-3 | VUS                   | VUS | 1 |
| 303       | BRCA1| 12          | c.4132G>A                                  | p.(Val1378Ile) | missense      | Class-3 | VUS                   | VUS | 1 |
| 1013      | BRCA1| 16          | c.4986 + 47A > G (IVS16+47A > G)             | -             | Intron        | Class-3 | -                     | - | 1 |
| 635       | BRCA1| 16          | c.4843G>A                                  | p.(Ala1615Thr) | missense      | Class-3 | VUS                   | VUS | 1 |
| 272,478   | BRCA1| 20          | c.5277 + 60_5277 + 61insGTATTCCAGCTCC          | -             | Intron        | Class-3 | VUS Benign/VUS        | 2 |
| 1012      | BRCA1| 23          | c.5407-72delAAAA                           | -             | Intron        | Class-3 | -                     | - | 1 |
| 527       | BRCA2| 2           | c.67 + 62T > G (IVS2+62T > G)               | -             | Intron        | Class-3 | VUS Benign/VUS        | 1 |
| 553       | BRCA2| 10          | c.1181A>C                                   | p.(Glu394Ala) | missense      | Class-3 | VUS                   | VUS | 1 |
| 886,930   | BRCA2| 11          | c.4928T>C                                    | p.(Val1643Ala) | missense      | Class-3 | VUS                   | VUS | 2 |
| 633       | BRCA2| 11          | c.4504C>A                                   | p.(Gln1502Lys) | missense      | Class-3 | -                     | - | 1 |
Table 2. Cont.

| Sample ID          | Gene | Exon/Intron | HGVS cDNA (BRCA1 NM_007294.3, BRCA2 NM_000059.3) | HGVS Protein | Variant Type | IARC Classification | Clin Var | BRCA Share-BIC-LOVD | N.       |
|-------------------|------|-------------|-----------------------------------------------|--------------|--------------|---------------------|----------|---------------------|---------|
| 399,532,558,635,679 | BRCA2 | 11          | c.5972C > T                                   | p.(Ala1991Val) | missense     | Class-3             | VUS      | VUS                | 5       |
| 212,309           | BRCA2 | 11          | c.6131G > C                                   | p.(Gly2044Ala) | missense     | Class-3             | VUS      | VUS                | 2       |
| 423               | BRCA2 | 11          | c.6441C > G                                   | p.His2147Gln  | missense     | Class-3             | VUS      | VUS                | 1       |
| 259,296           | BRCA2 | 11          | c.6461A > C                                   | p.(Tyr2154Ser) | missense     | Class-3             | VUS      | VUS                | 2       |
| 752               | BRCA2 | 11          | c.6641C > T                                   | p.(Thr2144Le)  | missense     | Class-3             | VUS      | VUS                | 1       |
| 518               | BRCA2 | 15          | c.7505G > A                                   | p.(Arg2502His) | missense     | Class-3             | VUS      | VUS                | 1       |
| 367               | BRCA2 | 16          | c.7618-11delATTTT                             | -             | Intron       | Class-3             | -        | -                  | 1       |
| 571               | BRCA2 | 25          | c.9275A > G                                   | p.(Tyr3092Cys) | missense     | Class-3             | VUS      | VUS                | 1       |
| 1012              | BRCA2 | 25          | c.9501 + 3A > T                               | -             | Intron       | Class-3             | VUS      | VUS                | 1       |
| 786               | BRCA2 | 26          | c.9648 + 84G > A                              | -             | Intron       | Class-3             | VUS      | Likely Benign/VUS   | 1       |
| 64                | BRCA2 | 27          | c.10024G > A                                  | p.(Glu3342Lys) | missense     | Class-3             | VUS      | VUS                | 1       |
| 1016              | BRCA2 | 27          | c.10095delinsGAATTATATCT                       | p.(Ser3366fs) | Frameshift deletion | Class-3     | VUS      | Benign/VUS         | 1       |

Abbreviations: HGVS, Human Genome Variation Society; cDNA, coding DNA; IARC, International Agency for Research on Cancer; BIC, Breast Cancer Variant Data Base; LOVD, Leiden Open Variation Database; VUS, Variant of Uncertain Significance. *** the individuals were all Caucasians.
3.5. Characteristics of Breast Cancer in BRCA Carrier Patients

Table 3 describes the characteristics of BC in patients with pathogenic/likely pathogenic BRCA1/2 variants in comparison with patients with BRCA1/2-VUS and without BRCA1/2 variants. Median age of the 33 patients with pathogenic/likely pathogenic BRCA1/2 variant was 46 years (range 27–65). The most frequent histology was ductal (n = 21, 63.6%), followed by lobular in seven (21.2%) patients and other invasive histotypes in five (15.2%) (p = 0.005, Fisher Exact test). VUS BRCA2 variants were observed with significant differences in patients with invasive tumor with respect to patients with in situ carcinoma (70% vs. 30% respectively, p = 0.014 Fisher Exact test). According to surrogate definitions of intrinsic subtypes of breast cancer, 36.4% of tumors were classified as triple negative, 45.5% as luminal a-like breast cancer and 3.0% as luminal b-like. The number of triple-negative BCs (TNBCs) was significantly higher in patients with pathogenic BRCA1/2-variant (36.4%) than in BRCA1/2 VUS (16.0%) and BRCA1/2 wild type patients (10.7%) (p < 0.001, Fisher Exact test). No enriched HER-2 was found in patients with pathogenic BRCA1/2 variant. In situ carcinoma was significantly observed in 32% of patients with BRCA1/2 VUS with respect to the 11.2% of patients without BRCA1/2 variant (p = 0.005, Fisher Exact test). The pathogenic BRCA1/2 variant was observed more often in patients with high Ki67 (81.8%) than in those with BRCA1/2-VUS (44.0%) and in those without BRCA1/2 variant (52.7%) (p = 0.008, Fisher Exact test). No significant differences were detected in terms of median age, stage, grading, and exitus. An example is shown in Figure 1: the family members of the proband harboring the pathogenic variant c.6313delA in the BRCA2 gene. As shown in the pedigree, the proband diagnosed with bilateral breast cancer at the age of 38 carried the pathogenic variant in BRCA2. She had a first-degree relative with both ovarian and breast cancer and a second-degree relative with bilateral breast cancer. Estimated variant probability for BRCA1/2 before genetic testing was 26.6% by Myriad and 18.4% by BRCA PRO. Genetic testing was performed on her two cousins with breast cancer who carried a BRCA2 gene with the same pathogenic variant. Her two daughters without breast cancer had the same pathogenic variant.

![Pedigree of patient ID 48 with c.6313delA p.(Ile2105Tyrfs) pathogenic variant in the BRCA2 gene. The proband is indicated by a black arrow. Cancer Type and age at cancer diagnosis is indicated in the legend. Symbols: squares = males, circles = females; quadrant shading = cancer affected; slash through square or circle = deceased.](image-url)
Table 3. Clinical features and BRCA status in BC.

| BRCA1 | BRCA2 | BRCA1/2 |
|-------|-------|---------|
| Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Overall Central Italian individuals (N. %) | | | | | | | | |
| Median | Range(Min-Max) | | | | | | | |
| BRCA1 | BRCA2 | BRCA1/2 |
| Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Age at diagnosis, years | Median | Range(Min-Max) | | | | | | |
| p-value | | | | | | | | |
| Histology | | | | | | | | |
| In situ carcinoma | | | | | | | | |
| Invasive ductal carcinoma | | | | | | | | |
| Invasive lobular carcinoma | | | | | | | | |
| Other invasive histotypes | | | | | | | | |
| p-value | | | | | | | | |
| Grading | | | | | | | | |
| Well-differentiated | | | | | | | | |
| Moderately differentiated | | | | | | | | |
| Poorly differentiated | | | | | | | | |
| Missing | | | | | | | | |
| p-value | | | | | | | | |
| Stage | | | | | | | | |
| 0 | | | | | | | | |
| I | | | | | | | | |
| II | | | | | | | | |
| III | | | | | | | | |
| IV | | | | | | | | |

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**Table 3. Clinical features and BRCA status in BC.**

| BRCA1 | BRCA2 | BRCA1/2 |
|-------|-------|---------|
| Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Overall Central Italian individuals (N. %) | | | | | | | | |
| Median | Range(Min-Max) | | | | | | | |
| BRCA1 | BRCA2 | BRCA1/2 |
| Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Age at diagnosis, years | Median | Range(Min-Max) | | | | | | |
| p-value | | | | | | | | |
| Histology | | | | | | | | |
| In situ carcinoma | | | | | | | | |
| Invasive ductal carcinoma | | | | | | | | |
| Invasive lobular carcinoma | | | | | | | | |
| Other invasive histotypes | | | | | | | | |
| p-value | | | | | | | | |
| Grading | | | | | | | | |
| Well-differentiated | | | | | | | | |
| Moderately differentiated | | | | | | | | |
| Poorly differentiated | | | | | | | | |
| Missing | | | | | | | | |
| p-value | | | | | | | | |
| Stage | | | | | | | | |
| 0 | | | | | | | | |
| I | | | | | | | | |
| II | | | | | | | | |
| III | | | | | | | | |
| IV | | | | | | | | |

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**Table 3. Clinical features and BRCA status in BC.**

| BRCA1 | BRCA2 | BRCA1/2 |
|-------|-------|---------|
| Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Overall Central Italian individuals (N. %) | | | | | | | | |
| Median | Range(Min-Max) | | | | | | | |
| BRCA1 | BRCA2 | BRCA1/2 |
| Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Age at diagnosis, years | Median | Range(Min-Max) | | | | | | |
| p-value | | | | | | | | |
| Histology | | | | | | | | |
| In situ carcinoma | | | | | | | | |
| Invasive ductal carcinoma | | | | | | | | |
| Invasive lobular carcinoma | | | | | | | | |
| Other invasive histotypes | | | | | | | | |
| p-value | | | | | | | | |
| Grading | | | | | | | | |
| Well-differentiated | | | | | | | | |
| Moderately differentiated | | | | | | | | |
| Poorly differentiated | | | | | | | | |
| Missing | | | | | | | | |
| p-value | | | | | | | | |
| Stage | | | | | | | | |
| 0 | | | | | | | | |
| I | | | | | | | | |
| II | | | | | | | | |
| III | | | | | | | | |
| IV | | | | | | | | |

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**Table 3. Clinical features and BRCA status in BC.**

| BRCA1 | BRCA2 | BRCA1/2 |
|-------|-------|---------|
| Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Overall Central Italian individuals (N. %) | | | | | | | | |
| Median | Range(Min-Max) | | | | | | | |
| BRCA1 | BRCA2 | BRCA1/2 |
| Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Age at diagnosis, years | Median | Range(Min-Max) | | | | | | |
| p-value | | | | | | | | |
| Histology | | | | | | | | |
| In situ carcinoma | | | | | | | | |
| Invasive ductal carcinoma | | | | | | | | |
| Invasive lobular carcinoma | | | | | | | | |
| Other invasive histotypes | | | | | | | | |
| p-value | | | | | | | | |
| Grading | | | | | | | | |
| Well-differentiated | | | | | | | | |
| Moderately differentiated | | | | | | | | |
| Poorly differentiated | | | | | | | | |
| Missing | | | | | | | | |
| p-value | | | | | | | | |
| Stage | | | | | | | | |
| 0 | | | | | | | | |
| I | | | | | | | | |
| II | | | | | | | | |
| III | | | | | | | | |
| IV | | | | | | | | |
| Gene       | BRCA1 | BRCA2 | BRCA1/2 |
|------------|-------|-------|---------|
|            | Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants | Variants | VUS | No Pathogenic Variants |
| Missing    | 35    | 3    | 2     | 30    | 0    | 4     | 31    | 3    | 6    | 26 |
| p-value    | * 0.619 | 0.134 | 0.288 |
| Tumor invasiveness |       |       |       |       |       |       |       |       |       |       |
| In situ    | 31    | 0    | 2     | 29    | 0    | 6     | 25    | 0    | 8    | 23 |
| p-value    |       | 0.106 | 0.014 | 0.001 |
| Invasive   | 232   | 19   | 5     | 208   | 14   | 14    | 203   | 33   | 17   | 182 |
| p-value    |       | 0.014 | 0.001 |       |
| Ki67       |       |       |       |       |       |       |       |       |       |       |
| High (≥14) | 146   | 15   | 3     | 128   | 13   | 10    | 123   | 27   | 11   | 108 |
| p-value    | * 0.149 | 0.014 | 0.001 |
| Low (<14)  | 56    | 0    | 2     | 54    | 1    | 3     | 52    | 1    | 5    | 50 |
| Missing    | 61    | 4    | 2     | 55    | 1    | 7     | 53    | 5    | 9    | 47 |
| p-value    | * 0.149 | 0.094 | 0.008 |
| St. Gallen subtype |       |       |       |       |       |       |       |       |       |       |
| Luminal A  | 78    | 5    | 2     | 71    | 11   | 7     | 60    | 15   | 7    | 56 |
| p-value    |       | 0.094 |       |       |       |       |       |       |       |       |
| Luminal B  | 46    | 0    | 1     | 45    | 1    | 3     | 43    | 3    | 2    | 42 |
| HER2 +     | 13    | 0    | 0     | 13    | 0    | 0     | 13    | 0    | 0    | 13 |
| Triple negative | 38 | 10 | 1 | 27 | 2 | 3 | 33 | 12 | 4 | 22 |
| Missing    | 88    | 4    | 3     | 81    | 1    | 8     | 79    | 5    | 11   | 72 |
| p-value    | * 0.001 | 0.02 | <0.001 |
| Exitus     |       |       |       |       |       |       |       |       |       |       |
| Living     | 250   | 17   | 7     | 226   | 14   | 18    | 218   | 30   | 23   | 197 |
| p-value    | * 0.434 | 0.513 | 0.382 |
| Dead       | 13    | 2    | 0     | 11    | 1    | 2     | 10    | 3    | 2    | 8 |
| p-value    | * 0.434 | 0.513 | 0.382 |

Abbreviations: VUS, Variant of Uncertain Significance; HER2, Human Epidermal Growth Factor Receptor 2. * Pearson Chi-square test or the Fisher Exact test, as appropriate. ** One patients possess the pathogenic variants of the both BRCA1 and BRCA2 genes simultaneously (ID 606). *** the individuals were all Caucasians.
3.6. Characteristics of Breast Cancer in Patients with VUS

Mean age of the 25 patients with BRCA1/2 VUS was 48 years (range 34–68) and the most frequent histology was ductal (40.0%), followed by lobular 4.0% with other invasive histotypes 24.0%. Grade 1 was detected in 24.0% of breast cancer, G2 in 36.0%, G3 in 24.0%; information about grading was missing in 16.0% of cases. According to surrogate definitions of intrinsic subtypes of breast cancer [20], 16.0% of tumors were classified as triple negative, 28.0% as luminal a-like breast cancer and 12.0% as luminal b-like. No enriched HER-2 was found in patients with BRCA1/2 VUS. Figure 2 shows the pedigree of a family with VUS. The proband harboring the c.4928T > C variant in the BRCA2 gene was diagnosed with breast cancer at the age of 39; her mother suffered from bilateral BC and carried the same VUS. Her aunt (mother’s sister) died of breast cancer as did her grandmother (BRCA test not performed). This VUS seems representative of the hereditary factor of BC due to the frequency of cases with bilateral breast cancer and the onset in youth in three relatives present in the maternal line (mother, aunt and maternal grandmother).

Figure 2. Pedigree of patient ID 886 with c.4928T>C, p.(Val1643Ala) Unclassified variant in BRCA2 gene. The proband is indicated by a black arrow. Cancer Type and age at cancer diagnosis is indicated in the legend. Symbols: squares = males, circles = females; quadrant shading = cancer affected; slash through square or circle = deceased.

4. Discussion

This is a Central Italian study evaluating the prevalence and spectrum of BRCA1/2 variants. We focused our study on variant detection rates and genetic characteristics associated with specific selection criteria for BRCA1/2 testing in high-risk families and patients affected by breast cancer, whereas other authors evaluated clinical implications and strategy of surveillance of women at high risk. Thirteen percent of the individuals evaluated were carriers of a pathogenic variant, according to the range shown in other countries [27–30], excluding Ashkenazi Jewish ancestry in which founder variants were prevalent [31]. The incidence of BRCA1 and BRCA2 variants was 7.7% and 6.3%,
respectively. According to the literature, we report an incidence of TNBC in BRCA-carriers (36.4%) about 2-fold higher than that found in sporadic breast cancer. TNBC has been reported to account for 12–24% of all BCs and is associated with an hereditary disease cause [32,33]. Approximately 70% of BCs found in BRCA1 variant carriers and up to 23% of BCs in BRCA2 carriers are triple-negative [34]. Therefore, according to national and international guidelines, women with TNBC diagnosed at an age \( \leq 50–60 \) years, irrespective of a positive cancer family history, are eligible for germline BRCA testing [11–13]. As reported in the literature [35,36], BRCA-mutated BC patients showed a significant number of triple-negative cancers \( (p < 0.001) \) and higher Ki-67 expression \( (p = 0.008) \) than in other patients (Table 3), which represents the higher aggressiveness of the disease. BRCA1 pathogenic/likely pathogenic variants reported in our study were higher than BRCA2 variants (54.9% and 45.0%, respectively). More than 2000 different variants have been identified in BRCA1/2 genes and in some populations, founder variants are the most prevalent ones. For example, up to 2.5% of the general Ashkenazi Jewish population will harbor variants in BRCA1 (already present in commercial panels) should also be investigated by next-generation sequencing. (Figure 2). Segregation analysis and functional studies should be further performed in this family due to variant carriers, as reported in a previous Italian study [39].

We observed 30 distinct pathogenic/likely pathogenic BRCA variants (14 in BRCA1 and 16 in BRCA2) and while 23 were observed only once, 5 in BRCA1 and 2 in BRCA2 variants were detected at least two or more times. These seven variants were detected in 23.3% of all the patients with pathogenic BRCA variant and almost all of them were observed in exon 20 of BRCA1 and exon 11 of BRCA2. It is important to screen individual populations and ethnic groups to evaluate the true prevalence of BRCA germline variants [38], as the frequency and type of BRCA variants vary significantly depending on ethnicity and race. To our knowledge, our BRCA study on an Italian population (breast/ovarian cancer patients and healthy population) showed that when several recurrent pathogenic variants are detected, these may be considered as founder variants for this population. If confirmed by further studies, this could have significant implications for preventive population screening and targeted treatments with PARP inhibitors. In our cohort, the BRCA1 c.5266dupC (also known as 5382insC) or BRCA2 c.5946delT (also known as 6174delT) [37].

In our study, of the 30 pathogenic-likely pathogenic variants observed, 2 (6.6%) are novel and it will be necessary to evaluate their level of penetration in carrier families.

Moreover, different BRCA variants lead to protein alterations that could have a different impact on the risk of developing tumors in BRCA variant carriers [40].

If a high risk BRCA variant should be detected, it is important to perform genetic counselling to guide patients and their families regarding risk reduction options and treatment. In our study, we have reported a list of the VUS identified (mostly missense variants) and we note a lack of consensus about their biological/clinical significance among the different databases. Based on the frequency or the co-occurrence of pathogenic variants of these VUS, found in the small number of cases tested in our center, it was not possible to classify these variants. Even though clinician’s decisions cannot be made based on VUS, some of our findings are worthy of attention and deserve further investigation. This is the case, for example, of the young patient (39 years old) with the variant c.4928T \( \rightarrow \) C reported in BRCA2 (Figure 2). Segregation analysis and functional studies should be further performed in this family due to the absence of consensus among databases. Moreover, other breast/ovarian cancer predisposition genes (already present in commercial panels) should also be investigated by next-generation sequencing.

A strength of our study is that it considers not only the affected individuals but also healthy people considered at risk on the basis of the Cuzick–Tyrer program (life-time risk cut off: 10%). Indeed, studies evaluating only patients affected might lead to an overestimate of probability of detecting a variant.

A possible limitation of our study is the selection of individuals for testing. Women should probably not be selected for BRCA testing using only protocols based on risk evaluation tools and strict probability thresholds. Furthermore, there are several different tools to evaluate BRCA risk, and we do not know which is best. Of course, programs with a proactive approach of genetic counseling...
probably need to enforce rigid selection criteria based on probability threshold in order to contain costs and safeguard their feasibility and ethical sustainability. Besides the variant risk, a woman’s personal motivation and the potential utility of test results for the family should be considered. Another limitation of our study is the absence of segregation analysis within family members that could facilitate follow up of people at high risk of disease and their relatives.

Notwithstanding these limitations, our study provides the identification of patients with heterozygous variants of both BRCA1 and BRCA2, along with individuals carrying one variant and a VUS, underlining the necessity of complete BRCA1/2 testing, which should be offered to all eligible individuals. The increase of genetic testing leads to the probability of having an non-informative result or VUS. For the management of VUS, it is important to evaluate family history, clinical factors and functional studies on BRCA protein.

Because this information can be confusing and anxiety-provoking to patients, international collaborative efforts are strongly encouraged to ensure that data pertaining to VUS are publicly available.

5. Conclusions

Our study reveals that the overall frequency of BRCA germline variants in the selected high-risk central Italian population (BC or OC patients and healthy individuals with elevated risk of hereditary BC or OC) is about 13.8%. Further, several recurrent pathogenic variants detected could be considered as founder variants, if confirmed by further studies. We believe that our results could have significant implications for preventive strategies for unaffected BRCA-carriers and effective targeted treatments such as PARP inhibitors for patients with BC or OC.

Author Contributions: Conceived and designed of the study: J.F., V.L., L.P.; involved in the conduct of the study A.M., E.M., P.A., C.M., A.A.-R., F.R.; data interpretation and statistical analysis: F.B., F.S., V.L.; performed sequencing analysis: L.P., M.S.R., F.R.T., V.L.; wrote the paper: J.F., V.L., F.B., L.P., M.S.R. All authors revised and approved the final manuscript.

Funding: This research was supported in part (reagents for BRCA analysis) by the Umbria Association Against Cancer (AUCC).

Acknowledgments: We thank all individuals who participated at the study.

Conflicts of Interest: The authors declare no conflict of interest.

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