Case Report

Double Heterozygosity for BRCA1 Pathogenic Variant and BRCA2 Polymorphic Stop Codon K3326X: A Case Report in a Southern Italian Family

Raffaele Palmirotta *, Domenica Lovero, Luigia Stefania Stucci, Erica Silvestris, Davide Quaresmini, Angela Cardascia and Franco Silvestris

Department of Biomedical Sciences and Human Oncology, University of Bari Aldo Moro, 70124 Bari, Italy; dom.lovero@gmail.com (D.L.); stuccistefania@gmail.com (L.S.S.); ericasilvestris85@gmail.com (E.S.); davide.quaresmini@hotmail.it (D.Q.); angelcard@hotmail.it (A.C.); francesco.silvestris@uniba.it (F.S.)

* Correspondence: raffaelepalmirotta@gmail.com; Tel.: +39-080-547-8674; Fax: +39-080-547-8831

Received: 15 December 2017; Accepted: 16 January 2018; Published: 18 January 2018

Abstract: Here, we describe a patient with bilateral breast cancer and melanoma, and with a concomitant double variant, namely p.Gln563Ter in BRCA1 and p.Lys3326Ter in BRCA2. The BRCA2 p.Lys3326Ter (K3326X) (rs11571833) mutation identified in our patient is a debated substitution of thymidine for adenine which is currently regarded as benign polymorphism in main gene databases. Recent studies, however, describe this variant as associated with breast and ovarian tumors. Based on the observation of the cancer’s earliest age of onset in this subject, our purpose was to reevaluate this variant according to recent papers indicating a role of powerful modifier of the genetic penetrance. Genetic testing was performed in all consenting patient’s relatives, and in the collection of the clinical data particular attention was paid to the age of onset of the neoplasia. Following our observation that the our patient with double heterozygosis had an early age of onset for cancer similar to a few rare cases of double mutation for BRCA1 and BRCA2, we also performed an extensive review of the literature relative to patients carrying a double heterozygosity for both genes. In line with previous studies relative to the rare double heterozygosity in both BRCA1/2 genes, we found the earlier onset of breast cancer in our patient with both BRCA1/2 mutations with respect to other relatives carrying the single BRCA1 mutation. The presence of the second K3326X variant in our case induces a phenotype characterized by early onset of the neoplasia in a manner similar to the other cases of double heterozygosity previously described. Therefore, we suggest that during the genetic counseling, it should be recommendable to evaluate the presence of the K3326X variant in association with other pathogenic mutations.

Keywords: BRCA1/BRCA2; mutational analysis; double heterozygosity; age of onset; K3326X

1. Introduction

Both BRCA1 and BRCA2 are onc suppressor genes involved in DNA repair and are commonly mutated in a number of cancers. The identification of molecular mechanisms in inherited diseases as hereditary breast and ovarian cancer syndrome (HBOC) has emphasized the role of these genes in cancer, allowing subjects at risk of cancer to be screened for prevention and, hopefully, early diagnosis [1].

Although mostly somatically mutated, in a relevant number of subjects these genes are expressed with germline mutations that, however, recur within the general population with a prevalence variable equal to 1/400 up to 1/800 individuals [1–3]. Several ethnic groups carrying constitutional mutations show significantly higher prevalence, such as the Ashkenazi Jewish with increased prevalence equal to 1/40 subjects of 185delAG and 5382insC and 617delT founder mutations in both BRCA1/2 genes [1]. For the same reasons, the presence of double mutations of BRCA1 and BRCA2 is extremely rare in the...
general population, although not exceptional in Ashkenazi breast-cancer patients [4]. Compared to the huge amount of mutational analysis data for BRCA1 and BRCA2 genes, only a few studies report dual mutations in non-Ashkenazi subjects, mostly consisting in the description of a single or of a small number of families [4–9].

In the present study, we describe a case of coexisting BRCA1 p.Gln563Ter and BRCA2 p.Lys3326Ter double variant in a southern Italian family. The BRCA2 variant, also known as K3326X, was firstly interpreted as pathogenic, but its identification in control populations with no increase in breast or ovarian cancer occurrence led to its reinterpretation of benign polymorphism [10]. Interestingly, new evidence of a significant association with cancer risk has been described [10] and recent studies reassessed the association of K3326X BRCA2 mutation with the risk of developing melanoma, urothelial, pancreatic, breast and ovarian cancers [11–14], while its role as modifier of genetic penetrance has been also reported [15]. By exploring the effect of this variant in our patient, we revealed clinical aspects apparently common to those previously described in patients with double BRCA1/BRCA2 heterozygosity, including the earlier age of onset of cancer.

Therefore, we reviewed the literature with regard to double mutations on BRCA1 and BRCA2 genes in order to search for similarities in genotype–phenotype correlations and corresponding diagnostic and clinical implications.

2. Results

Molecular analysis of the proband showed BRCA1 c.1687C>T (p.Gln563Ter, rs80356898) and BRCA2 c.9976A>T (p.Lys3326Ter; K3326*, rs11571833) heterozygous mutations bearing in both cases a premature stop codon in BRCA1 at exon 10, and BRCA2 at exon 27, respectively (Figure 1). The mutations recurred by genetic transmission to the patient’s asymptomatic daughters (Figure 2): the first, namely a 36-year-old (IV.1) inherited both BRCA1 and BRCA2 mutations; whereas the younger, aged 30 (IV.3) showed only the BRCA2 variant. The proband’s asymptomatic 91-year-old mother (II.9) bore only the mutation in BRCA1 in a similar fashion to the sister with breast cancer who died at 56 years of age (III.3), and for whom the analysis was performed on formalin-fixed, paraffin-embedded (FFPE) tissue. Mutational analysis, which turned out to be negative, was later extended to the proband’s 70-year-old husband, since he was reported as having a degree of relationship (first-degree cousins) with his mother (II.7), who died of breast cancer at the age of 70, and with the maternal grandmother of the proband (I.3), who died also at the age of 70 of breast cancer.

Figure 1. Direct sequence analysis of the BRCA1 c.1687C>T and BRCA2 c.9976A>T variants.
According to the data shown on the variant annotation file generated by analysis on Ion Reporter v.5.0 (Thermo Fisher Scientific Inc.), the somatic mutation c.9257-16T>C (IVS24-17T>C) mutation [16], we extended the analysis of both variants to all predicted to be possibly damaging, with a score of 0.777 (sensitivity: 0.85; specificity: 0.92); while using another web tool SIFT Human Coding SNPs (http://sift.jcvi.org/www/SIFT_chr_coords_submit.html) this mutation is predicted to be tolerated, with a SIFT score of 0.23. This variant is classified in the COSMIC database (http://cancer.sanger.ac.uk/cosmic) (COSM5842418) and described with a functional analysis through hidden Markov models (FATHMM) prediction as pathogenic (score 0.72).

Genetic testing was also performed in other consenting relatives, and the following data refer to the presence of germinal BRCA1 or BRCA2 gene mutation. The mother’s sister had died at 60 years of age for a metastatic ovarian cancer (II.10) one year earlier. She had three children: a 53-year-old son (III.4), who had received a diagnosis of cutaneous melanoma two years before, but with no mutations; a 51-year-old daughter (III.5) carrying no mutations; and an asymptomatic 48-year-old daughter (III.6) positive to the mutation of the BRCA1 gene. An additional sister of the mother’s proband (II.11) died at 80 years of age for causes unrelated to cancer, while her son died at 55 years of age of pancreatic cancer (III.9). Two other sons of 70 (III.7) and 64 (III.8) years of age were apparently asymptomatic, but both of them bore mutations in the BRCA1 gene. Furthermore, three more cousins, daughters of the asymptomatic 80-year-old sister of the mother’s proband (II.13) of 57 (III.13), 55 (III.14) and 45 (III.15) years of age, were negative to the mutational analysis.

Since the BRCA2 p.Lys3326* mutation has often been found in linkage disequilibrium with the presence of a pathogenic frameshift BRCA2 c.6275_6276delTT (Leu2092ProfsTer7) and a non-pathogenic BRCA2 c.9257-16T>C (IVS24-17T>C) mutation [16], we extended the analysis of both variants to all consenting subjects. None of the analyzed subjects presented these sequence variants. The sequence analysis performed on the proband paraffin-embedded melanoma samples revealed the presence of the two BRCA1 and BRCA2 variants and a novel BRCA2 c.4297G>A somatic variant (p.Gly1433Arg). According to the data shown on the variant annotation file generated by analysis on Ion Reporter v.5.0 (Thermo Fisher Scientific Inc.), the somatic mutation BRCA2 c.4297G>A has a PolyPhen score of 0.916 and a SIFT score of 0.22. Using the WEB-tool PolyPhen-2 (genetics.bwh.harvard.edu/pph2/) this mutation is predicted to be possibly damaging, with a score of 0.777 (sensitivity: 0.85; specificity: 0.92); while using another web tool SIFT Human Coding SNPs (http://sift.jcvi.org/WWW/SIFT_chr_coords_submit.html) this mutation is predicted to be tolerated, with a SIFT score of 0.23. This variant is classified in the COSMIC database (http://cancers.sanger.ac.uk/cosmic) (COSM5842418) and described with a functional analysis through hidden Markov models (FATHMM) prediction as pathogenic (score 0.72).
Finally, molecular screening of CDKN2A, p14 (specific exon-1β) and CDK4 genes, which are involved in approximately 20–40% of large, high-risk families, performed both on the proband and on the cousin affected by melanoma (III.4), did not identify any pathogenetic sequence variant.

We further completed an extensive review of world-wide literature using the PubMed database (https://www.ncbi.nlm.nih.gov/pubmed) and reviewing the references of retrieved articles. All studies involving Jewish Ashkenazi patients were deliberately excluded. In two papers we have amended the calculation of two patients (Table 1) [17–19]. From 1998 to 2017, 19 articles described 33 families with 54 subjects carrying a double BRCA mutation [2,8,9,17,19–33]. Table 1 summarizes the main data, including ethnicity, description of mutations, matrilineal or patrilineal transmission of variants, cancer type and age of onset, for a total of 56 cases (34 probands and 22 relatives), with the addition of our current report.

The types of mutations and combinations of mutations on BRCA1 and BRCA2 did not occur in any of the probands and/or families studied. In 18 cases out of 34, the paternal and maternal transmission is not determined. Analysis of data, documented with molecular analysis, shows that in 8 cases one of the parents transmitted both variants, in 3 cases both parents had a mutation, and in 2 cases a single variant was transmitted by one parent while the other one was wild-type. Finally, in 2 cases one of the mutations had been identified in one of the parents while the other had not been analyzed. The phenotypic expression in double heterozygosity varied from unilateral breast cancer at the age of 26, to asymptomatic at the age of 72. Forty-two cases (75%) had a primary cancer: 35 breast, 2 ovary, 2 prostate, and 1 case for cervix, caecum and stomach, respectively. Fourteen subjects (25.0%) were asymptomatic (ranging from 30 to 72 years of age), 8 of whom (14.2%) were older than 40. A secondary neoplasia was detected in 14 out of 42 patients.

Finally, the median age at diagnosis for breast cancer was 38 years (range 26 to 76), while the median age at diagnosis calculated for all primary cancers was 40 (range 26 to 76). In very few cases was it possible to compare the age at the onset of the first cancer of subjects carrying the double mutations, with their single heterozygous relatives. In particular, Heidemann et al., described six German non-Jewish subjects with double mutation in whom the onset of the first breast cancer was at 39.6 years mean age, compared to their single heterozygous female relative with a mean age of 52.4 years [32]. Expanding the analysis to the available literature on Caucasian double heterozygote (DH) patients, the authors confirmed the occurrence of the first cancer at a mean age of 41.4 years in patients with double mutation in contrast to 53.0 years in the relatives carrying a single BRCA mutation. The 95% confidence intervals between double heterozygotes and their single heterozygous relatives did not overlap [32].
Table 1. Data of females with BRCA1 and BRCA2 double heterozygosity published in the literature. WT: Wild Type; ND: Not Determined; DH: double heterozygosity; breast u.: breast unilateral; breast b.: breast bilateral; ovarian b.: ovarian bilateral; Mel: melanoma.

| Geographical Localization/Ethnic Group | BRCA1 Mutation | BRCA2 Mutation | Sex | Inheritance | Mother | Father | Proband Cancer/Age of Onset (Years) [Relative with DH] | References |
|---------------------------------------|----------------|----------------|-----|-------------|--------|--------|-------------------------------------------------------|------------|
| Scottish                              | c.2380G>T      | c.2067_2068insA| F   | WT          | ND     |        | Breast 35                                             | [20]       |
| German descent                       | c.5090G>T      | c.6409_6410delCTAAA| M   | BRCA1       | ND     |        | Asymptomatic 36 [Sister asymptomatic 34] [Brother asymptomatic 30] | [21]       |
| Australia (no Jewish ancestry)       | c.5769_3770delGA | c.5946_5946delT| F   | WT          | BRCA2  |        | Breast c.40                                            | [22]       |
| Spain                                | c.5123C>A      | c.6275_6276delTT| F   | BRCA1 BRCA2 | -      |        | Breast 28 [Mother asymptomatic 70] [Sister asymptomatic 40] [Cousin asymptomatic 47] [Cousin asymptomatic 41] [Uncle prostate 66] [Aunt breast 70] [Aunt breast 66] | [23]       |
| Korea                                | c.4981G>T      | c.5946_5946delTGCA| F   | BRCA1 BRCA2 | -      |        | Breast 33 [Mother stomach 62]                         | [17]       |
| Spain                                | c.1516_1520delT | c.2798_2799delCA| F   | ND          | ND     |        | Breast 26                                             | [8]        |
| Korea                                | c.1656_1660delT | c.4599A>C      | F   | ND          | ND     |        | Breast 37                                             | [17]       |
| Netherlands                          | c.2605_2606delAA| c.3487_3488delRG| F   | ND          | ND     |        | Ovarian 40, breast 45                                 | [5]        |
| Netherlands                          | c.2605_2606delAA| c.4449_4450delA| F   | ND          | ND     |        | Breast 28                                             | [17]       |
| European                             | c.962C>A       | c.3170_3171delAGAA| F   | ND          | ND     |        | Breast 37                                             | [2]        |
| Italy                                | c.4285_4286insG | c.7786C>T     | F   | ND          | ND     |        | Breast 37                                             | [34]       |
| Australia                            | c.3351_3361delCAAG| c.651_652delG| F   | ND          |        |        | Breast 34, colon 35, breast 53 [Sister asymptomatic 65] | [25]       |
| Italy                                | c.5263_5264insC | c.5796_5797delEA| F   | BRCA1       | BRCA2  |        | Breast 36, ovarian 42                                 | [28]       |
| Italy                                | c.389_835delC  | c.1835T>G     | F   | ND          | ND     |        | Breast 43                                             | [27]       |
| Italy                                | c.3916_3917delTT| c.5379_5380delRG| F   | WT          | ND     |        | Breast 30, ovarian 36                                 | [27]       |
| Italy                                | c.1670C>T      | c.6409C>T     | F   | ND          | ND     |        | Breast 46, ovarian 58                                 | [27]       |
| Italy                                | c.2405_2406delTG| c.4249_4250insT| F   | ND          | ND     |        | Breast and ovarian 52                                 | [26]       |
| Denmark                              | c.5096G>A      | c.631_641insC | F   | -           | BRCA1  | BRCA2  | Breast 53, ovarian 59 [Father breast 76] [Son and daughter asymptomatic] | [28]       |
| Caucasian                            | c.1961_1962delA| c.1444_1445delC| F   | ND          | ND     |        | Ovarian b. 50                                        | [19]       |
| Caucasian (maternal Ashkenazi ancestry)| c.5265_5266insC| c.4828_4830delTG| F   | ND          | ND     |        | Breast u. 40                                         | [29]       |
| Korea                                | c.3627_3628insA| c.6724_6725delRG| F   | ND          | ND     |        | Breast 26                                             | [19]       |
| Korea                                | c.390C>A       | c.3018_3019delA| F   | ND          | ND     |        | Breast 45                                             | [19]       |
| Korea                                | c.5030_5031delCTAAA| c.1399A>T    | F   | BRCA1       | BRCA2  |        | Breast 35                                             | [20]       |
| Japan                                | c.188T>A       | c.5578_5579delTTAA| F   | BRCA1 BRCA2 | -      |        | Breast 55 [Father asymptomatic 51] [Cousin breast 41] Endometrial cancer 46 | [30]       |
| Afrikaners                           | c.2635G>T      | c.7934_7935delG| F   | BRCA1       | BRCA2  |        | Breast 42 [Healthy second cousin 49]                 | [31]       |
| Germany                              | c.5261_5263insC| c.5645C>A      | F   | WT          | BRCA1  | BRCA2  | Breast b. 37, Ovarian b. 63 [Father prostate 68]       | [32]       |
| Germany                              | c.66_67delAG  | c.5722_5723delCCT| F   | BRCA1       | BRCA2  |        | Breast b. 31, 38 [Mother breast 40]                   | [32]       |
| Germany                              | c.963C>A      | c.2231C>G     | F   | BRCA1 BRCA2 | -      |        | Breast b. 31, 38 [Mother breast 40]                   | [32]       |
| Germany                              | c.3910_3911delG| c.2803A>T     | F   | BRCA1       | BRCA2  |        | Breast u. 39 [Mother breast 34; another cancer not reported 35] | [32]       |
| Germany                              | c.5193_5194delKL| c.658_659delCT| F   | ND          | ND     |        | Cervix 58, ovarian 61                                 | [32]       |
| Germany                              | c.3703_3704delCTAAA| c.1813_1814insA| F   | ND          | ND     |        | Cervix 26, breast 40                                  | [32]       |
| Italy                                | c.547_547+1T-A | c.2803A>T    | F   | -           | BRCA1  | BRCA2  | Breast 35 [Father asymptomatic 72]                    | [33]       |
| France                               | c.1016_1017insA| c.6814_6815delA| F   | BRCA1       | WT     |        | Breast 46                                             | [9]        |
| Italy                                | c.1670C>T      | c.9976A>T     | F   | BRCA1       | ND     |        | Breast u. (40), breast u (47), breast b (54), Mel (54) [Asymptomatic daughter (36)] | This report |
3. Discussion

BRCA1 c.1687C>T (p.Gln563Ter/Q563X, previously named 1806C>T) variant is a well-known and fully characterized mutation which is particularly common in European populations. In Austria and Slovenia, for example, it recurs approximately in 15% and 26% respectively of all BRCA1 pathogenic variants, and haplotype analysis suggests a potential Austrian origin [34,35]. This mutation, related to lung, gastric and bilateral breast cancers, was found with remarkable frequency in Spain [36] and with a strong founder effect in hereditary ovarian/breast cancer Polish patients [37]. In 2003, Johannsson O.T. et al., linearized a breast lymph node metastasis from a 53-year-old Swedish woman carrying the Q563X mutation [38]. The histological analysis revealed a poorly differentiated adenocarcinoma, negative for estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER-2) and epidermal growth factor receptor (EGFR) [38]. A complete expression dataset is on the website Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM155216).

The BRCA2 c.9976A>T (p.Lys3326*) variant was identified for the first time in 1996 during a mutational screening program completed on families with recurrent breast cancer in Nebraska. The study was carried out on 513 breast cancer patients and concluded that this mutation does not confer a highly penetrant susceptibility for inherited breast cancer [39]. Despite a number of observations supporting the theory of a close association between this variant and breast, pancreas, esophageal and squamous-cell lung cancers, parallel equally detailed studies supported the non-pathogenicity of this variant [12,16]. In particular, in a study of 95 families carrying the mutation, Higgs et al., showed that the previously reported associations with increased cancer risk are in many cases due to the concomitant presence of a pathogenic frameshift BRCA2 mutation c.6275_6276delTT (Leu2092ProfsTer7) as well as to the non-pathogenic BRCA2 c.9257-16T>C (IVS24-17T>C) that are often found in linkage disequilibrium with p.Lys3326* [16]. In 2015 Thompson et al., on the basis of a study performed on 2634 Australian breast and/or ovarian cancer patients, provided evidence on the association with a low to moderate increase of breast cancer risk [10]. More recently, a molecular screening of Swedish cutaneous malignant melanoma patients reported the presence of the p.Lys3326* mutation in 12 out of 452 unrelated patients, 8 of them with obvious familial transmission, and 4 with sporadic melanoma [11]. Finally, a further study performed on a Chinese population of 6064 patients and 8661 controls showed that BRCA2 p.Lys3326* plays an important role in the genetic susceptibility to urinary tract cancers since it is apparently associated with increased risk of bladder cancer [13].

More recently, a massive epidemiological study evaluated the association between this BRCA2 variant and the risk of breast, ovarian and prostate cancers. In this regard, researchers at the Collaborative Oncological Gene-Environment Study (COGS), analyzed data from 76,637 breast/prostate patients, including 7183 BRCA1 and 5101 BRCA2 mutation carriers in comparison with 83,796 control subjects. The study confirmed the association of the BRCA2 K3326X variant with an increased risk of developing triple-negative breast and serous ovarian cancers irrespective of additional BRCA2 pathogenic variants, while no association with prostate cancer risk was found [12]. Finally, in a study of 48 women with ovarian cancer and high risk of genetic inheritance but negative for both BRCA1 and BRCA2 mutations, Stafford et al., observed two patients carrying the K3326X variant, one with a concomitant RAD51D nonsense mutation and the other with an ATM frameshift mutation, having both developed breast and ovarian cancer [15]. Therefore, the authors argued that this variant is of minimal risk when inherited alone, but may acquire the function of strong modifier of penetrance when concomitant with a second BRCA pathogenic mutation [15].

In relation to the effect on cellular and biochemical properties of this variant in cancer cell lines, several complete studies have already been reported. In some cases the results show that the K3326X variants had no effect on the BRCA2 function and indicate it as “neutral variant” [40,41]. However, a study performed in mouse cells deleted for the COOH terminus of BRCA2 (amino acids 3140–3328) and irradiated with γ-radiation shows that the deletion of BRCA2 could accelerate cell proliferation and stimulate cancer by defective MmRadSl-mediated DNA repair [42].
However, to date in the Breast Cancer Information Core database (https://research.nhgri.nih.gov/projects/bic/), the K3326X variant, which is found with a frequency of about 1% in the population, is still clinically classified in the “pending” category.

In our study, we have identified double heterozygosity in the proband and in her asymptomatic daughter. The asymptomatic mother of the proband has transmitted only the BRCA1 mutation and, since the father’s sampling was not available, we cannot argue whether or not the BRCA2 K3326X was due to a “de novo” variant, or to a patrilineal transmission. Unfortunately, all relatives of the father died without leaving children. The proband developed the first breast cancer at the age of 40, while the sister, with only the BRCA1 mutation, had an age of diagnosis of 56 years. Furthermore, the maternal aunt and the maternal grandmother, both obligate carriers for the BRCA1 variant, developed an ovarian cancer at the age of 59 and a breast cancer at 70, respectively.

From the overall examination of the mutational state and the pedigree, we have drawn some considerations. First, we observed that the proband and her sister, both suffering from ductal breast carcinoma, had a very different age of onset with a marked difference of about 16 years. Neither had preventive mastectomy or oophorectomy and the two patients lived in the same household with the same eating habits, environmental and lifestyle factors. The observation of all other relatives only carrying BRCA1 mutations leads us to consider that the pathogenic variant p.Gln563Ter, despite bearing a premature stop codon at exon 10 in the BRCA1 gene, is present with a low penetrance in this family. A paradigmatic example is offered by the mother of the two sisters (II9), a carrier of the mutation but not affected by any neoplasia despite her age of 90 years, and the maternal aunt (II10), an obligate carrier and with a diagnosis of ovarian cancer at 59 years of age. These differences cannot only be explained by anticipation, since the proband’s cousins, III6, III7 and III8, are also carriers of the BRCA1 mutation but not affected by neoplasia at 48, 70 and 64 years, respectively (mean age 60.6 years).

We focused on the cancer’s earliest age of onset in the subject with double heterozygosity and a careful examination of literature revealed that this is in agreement with previous findings and was not unexpected in patients carrying double heterozygosity for BRCA1 and BRCA2 genes [32]. Similarly, a few published works also report earlier age of breast cancer onset in women with a double mutation for both BRCA genes while, to our knowledge, there are no similar data concerning a double mutation involving the K3326X BRCA2 variant.

The first observations of a double mutation in the BRCA1 and BRCA2 genes was found in 1997 in a casistica of patients of Ashkenazi origin affected by breast and ovarian cancer carrying both the 185delAG in the BRCA1 and the 6174delT BRCA2 gene mutations [5,6]. In this population, three founder mutations, BRCA1 185delAG, BRCA1 5382insC and BRCA2 6174delT, are particularly common with an approximate frequency of 1%, 0.1%, and 1.4%, respectively [6,7,43]. Therefore, on the basis of predictive statistical calculations, the population carrier frequency of BRCA1 and BRCA2 mutations in the Ashkenazi population should be approximately 3%, with a presumable occurrence of double heterozygosity of 1 in 1800 individuals [8,9]. In a BRCA1 and BRCA2 mutational study performed on 10,000 subjects, including 3022 Ashkenazi, Frank et al., identified double mutations exclusively in the Ashkenazi population with a frequency of 11 of the 3022 tested individuals (0.36%), and 11 on 617 positive cases (1.8%) [44]. In a review of the literature on 34 Ashkenazi ancestry patients with double heterozygosity for BRCA1 and BRCA2, Leegte et al., described no genotype–phenotype correlation between the double mutational status and the age of onset, the cumulative lifetime risks and the presence of multiple primary tumors [9]. In an additional Ashkenazi cohort of 1191 BRCA mutations, with carriers from 567 families, Lavie et al., identified 22 subjects (1.84%) from 17 families (2.9%) bearing the double BRCA1/BRCA2 mutation [4]. However, all 22 subjects were females, 7 of whom had breast cancer (46%) and 1 had ovarian cancer (6.6%). The authors noted a younger age at the occurrence of cancer in patients carrying the double mutation (44.6 ± 13 years) with respect to patients with a single mutation (48.1 ± 13 years), deducing from the absence of other clinical differences among the two groups of patients that none of them would have benefited from dedicated diagnostic or therapeutic measures [4].
Conversely, in the non-Ashkenazi population the prevalence of BRCA1 and BRCA2 mutation carriers is approximately 0.24% and double heterozygosity is a rare phenomenon recurring in approximately 1 in 190,000 subjects [8,9,45]. However, data relative to carriers of the double mutation are available only in a very limited number of studies that usually describe only single case reports. As accurately described in the results section, after a careful examination of the literature we have identified 19 articles describing 33 families with 54 subjects carrying a double BRCA mutation.

The examination of these data therefore suggests that the presence of the second K3326X variant in our case induces a phenotype characterized by early onset of the neoplasia in a manner similar to the other cases of double heterozygosity previously described. However, we must also keep in mind data from recent studies describing the mild association with the risk of developing breast and ovarian cancers [12] and assigning to this variant the role of a strong modifier of penetrance [15]. These findings should be considered during the counseling of carriers of a pathogenic mutation and a concomitant K3326X variant for a potentially earlier age of onset of the first cancer, as suggested also in other studies [32].

Finally, we would like to focus the melanoma found in our patient, in which next-generation sequencing (NGS) detected a somatic BRCA2 variant c.4297G>A (p.Gly1433Arg) reported as pathogenic by the COSMIC database. As mentioned above, a recent study showed a significant association between the K3326X variant allele and melanoma [11]. Intriguingly, this missense mutation identified in our work has been previously reported as a somatic mutation in genetic screening performed on samples from patients affected by metastatic melanoma [46]. To date, the association between mutations of the BRCA1/BRCA2 genes and the onset of melanoma is still being studied with the presence in literature of controversial or inconclusive evidence [47]. However, as suggested by other authors, patient carriers of a BRCA1/BRCA2 mutations should be informed about the risk of skin cancer and be subjected to a periodic dermatological control [48].

We hope that our observations may add knowledge to this topic and contribute to further studies needed to verify these findings.

4. Materials and Methods

A 65-year-old woman was referred to our Oncogenomic Research Center for genetic counseling, including a pedigree analysis and a complete medical examination. The patient reported her personal and familial history of breast cancer since, at age of 40 years, she was diagnosed as having breast cancer on the left, and underwent a quadrantectomy. The pathologist described undifferentiated ductal, infiltrating carcinoma of the breast (G3—pT1N0M0), showing the estrogen receptor (ER) at 25% in tumor cells whereas the progesterone receptor (PgR), proliferation-related Ki-67 antigen (Ki-67) expression and HER-2 were undetectable. Seven years later, the patient developed a second ductal cancer still on the residual left breast with peculiar molecular aspects, namely triple-negative with a Ki-67 of 30%. At the age of 54, a further cancer on the right breast was diagnosed, and the patient underwent a mastectomy. The tumor was still triple negative, and showed Ki-67 expression on 48% of tumor cells. Together with the third breast cancer, the patient developed a pigmented flat cutaneous neoplasm of 1.1 cm in diameter, with irregular margins, that was surgically removed and described by the pathologist as melanoma in situ. A paraffin-embedded sample of the melanoma was recruited. Germline molecular analyses with sequencing of both BRCA1 and BRCA2 genes were completed.

Once mutational data were available, we extended the genetic screening to all family members to complete a co-segregational analysis (Figure 1). Written informed consent was obtained from each participating individual, and the study was performed under the Ethics Committee of the University of Bari approvals (number 5329, 5 July 2017) and in accordance with the principles embodied in the Declaration of Helsinki.

The patient’s sister also developed a breast cancer, histologically documented as a moderately differentiated ductal left breast carcinoma (G2—pT1Nsn0), with Ki67 at 60% and HER-2 at 90% on tumor cells, while both ER and PgR were not expressed.
Genomic DNA was isolated from the peripheral blood of the patient using the DNeasy® blood and tissue Kit (QIAGEN Inc., Chatsworth, CA, USA) and quantified with a Qubit® 3.0 fluorometer (Life Technologies™, Carlsbad, CA, USA). We employed 10 ng of DNA to prepare the barcoded library using the Ion AmpliSeq™ Library kit 2.0 and the Ion Xpress™ barcode adapters (Life Technologies™) on the Ion AmpliSeq™ BRCA1 and BRCA2 Panel (Life Technologies™) according to the manufacturer’s instructions. The library was purified with Agentcourt AMPure XP reagent (Beckman Coulter, Beverly, MA, USA) and quantified with the Ion Library Quantitation Kit (Life Technologies™) on the StepOne Plus system (Applied Biosystem, Foster City, CA 94404, USA). Template preparation was performed with the Ion OneTouch™ 2 System (Life Technologies™) and Ion OneTouch™ ES. Finally, sequencing was performed on a personal genome machine (PGM) using the Ion PGM™ Hi-Q™ View Sequencing kit (Life Technologies™) on the Ion 314 chip v2 set (Life Technologies™) at the 500 flows standard. The results of sequence analysis were analyzed using Torrent Suite Software 5.0.4 (Life Technologies™) aligning all reads to the human reference hg19 genome. Variant calling was performed running the Torrent Variant Caller plugin version 5.0.4.0.

Specific sequencing reactions to confirm NGS data were performed using a Big Dye Terminator on a 3500 Series Genetic Analyzer (Thermo Fisher Scientific Inc., Foster City, CA 94404, USA). In order to exclude pre-analytical and analytical errors, PCR reactions and sequencing analyses were carried out on two different DNA extractions. For the control of both primer design and results, we referred to the ENSEMBL sequence BRCA1 (ENST00000357654.7) and BRCA2 (ENST00000380152.7).

We evaluated possible genetic factors contributing to the hereditary melanoma. For this reason, molecular screening for the CDKN2A with p14 (specific exon-1β) and CDK4 genes was performed both on the proband and the cousin (III.4) affected by melanoma, as previously described [49,50].

Each identified variant was investigated in its potential pathogenic role using prediction algorithms such as SIFT and Polyphen, and web databases such as the Breast Cancer Information Core (http://research.nhgri.nih.gov/bic/), COSMIC (http://cancer.sanger.ac.uk/cosmic), Leiden Open Variation Database (http://www.lovd.nl/3.0/home), dbSNP (http://www.ncbi.nlm.nih.gov/snp/), Ensembl (http://www.ensembl.org/index.html) and the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php).

Acknowledgments: The present study was partially supported by a grant from the Italian Association for Cancer Research (grant no. IG11647) (Franco Silvestris) and from the Apulia Region Oncogenomic Project (grant no. 2582-30-12-2013). The authors wish to thank Paola Ghiorzo for melanoma molecular screening. The authors would also like to thank the Associazione per la Ricerca Biomolecolare Onlus for supporting the publication of this paper.

Author Contributions: Raffaele Palmiotto and Franco Silvestris: principal investigators, study conception and design; Domenica Lovero and Davide Quaresmini: sample collection and molecular analysis; Luigia Stefania Stucci, Erica Silvestris and Angela Cardascia: patient management, analysis and interpretation of data. All authors were involved in drafting the article and/or revising it critically for important intellectual content, and all authors approved the final version to be published.

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

References
1. Petrucelli, N.; Daly, M.B.; Feldman, G.L. Hereditary breast and ovarian cancer due to mutations in BRCA1 and BRCA2. Genet. Med. 2010, 12, 245–259. [CrossRef] [PubMed]
2. Claus, E.B.; Petruzella, S.; Matloff, E.; Carter, D. Prevalence of BRCA1 and BRCA2 mutations in women diagnosed with ductal carcinoma in situ. JAMA 2005, 293, 964–969. [CrossRef] [PubMed]
3. Claus, E.B.; Schildkraut, J.M.; Thompson, W.D.; Risch, N.J. The genetic attributable risk of breast and ovarian cancer. Cancer 1996, 77, 2318–2324. [CrossRef]
4. Lavie, O.; Narod, S.; Lejbkowicz, F.; Dishon, S.; Goldberg, Y.; Gemer, O.; Rennert, G. Double heterozygosity in the BRCA1 and BRCA2 genes in the Jewish population. Ann. Oncol. 2011, 22, 964–966. [CrossRef] [PubMed]
5. Ramus, S.J.; Friedman, L.S.; Gayther, S.A.; Ponder, B.A.; Bobrow, L.; van der Looji, M.; Papp, J.; Ohlaj, E. A breast/ovarian cancer patient with germline mutations in both BRCA1 and BRCA2. Nat. Genet. 1997, 15, 14–15. [CrossRef] [PubMed]

6. Gershoni-Baruch, R.; Dağan, E.; Kepten, I.; Freid, G. Co-segregation of BRCA1 185delAG mutation and BRCA2 6174delT in one single family. Eur. J. Cancer 1997, 33, 2283–2284. [CrossRef]

7. Randall, T.C.; Bell, K.A.; Rebane, B.A.; Rubin, S.C.; Boyd, J. Germline mutations of the BRCA1 and BRCA2 genes in a breast and ovarian cancer patient. Gynecol. Oncol. 1998, 70, 432–434. [CrossRef] [PubMed]

8. Meynard, G.; Mansi, L.; Lebahar, P.; Villanueva, C.; Klajer, E.; Calcagno, F.; Vivalta, A.; Chaix, M.; Collonge-Rame, M.A.; Populaire, C.; et al. First description of a double heterozygosity for BRCA1 and BRCA2 pathogenic variants in a French metastatic breast cancer patient: A case report. Oncol. Rep. 2017, 37, 1573–1578. [CrossRef] [PubMed]

9. Leegte, B.; van der Hout, A.H.; Deffenbaugh, A.M.; Bakker, M.K.; Mulder, L.M.; ten Berge, A.; Leenders, E.P.; Wesseling, J.; de Hullu, J.; Hoogerbrugge, N.; et al. Phenotypic expression of double heterozygosity for BRCA1 and BRCA2 germline mutations. J. Med. Genet. 2005, 42, e20. [CrossRef] [PubMed]

10. Thompson, E.R.; Gorringe, K.L.; Rowley, S.M.; Li, N.; McInerny, S.; Wong-Brown, M.W.; Devereux, L.; Li, J.; Lifepool, I.; Trainer, A.H.; et al. Reevaluation of the BRCA2 truncating allele c.9976A > T (p.Lys3326Ter) in a familial breast cancer context. Sci. Rep. 2015, 5, 14800. [CrossRef] [PubMed]

11. Tuominen, R.; Engstrom, P.G.; Helgadottir, H.; Eriksson, H.; Unneberg, P.; Kjellqvist, S.; Yang, M.; Linden, D.; Edsgard, D.; Hansson, J.; et al. The role of germline alterations in the DNA damage response genes BRIP1 and BRCA2 in melanoma susceptibility. Genes Chromosom. Cancer 2016, 55, 601–611. [CrossRef] [PubMed]

12. Meeks, H.D.; Song, H.; Michailidou, K.; Boila, M.K.; Dennis, J.; Wang, Q.; Barrowdale, D.; Frost, D.; Embrace; McGuffog, L.; et al. BRCA2 Polymorphic Stop Codon K3326X and the Risk of Breast, Prostate, and Ovarian Cancers. J. Natl. Cancer Inst. 2016, 108. [CrossRef] [PubMed]

13. Ge, Y.; Wang, Y.; Shao, W.; Jin, J.; Du, M.; Ma, G.; Chu, H.; Wang, M.; Zhang, Z. Rare variants in BRCA2 and CHEK2 are associated with the risk of urinary tract cancers. Sci. Rep. 2016, 6, 33542. [CrossRef] [PubMed]

14. Martin, S.T.; Matsubayashi, H.; Rogers, C.D.; Philips, J.; Couch, F.J.; Brune, K.; Yeo, C.J.; Kern, S.E.; Hruban, R.H.; Goggins, M. Increased prevalence of the BRCA2 polymorphic stop codon K3326X among individuals with familial pancreatic cancer. Oncogene 2005, 24, 3652–3656. [CrossRef] [PubMed]

15. Stafford, J.L.; Dyson, G.; Levin, N.K.; Chaudhry, S.; Rosati, R.; Kaipage, H.; Wernette, C.; Petrucelli, N.; Simon, M.S.; Tainsky, M.A. Reanalysis of BRCA1/2 negative high risk ovarian cancer patients reveals novel germline risk loci and insights into missing heritability. PLoS ONE 2017, 12, e0178450. [CrossRef] [PubMed]

16. Higgs, J.E.; Harkness, E.F.; Bowers, N.L.; Howard, E.; Wallace, A.J.; Laloo, F.; Newman, W.G.; Evans, D.G. The BRCA2 polymorphic stop codon: Stuff or nonsense? J. Med. Genet. 2015, 52, 642–645. [CrossRef] [PubMed]

17. Choi, D.H.; Lee, M.H.; Bale, A.E.; Carter, D.; Haflity, B.G. Incidence of BRCA1 and BRCA2 mutations in young Korean breast cancer patients. J. Clin. Oncol. 2004, 22, 1638–1645. [CrossRef] [PubMed]

18. Choi, D.H.; Lee, M.H.; Haflity, B.G. Double heterozygotes for non-Caucasian families with mutations in BRCA1 and BRCA2 genes. Breast J. 2006, 12, 216–220. [CrossRef] [PubMed]

19. Noh, J.M.; Choi, D.H.; Nam, S.J.; Lee, J.E.; Kim, J.W.; Kim, S.W.; Kang, E.; Lee, M.H.; Ahn, S.H.; Kim, K.S.; et al. Characteristics of double heterozygosity for BRCA1 and BRCA2 germline mutations in Korean breast cancer patients. Breast Cancer Res. Treat. 2012, 131, 217–222. [CrossRef] [PubMed]

20. Liede, A.; Rehal, P.; Vesprini, D.; Jack, E.; Abrahamson, J.; Narod, S.A. A breast cancer patient of Scottish descent with germ-line mutations in BRCA1 and BRCA2. Am. J. Hum. Genet. 1998, 62, 1543–1544. [CrossRef] [PubMed]

21. Loader, S.; Rowley, P.T. Deleterious mutations of both BRCA1 and BRCA2 in three siblings. Genet. Test. 1998, 2, 75–77. [CrossRef] [PubMed]

22. Tesoriero, A.; Andersen, C.; Southey, M.; Somers, G.; McKay, M.; Armes, J.; McCredie, M.; Giles, G.; Hopper, J.L.; Venter, D. De novo BRCA1 mutation in a patient with breast cancer and an inherited BRCA2 mutation. Am. J. Hum. Genet. 1999, 65, 567–569. [CrossRef] [PubMed]

23. Caldes, T.; de la Hoya, M.; Tosar, A.; Sulleiro, S.; Godinho, J.; Ibanez, D.; Martin, M.; Perez-Segura, P.; Diaz-Rubio, E. A breast cancer family from Spain with germline mutations in both the BRCA1 and BRCA2 genes. J. Med. Genet. 2002, 39, e44. [CrossRef] [PubMed]
24. Musolino, A.; Naldi, N.; Michiara, M.; Bella, M.A.; Zanelli, S.; Bell, R.; Devery, S.; Andrieska, N.; Winship, I. Familial breast cancer: Double heterozygosity for BRCA1 and BRCA2 mutations with differing phenotypes. *Biol. Reprod.* 2008, 7, 119–124. [CrossRef] [PubMed]

25. Pilato, B.; de Summa, S.; Danza, K.; Lambo, R.; Paradiso, A.; Tommasi, S. Maternal and paternal lineage double heterozygosity alteration in familial breast cancer: A first case report. *Breed Sci. Res. Treat.* 2010, 124, 875–878. [CrossRef] [PubMed]

26. Zurradelli, M.; Peisell, B.; Manoukian, S.; Zaffaroni, D.; Barsie, M.; Pensotti, V.; Cavallari, U.; Masci, G.; Mariette, F.; Benski, A.C.; et al. Four new cases of double heterozygosity for BRCA1 and BRCA2 gene mutations: Clinical, pathological, and family characteristics. *Breed Sci. Res. Treat.* 2010, 124, 251–258. [CrossRef] [PubMed]

27. Steffensen, A.Y.; Jonson, L.; Ejlertsen, B.; Gerdes, A.M.; Nielsen, F.C.; Hansen, T.V. Identification of a Danish breast/ovarian cancer family double heterozygote for BRCA1 and BRCA2 mutations. *Fam. Cancer* 2010, 9, 283–287. [CrossRef] [PubMed]

28. Augustyn, A.M.; Agostino, N.M.; Namey, T.L.; Nair, S.; Martin, M.A. Two patients with germline mutations in both BRCA1 and BRCA2 discovered unintentionally: A case series and discussion of BRCA testing modalities. *Breed Sci. Res. Treat.* 2011, 119, 629–634. [CrossRef] [PubMed]

29. Nomizu, T.; Matsuaki, M.; Katagata, N.; Kobayashi, Y.; Sakuma, T.; Monna, T.; Saito, M.; Watanabe, F.; Midorikawa, S.; Yamaguchi, Y. A case of familial breast cancer with double heterozygosity for BRCA1 and BRCA2 genes. *Breed Sci. Res. Treat.* 2015, 22, 557–561. [CrossRef] [PubMed]

30. Loubser, F.; de Villiers, J.N.; van der Merwe, N.C. Two double heterozygotes in a South African Afrikaner family: Implications for BRCA1 and BRCA2 predictive testing. *Clin. Genet.* 2012, 82, 599–600. [CrossRef] [PubMed]

31. Heidemann, S.; Fischer, C.; Engel, C.; Fischer, B.; Harder, L.; Schlegelberger, B.; Niederacher, D.; Goecke, T.O.; Doelken, S.C.; Ditke, N.; et al. Double heterozygosity for mutations in BRCA1 and BRCA2 in German breast cancer patients: Implications on test strategies and clinical management. *Breed Sci. Res. Treat.* 2012, 134, 1229–1239. [CrossRef] [PubMed]

32. Vietri, M.T.; Molinari, A.M.; Caliendo, G.; De Paola, M.L.; Giovanna, D.; Gambardella, A.L.; Petronella, P.; Cioffi, M. Double heterozygosity in the BRCA1 and BRCA2 genes in Italian family. *Clin. Chem. Lab. Med.* 2013, 51, 2319–2324. [CrossRef] [PubMed]

33. Janavicius, R. Founder BRCA1/2 mutations in the Europe: Implications for hereditary breast-ovarian cancer prevention and control. *EPMA J.* 2010, 1, 397–412. [CrossRef] [PubMed]

34. Kraic, M.; Teugels, E.; Zgajnar, J.; Goelen, G.; Besic, N.; Novakovic, S.; Hocevar, M.; De Greve, J. Five recurrent BRCA1/2 mutations are responsible for cancer predisposition in the majority of Slovenian breast cancer families. *BMC Med. Genet.* 2008, 9, 83. [CrossRef] [PubMed]

35. Salazar, R.; Cruz-Hernandez, J.J.; Sanchez-Valdivieso, E.; Rodriguez, C.A.; Gomez-Bernal, A.; Barco, E.; Fonseca, E.; Portuguese, T.; Gonzalez-Sarmiento, R. BRCA1-2 mutations in breast cancer: Identification of nine new variants of BRCA1-2 genes in a population from central Western Spain. *Cancer Lett.* 2006, 233, 172–177. [CrossRef] [PubMed]

36. Kluska, A.; Balabas, A.; Paziewska, A.; Kulecka, M.; Nowakowska, D.; Mikula, M.; Ostrowski, J. New recurrent BRCA1/2 mutations in Polish patients with familial breast/ovarian cancer detected by next generation sequencing. *BMC Med. Genom.* 2015, 8, 19. [CrossRef] [PubMed]

37. Johannsson, O.T.; Staff, S.; Vallon-Christersson, J.; Kytola, S.; Gudjonsson, T.; Rennstam, K.; Hedenfalk, I.A.; Adeyinka, A.; Kjellen, E.; Wennerberg, J.; et al. Characterization of a novel breast cancer xenograft and cell line derived from a BRCA1 germ-line mutation carrier. *Lab. Invest.* 2003, 83, 387–396. [CrossRef] [PubMed]

38. Mazoyer, S.; Dunning, A.M.; Serova, O.; Dearden, J.; Puget, N.; Healey, C.S.; Gayther, S.A.; Mangion, J.; Stratton, M.; Lynch, H.T.; et al. A polymorphic stop codon in BRCA2. *Nat. Genet.* 1996, 14, 253–254. [CrossRef] [PubMed]

39. Kuznetsov, S.G.; Liu, P.; Sharan, S.K. Mouse embryonic stem cell-based functional assay to evaluate mutations in BRCA2. *Nat. Med.* 2008, 14, 873–881. [CrossRef] [PubMed]
41. Wu, K.; Hinson, S.R.; Ohashi, A.; Farrugia, D.; Wendt, P.; Tavtigian, S.V.; Deffenbaugh, A.; Goldgar, D.; Couch, F.J. Functional evaluation and cancer risk assessment of BRCA2 unclassified variants. *Cancer Res.* 2005, 65, 417–426. [PubMed]

42. Morimatsu, M.; Donoho, G.; Hasty, P. Cells deleted for Brca2 COOH terminus exhibit hypersensitivity to gamma-radiation and premature senescence. *Cancer Res.* 1998, 58, 3441–3447. [PubMed]

43. Friedman, E.; Bar-Sade Bruichim, R.; Kruglikova, A.; Risel, S.; Levy-Lahad, E.; Halle, D.; Bar-On, E.; Gershoni-Baruch, R.; Dagan, E.; Kepten, I.; et al. Double heterozygotes for the Ashkenazi founder mutations in *BRCA1* and *BRCA2* genes. *Am. J. Hum. Genet.* 1998, 63, 1224–1227. [CrossRef] [PubMed]

44. Frank, T.S.; Deffenbaugh, A.M.; Reid, J.E.; Hulick, M.; Ward, B.E.; Lingenfelter, B.; Gumpper, K.L.; Scholl, T.; Tavtigian, S.V.; Pruss, D.R.; 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. [CrossRef] [PubMed]

45. Peto, J.; Collins, N.; Barfoot, R.; Seal, S.; Warren, W.; Rahman, N.; Easton, D.F.; Evans, C.; Deacon, J.; Stratton, M.R. Prevalence of *BRCA1* and *BRCA2* gene mutations in patients with early-onset breast cancer. *J. Natl. Cancer Inst.* 1999, 91, 943–949. [CrossRef] [PubMed]

46. Van Allen, E.M.; Wagle, N.; Sucker, A.; Treacy, D.J.; Johannessen, C.M.; Goetz, E.M.; Place, C.S.; Taylor-Weiner, A.; Whittaker, S.; Kryukov, G.V.; et al. The genetic landscape of clinical resistance to RAF inhibition in metastatic melanoma. *Cancer Discov.* 2014, 4, 94–109. [CrossRef] [PubMed]

47. Wu, S. Do mutations in *BRCA1*/*BRCA2* confer a higher risk of skin cancer? *Br. J. Dermatol.* 2015, 172, 1473. [CrossRef] [PubMed]

48. Gumaste, P.V.; Penn, L.A.; Cymerman, R.M.; Kirchhoff, T.; Polsky, D.; McLellan, B. Skin cancer risk in *BRCA1/2* mutation carriers. *Br. J. Dermatol.* 2015, 172, 1498–1506. [CrossRef] [PubMed]

49. Leachman, S.A.; Lucero, O.M.; Sampson, J.E.; Cassidy, P.; Bruno, W.; Queirolo, P.; Ghiorzo, P. Identification, genetic testing, and management of hereditary melanoma. *Cancer Metastasis Rev.* 2017, 36, 77–90. [CrossRef] [PubMed]

50. Taylor, N.J.; Mitra, N.; Goldstein, A.M.; Tucker, M.A.; Avril, M.F.; Azizi, E.; Bergman, W.; Bishop, D.T.; Bressac-de Paillerets, B.; Bruno, W.; et al. Germline Variation at *CDKN2A* and Associations with Nevus Phenotypes among Members of Melanoma Families. *J. Investig. Dermatol.* 2017, 137, 2606–2612. [CrossRef] [PubMed]

© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).