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Review

Hereditary breast cancer: beyond *BRCA* genetic analysis; *PALB2* emerges

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Abstract

Despite the initial enthusiasm following the discovery of the association of *BRCA* germline mutations with hereditary breast and/or ovarian cancer, in many families affected by the syndrome no pathogenic mutations were detected in the two genes, although exhaustively searched. Many other genes have also been implicated due to their role in the same pathway of DNA repair where the *BRCA1/2* genes are involved: homologous recombination (HR). Among them, *PALB2* clearly emerges as the third breast cancer susceptibility gene. Its mutations have been detected in most populations investigated so far, albeit rarely: in 1%–4% of families negative for *BRCA* mutations, with either partial or complete penetrance. In some populations, *PALB2* recurrent mutations have been identified and the estimated hazard risks are comparable to those of *BRCA* mutations. Since new effective targeted therapeutic options are becoming available ("synthetic lethality" with novel PARP inhibitors, etc.) that are applicable to all those patients with a defect in HR pathway, it is imperative to detect all these candidate patients. Data obtained from laboratory tests in the tumor (simple immunohistochemistry, gene expression analysis, etc.) can assist in the recognition of a specific pattern (*BRCA1*ness, HRless) so that even patients that look "sporadic" could benefit from these targeted therapies. Therefore, a genetic analysis algorithm is proposed, although with the advent of Next Generation Sequencing it is predicted that in the future most germline genetic alterations and also somatic or epigenetic events in the tumor of these genes will be detected.

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Introduction

After about 125 years from the first published description of hereditary breast cancer by French physician Paul Broca in his assay *Traité des Tumeurs* regarding his wife's family (1), the first culprit breast cancer susceptibility gene was discovered in 1994: *BRCA1* (2) and soon was followed by *BRCA2* (3). In this sense, his memory could be honored by paraphrasing the two genes to *BROCA1* and *2*. These genes are highly penetrant and pathogenic mutations in heterozygosity confer cumulative risks of breast cancer by age 70: 65% (95% CI 51–75) for *BRCA1* and 39% (95% CI 22–51) for *BRCA2* (4). Hereditary breast cancer is associated with ovarian cancer and *BRCA1* or *BRCA2* mutations confer also 45% (95% CI 33–54) or 11% (95% CI 4.1–18) cumulative risks of ovarian malignancy correspondingly by the same age (4). Families affected by mutations in these dominant genes are characterized by younger ages of onset compared to sporadic breast (and ovarian) cancer, increased numbers of cases with breast and ovarian cancer in the same patient or bilateral breast cancer in female carriers and rarely, breast cancer in male carriers. Occasionally, other cancers can also be found in family members, such as pancreatic, prostate, melanoma, etc. (5, 6).

Still though, there is a great percentage of these families (<50% depending on the population) where no deleterious mutations in the two high-risk *BRCA* genes have been detected in their peripheral blood DNA, despite technological improvements for mutation detection methods and exhaustive genetic analysis performed covering additionally for large genomic rearrangements and non-exonic sites.

Only a low percentage of the remaining families can be attributed to germline mutations in genes, such as *p53, PTEN, STK11, CDH1,* whose action can be easily recognized clinically since breast cancer is only part of a wider syndrome with multiple tumors in many sites and other pathologies as well: they cause Li-Fraumeni, Cowden, Peutz-Jeghers, diffuse gastric carcinoma, correspondingly. The percentage up to 100% for hereditary breast cancer families is predicted to be filled either by a combination of low-risk but common mutated alleles (to be discovered and validated through genome-wide association studies in high numbers of patients and controls) (7) or by intermediate- (or moderate)

risk genes, such as *ATM, CHEK2, BRIP1, PALB2* (8–12) and recently discovered *RAD51C* (13).

Biallelic mutations (either homozygous or compound heterozygous) in some of the aforementioned genes (*BRCA2, BRIP1, PALB2, RAD51C*) lead also to Fanconi anemia: an heterogeneous recessive disease characterized by developmental abnormalities, thrombopenia, bone marrow aplasia or failure, hematological cancers (leukemias, lymphomas) and usually lethal solid tumors early in adulthood (medulloblastoma, Wilms' tumor). This connection between breast cancer and Fanconi anemia was proven also by the investigation of the role of these four gene products in a common terminal pathway for DNA repair. This is the error-free homologous recombination (HR) mechanism and will be described in detail, updated with most recent knowledge, in the next paragraph of this review. The protein products of the majority of the aforementioned genes participate in the handling of DNA damages and/or in cell cycle regulation and their deficit causes tumorigenesis; therefore are classified clearly as tumor suppressors.

PALB2 emerges nowadays as the third breast cancer susceptibility gene and will be a main topic of this review. Since its discovery in 2006 (14), *PALB2* mutations have been detected – albeit at low percentages – in most breast cancer populations tested so far worldwide in families with either small or large number of cases. Studies with some of the recurrent *PALB2* mutations tested in patients unselected for family history have demonstrated risks and penetrances as high as those arising from *BRCA* mutations (15, 16). It also follows the BRCA pattern: although PALB2 protein is needed in all human cells for HR, its mutations lead to tumors mainly in breast tissue (and ovaries) and mainly in females.

The inclusion of *PALB2* gene (and other HR genes as well) in the panel of genes for genetic analysis of hereditary breast cancer is critically discussed. There is definitely the need to increase diagnostic sensitivity so that all carriers of mutations in these HR genes can be detected: patients and their progeny can benefit from increased surveillance and offer of suitable therapeutic options or even new approaches (such as "synthetic lethality") arising from basic research work. We should not forget that some of the "sporadic" cases are due to incomplete penetrance, small number of family members, paternal inheritance (and adoption in some cases). Both these cases but also those with modest family history can clearly benefit from data obtained from the tumor: either histological (grade, medullary subtype, etc.) or immunohistochemical (IHC) or, even more refined, from pattern recognition of gene expression in microarrays. The so-called triple-negative breast cancer (TNBC) could unmask cases with the presence of a *BRCA1* mutation (17): this is the most aggressive phenotype of *BRCA1* cancers (*BRCA1ness*) due most probably to the multiplicity of roles for the BRCA1 protein. Notably, the same aggressive phenotype can be achieved by HR deficit obtained by mutation(s) in other *HR* genes (single or in combination) and/or epigenetic silencing of their expression.

Connection of hereditary breast cancer with homologous recombination DNA repair and Fanconi anemia

Every day due to free radical formation and exposure to environmental extrinsic factors (X-rays, UV light, chemical substances, etc.) many DNA damages occur per cell per day. Double stranded breaks (DSBs) that are caused mainly by ionizing radiation (IR) occur at low frequency (eight events/day/cell, V.A. Bohr, pers. comm.) and are the most toxic ones: they should be repaired immediately and in an infallible way. The ideal method is the HR and not the error-prone non-homologous end joining (NHEJ).

DSBs are located via the multi-protein BASC complex (BRCA1-associated surveillance complex) and the information is passed to the transducer ATM protein with phosphorylation: then ATM approaches the damage area and "marks" the chromatin protein H2AX by phosphorylation in two residues (Figure 1, left part). This is recognized by the MDC1 complex that adds the sturdier poly-ubiquitylation marks at Lys-63 of Ub (these are localization signals and not degradation signals as those at Lys-48 where the protein is headed to proteasome for degradation). These marks attract the RAP80-ABRAXAS-BRCA1 complex because of the double ubiquitin interacting motif (UIM) domain possessed by receptor associated protein (RAP)80. Then after BRCA1 SUMOylation and with the help of its two BRCT transcactivation domains, the MRN (MRE11-RAD50-NBS1) complex, CtBP (C-terminal binding protein) and BRIP1 appear successively and prepare single-stranded extensions covered and protected by RPA (replication protein A). In the meantime, BRCA1 heterodimerizes also with BARD1 (through its RING domain) and exerts G1/S phase control and other regulatory functions. Finally, binding of BRCA1 to PALB2 localizes BRCA2 to the repair foci. Eight RAD51 molecules bind with the eight BRC repeat domains of BRCA2 and displace RPA (18) and then assisted possibly by RAD51C, they perform strand invasion, Holliday junction and eventually HR (19, 21, 22).

Another type of damage, inter-strand crosslinks (ICLs), induced by chemicals, such as alkylating agents (platinum and derivatives), mitomycin C, diepoxy-butane (DEB), etc., need the terminal HR mechanism as well. However, they must be detected first and then by-passed using a combination of repair mechanisms like nucleotide excision repair (NER) and trans-lesion synthesis (TLS) that may introduce errors (point mutations) before finally being repaired by HR. The ICL damage is detected via ATR by FANCM helicase and AP24 protein and then the Fanconi anemia nuclear core complex is formed composed by proteins FANCA, -G, -F, -C, -B, AP100, -E and -L (23) (Figure 1, right part). The last two possess E3 ubiquitin ligase activity and monoubiquitylate FANCD2 and FANCI via UBET2T (20, 24). The Fanconi anemia core complex is then removed and FANCD2 and FANCI recruit HR proteins BRCA2 (FAND1), BRIP (FANCJ), PALB2 (FANCN) and probably RAD51C (FANCO) and newly found SLX4 (FANCP). The FANCD2 and FANCI are deubiquitylated then via USP1. Biallelic mutations in the genes of most of the aforementioned proteins that repair ICL lesions cause

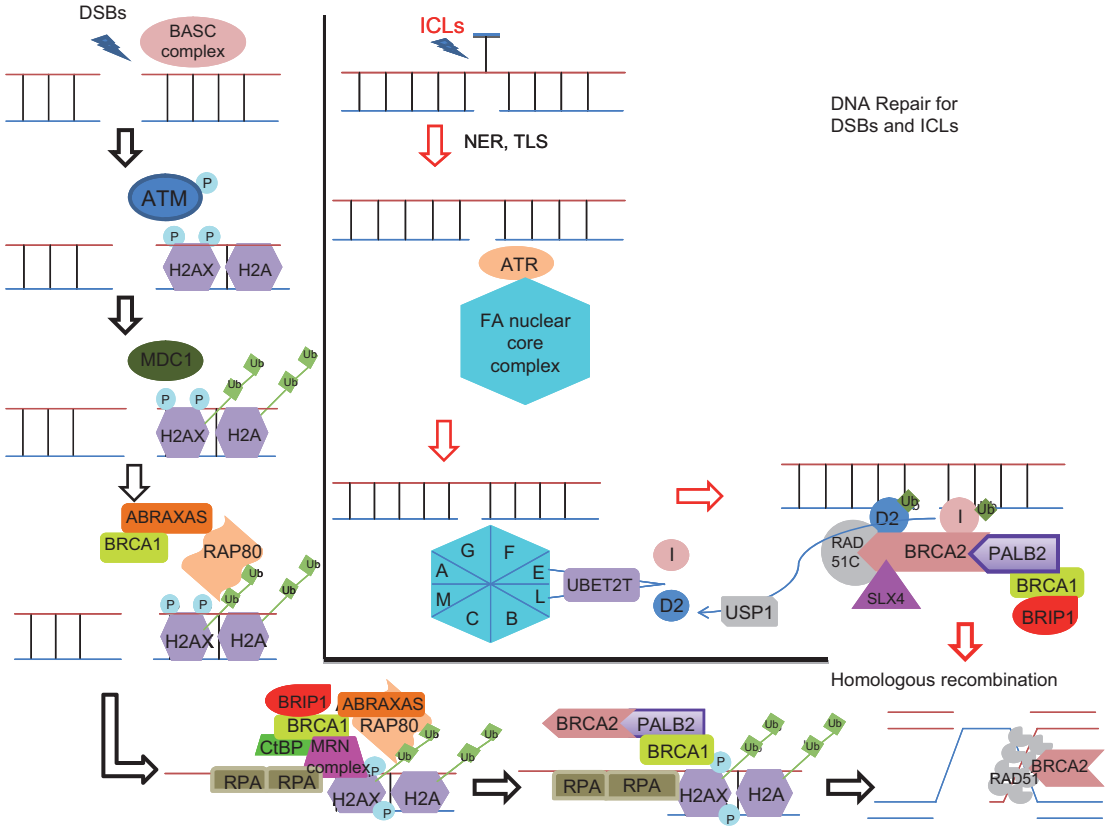

Figure 1 Network of proteins involved in DNA repair of interstrand cross-links (ICLs) and double-strand breaks (DSBs) through the homologous recombination (HR) mechanism. Defects in HR lead to error-prone NHEJ and either apoptosis or susceptibility to tumors later on [compiled and adapted from references (11, 12, 18–20)].

different forms of Fanconi anemia. From the total 15 groups of Fanconi anemia, FANCA is the most prevalent (66%) followed by FANCC (10%) and FANCG (9%) (23). In this model, RAD51 is again the final effector of HR and is not clear whether BRCA1 participates since no biallelic *BRCA1* mutations have been found in Fanconi anemia (could be very well be that biallelic inactivation of *BRCA1,* but *RAD51* as well, could be extremely lethal for any human cell).

From these suggested repair models for DSB and ICL damages, it is sufficiently explained why mutations in certain genes result only in Fanconi anemia, in other genes result only in breast and/or ovarian cancer, while in some genes result in both syndromes. In this last category, biallelic carriers develop more severe and lethal Fanconi anemia earlier in life and heterozygotes develop breast/ovarian cancer usually during the 4th–6th decade of life (25).

Cells with HR deficit due to either DNA repair gene mutations or loss of expression of these genes will undergo either apoptosis (hence the developmental defects in Fanconi patients) or carcinogenicity due to broken chromosomes and aneuploidy or accumulation of point mutations from errors that occurred during NHEJ. Therefore, the ideal laboratory test would not be the cumbersome mutation scanning for all mentioned DNA repair genes, but rather, as recently suggested, a competence test for HR like the RAD51 score, an immunofluorescent measurement of formed RAD51 foci after damage induction (26). This test though, is not yet standardized and commercialized and only few reference laboratories could provide for it.

PALB2

PALB2 gene (Gene ID#79728, OMIM #610355), was located in a 38.2 Kb area on chromosome 16 (16p12.1) (14). It consists of 13 exons transcribing approximately 3.5 Kb mRNA, which encodes a protein of 1186 amino-acids (131 KDa) with pI 6.4 and a charge of –2. Exons 4 and 5 are much larger than all others (genomic sequence NG_007406.1, mRNA sequence NM_024675.3). C-terminal end of PALB2 protein through seven-bladed β-propeller WD40 (tryptophan-aspartic acid rich) domains (AA 836-1186), is anchoring the N-terminal end of BRCA2 protein (AA 10-40), as was shown elegantly in crystallization experiments, and apparently assists in localizing BRCA2 in nuclear chromatin structures; thus justifying the initials of *PALB2* gene: partner and localizer of BRCA2 (14, 27). Co-localization of PALB2 and BRCA2 in DNA repair foci was proven via immunoprecipitation and immunofluorence experiments following DNA damage DSBs induced by IR. Moreover, a deficit of HR DNA repair was reported

after *PALB2* siRNA transfection: *BRCA2* mRNA was fully expressed but the protein was unable to enter the nucleus and remained in the cytoplasmic portion as found in fractionation experiments. In addition, it was recently discovered that the N-terminal end of PALB2 also interacts with BRCA1 protein via the coiled coil regions (PALB2 AA 9-44 with BRCA1 1393-1424, few residues before the two BRCT domains) (28). The terminal HR is only performed via extremely large molecular complexes and apparently the axis BRCA1-PALB2-BRCA2 is very central to this mechanism (29). PALB2 interacts also with RAD51 (AA 1-200, 836-1186) and most probably binds directly with DNA at AA 1-200 (30–32) (Figure 2, data derived also from GenBank NP_078951.2 and UniProKB/Swiss-Prot Q86YC2).

PALB2 mutations were detected for the first time in 2007; in heterozygosity in families with hereditary breast cancer (33) and biallelic in cases of Fanconi anemia and childhood tumors (34, 35). Restoration of *PALB2* gene expression restores the cellular defect of the Fanconi anemia complementation group N and this is the reason that the name *FANCN* was assigned in parallel (34). In older studies, *BRCA2* gene was found to correspond to *FANCD1* and *BRIP1* gene (earlier *BACH1*) to *FANCJ*, respectively (36).

Most pathogenic *PALB2* mutations detected so far are truncating frameshift or stop codons and are scattered throughout the entire gene region with no hot-spot areas. Even mutations affecting the C-terminus could still be deleterious, e.g., Y1183X in the 13th exon that removes the last four residues of the protein (35) abolishes the propeller structure of C-terminus of PALB2 and destabilizes the whole protein (27). Using multiplex ligation-dependent probe amplification (MLPA) methodology, mutations with large genomic rearrangements have also been reported (34, 37), however, they should be considered very rare since in other two studies results were not corroborated, although the same technique was used (38, 39). Until now, deleterious *PALB2* mutations are detected in various populations ranging from 1% to 2% of women with hereditary breast and ovarian cancer negative for mutations in *BRCA1/2* genes (31, 40–44) up to 3%–4% in one study in the US (45). In certain populations, such as Finnish or French Canadians, recurrent or founder mutations are detected, even in patients that had not been selected based on family history (39, 43). In the recurrent Finnish *PALB2* c.1592delT mutation the cumulative risk was estimated to be 40% (95% CI 17–77) by age 70 years comparable to *BRCA2* risks (15) while in another more recent investigation from Australia, the hazard ratio of *PALB2* c.3113G>A was estimated to reach a high 91% (95% CI 44–100) by the same age (16). Both studies were conducted in patients unselected for family history. Therefore, it seems that *PALB2* is a moderate risk gene of a "higher order" compared to the rest of this category. In studies with large pedigrees with detected *PALB2* mutations, there have been families with either complete or partial penetrance.

PALB2 mutations have also been detected in 1% of a population of male breast cancer (46, 47) and in increased percentage (2%) in *BRCA(-)* bilateral breast cancer patients (48) with one of the pedigrees including a stomach cancer case as well.

In 2009, a connection with familial pancreatic cancer was observed (49) and demonstrated further in larger studies where *PALB2* mutations were detected in 3%–4% of familial pancreatic cancer (50, 51) but not necessarily enriched in breast-pancreas families as one would expect (52). No association was found with melanoma (53).

PALB2 single nucleotide polymorphisms (SNPs) have been already examined for their association with breast cancer (54) and it would be interesting to see the results of any future studies relating them with expression levels.

Missense mutations detected so far are evaluated with the published sets of criteria (55, 56), e.g., mutation position

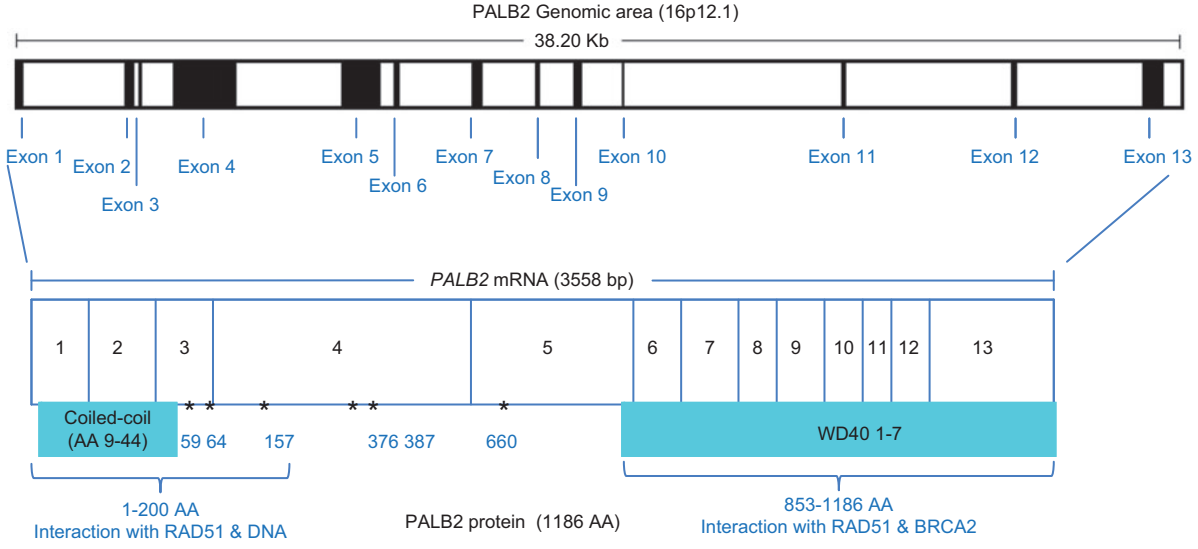

Figure 2 *PALB2* exonic structrure and interactions with other proteins: with BRCA1 through a coiled-coil structure (AA 9-44), with BRCA2 with seven WD-repeats (AA 836-1186) and with RAD51 (AA 1-200, 836-1186) or directly with DNA (AA 1-200) (30–32).
Sites for phosphorylation of serines are indicated with an asterisk (*).

is important: an alanine change at position 1025 of PALB2 protein abolished its binding to BRCA2 (27). Bioinformatics prediction tools that are freeware can also be used in their interpretation, such as Polyphen (www.genetics.bwh.harvard.edu/pph), SIFT (http://sift.jcvi.org) and Mutation taster (www.mutationtaster.org).

In general, significantly less ovarian cancer is seen in *PALB2* families compared to *BRCA1* and *BRCA2* families (31, 44) and it remains to be seen whether ovarian cancer risk is truly increased in *PALB2* mutation carriers. The average disease onset age is between those for *BRCA1* and *BRCA2*. Pathology data from *PALB2* tumors will be discussed later on, in the relevant section of this review.

Regarding loss of heterozygosity (LOH) in *PALB2* tumors, data is conflicting; it has been shown in two studies (41, 45) while in another two, no LOH was detected and this led investigators to propose that the model of haploinsufficiency could apply for this tumor suppressor gene (40, 43).

Somatic *PALB2* mutations have been reported in one study so far in the molecular profile of a metastatic lobular breast cancer (57).

Other risk genes common for breast cancer and Fanconi: *RAD51C, SLX4* and *BRIP1*

RAD51C also participates in DNA repair by HR and it is the gene product of one of the five *RAD51* paralogs (the rest of them are -B, -D, XRCC-2 and -3). The gene is located in position 17q23 and consists of 9 exons. Deleterious mutations of all types (stop codons, frameshift, missense, splicing) were detected in 1.3% of German females with the occurrence of both breast and ovarian cancer that were otherwise *BRCA1/2* negative (13) and also in ovarian-only cancer (58). A *RAD51C* hypomorphic missense mutation (R258H) in homozygosity was also detected in one consanguineous family that suffered from a Fanconi-anemia-like disorder with multiple congenital abnormalities but with no hematological malignancy (therefore proposed to be renamed to *FANCO)*. However, the frequency mentioned above was not verified in other populations (31, 59, 60). Another paralog, *RAD51D* was also recently associated with ovarian-only cancer families (61).

SLX4 is another gene with recently discovered mutations in Fanconi anemia (hence the proposed *FANCP*) (62, 63), however, when a population of familial German and Byelorussian breast cancer patients was examined, few missense mutations were detected and only one was predicted in silico to be deleterious (G700R) (64).

BRIP1 or *BACH1* gene is localized in chromosome 17 (17q22.2). It consists of 20 exons transcribed to an mRNA which encodes a 130 Kb protein containing 1249 amino acids. *BRIP1* encodes a helicase that contributes to chromosomal stability by interacting with the C-terminal domain of BRCA1 protein (BRCA1 Interacting Protein- terminal helicase 1) (65). Studies conducted so far, have reported that alterations affecting the BRIP1-BRCA1 interaction might be responsible for a very small percentage of hereditary breast and ovarian cancer (11, 31, 66–68). Biallelic mutations were identified in families with Fanconi anemia (but with no childhood solid tumors) that were classified to complementation group *FANCJ* (69). In male breast cancer, there have been no significant findings in *BRIP1* gene (70). Nevertheless, a stop codon mutation detected in exon 17 (P798X) is present in 0.3% of hereditary prostate cancer (71).

From the data presented in this section, one could conclude that these three recently investigated genes seem more "Fanconi" and "ovarian cancer" than "hereditary breast cancer" susceptibility genes – at least up to now.

Diagnostic hints from tumor pathology data – *BRCA1*ness phenotype

As noted in the introduction, it is imperative to detect all HR gene mutation carriers for appropriate monitoring and therapy selection. Earlier studies have observed significant differences between *BRCA* carriers and sporadic ones in the results of immunohistochemistry tests that are performed routinely in most tumor specimens. The most characteristic for almost 80% of *BRCA1* breast cancer tumors was triple negativity (TNBC) for estrogen receptor (ER), progesterone receptor (PgR) and amplification of *HER2* (*c-erbb2*) oncogene (72, 73).

In parallel, analogous results to these simple tests were obtained by far more expensive molecular profiles where a pattern of expression of 176 genes in certain tumors was able to clearly distinguish a "*BRCA1*" signature from *BRCA2* and sporadic tumors (74). With a wider and better selection of genes, all breast cancer tumors could be classified initially in four categories: the good prognosis luminal types A and B (usually ER/PgR positive), HER2-amplified type (responsive to herceptin mAb) and the basal type in 15% of cases (75). Lately, another poor prognosis type is added with epithelial to mesenchymal transition (EMT) features and claudin-low expression (76). A substantial majority of tumors with *BRCA1* germline mutations falls within the basal subtype and as mentioned previously belongs also to the simple TNBC classification; however the basal and TNBC groups overlap but they do not coincide (17). Lately, new approaches with MLPA or microarray have been developed to identify *BRCA1*ness (77, 78).

Unfortunately, the majority of patients cannot obtain detailed molecular portraits; few benefit from the FDA-approved commercial Onco*type* Dx and MammaPrint tests (21- and 70-gene expression arrays correspondingly) (more can be found in AACC-sponsored and EFCC/EDM-backed website www.labtestsonline.org when searching for multiparameter gene expression tests for breast cancer). The rest can benefit from the three cheap and simple IHC tests but also from additional parameters, e.g., IHC tissue staining for CK14 and CK5/6 antigens (basal type markers) increases the possibility of detecting a germline *BRCA1* mutation by 148 times when TNBC phenotype co-exists (79). A significant overrepresentation of medullary histology subtype is also remarkable (18%) in *BRCA1* tumors compared to the rest (3%) (72, 80–84). It seems that since BRCA1 is higher in

hierarchy in many mechanisms and cell regulations, disruption of its function through mutation and LOH results in a more aggressive phenotype in cancers of mutation carriers, especially at a younger age. This is the reason for *BRCA1* tumors being observed with higher grade histologically and with increased proliferation with either Ki-67 IHC staining or S-phase measurements with flow or image cytometry (73). In the effort to spot as many *BRCA1* carriers as possible, we have also been assisted by the presence of giant cells in two anecdotal cases with no apparent family history but with highly aneuploid tumors (see impressive Supplementary Figures S1 and S2) (81, 82). This is a rare feature and we report it here in order to instigate discussion about its association with *BRCA1* mutations (86, 87). This aggressive *BRCA1*ness phenotype can also be present in 25% of sporadic cancer since the same results can be achieved through, e.g., a combination of silencing *BRCA1* gene expression by promoter CpG island methylation and somatic mutations.

Where does *PALB2* stand? In patients with *PALB2* mutations tumor histopathological characteristics (histological type, IHC results for hormone receptors ER/PgR, and HER2 oncogene) resemble to cancers caused by either *BRCA1* or *BRCA2* mutations (31, 39–42, 48). Therefore, some *PALB2* tumors are TNBC (about 40%, Reis-Filho, pers. comm.). Only one study reported data for p53 and Ki-67 IHC from one tumor (41). Due to the limited availability of *PALB2* tumors features like aneuploidy, presence of medullary subtype and giant cells, pattern of gene expression (do they belong to the basal subtype?) are not available so far. At the moment, no safe conclusions can be drawn regarding whether pathology is affected by *PALB2* mutation position and/or combination of LOH and polymorphism in other genes.

Therapeutic options for the treatment of TNBC or cancer with homologous recombination deficit

In the era of Personalized Medicine, hereditary breast cancer patients should be offered the best therapeutic options according to their genetic profile. Patients with defects in HR could certainly benefit by using chemotherapeutics that increase the number of inflicted DSBs and ICLs, such as platinum derivatives and MMC (88). Both patients and mutation carriers should be monitored regularly with magnetic resonance imaging (MRI) for earlier detection of small malignant lesions and be advised about prophylactic bilateral mastectomy and/or salpingo-oophorectomy, especially after childbearing or reaching 40 years old (89–91). Chemoprevention strategies for carriers (e.g., with tamoxifen), special diets (92) and detection of circulating tumor cells as a prognostic tool for patients remain to be tested in large cohorts (93). So far no other specific guidelines regarding clinical management of *PALB2* or any other HR gene mutation carriers or patients exist besides those available for *BRCA* mutation carriers.

"Synthetic lethality" is a new concept derived from basic research and has been proposed to be suitable for carriers of a defect in one of the DNA repair mechanisms, e.g., HR in TNBC and/or *BRCA1*ness tumors (88). The principle is as follows: if only a single type of chemotherapy is administered (e.g., platinum) in patients bearing a mutation in this pathway, cancer cells may manage to survive by increasing activity of other repair paths, e.g., base excision repair (BER). However, if an inhibitor of this other pathway is co-administered, e.g., a poly (ADP-ribose) polymerase 1 (PARP1) inhibitor that blocks BER, then cancer cells will eventually die while normal cells will manage to survive since they have one of the repair mechanisms intact (22). First clinical trials with such inhibitors (BSI-201-Iniparib and AZD2281-Olaparib) have been performed in *BRCA* patients (94, 95) and in combination with chemotherapy in metastatic TNBC patients (96) with acceptable results and no adverse reactions. However, in larger studies both inhibitors failed to show clear statistical benefit in either progression-free survival or overall survival efficacy points and these trials ended. Reasons for these inhibitors not working could be due to lack of drug selectivity or even resistance due to a reverse *BRCA* mutation (97, 98). More knowledge regarding their mechanism of action should certainly be acquired (99). Possibly, novel inhibitors in the pipeline (AB-899-Neliparib, AG-014699-Rucaparib, MK4827 etc) or future production of more selective inhibitors for PARP1 (and not for PARP2) may show survival benefit for the patients and also provide appropriate chemoprophylaxis tools for carriers of *BRCA1* mutations. This should be applicable also for mutation carriers of other genes where there is also defect in the same DNA repair path, e.g., *PALB2,* (44) which was shown elegantly with a PALB2-deficient cell line treated with Olaparib (30). These observations and proposals can certainly be extended to tumors with hypermethylated promoters of these genes, e.g., *BRCA1* CpG island hypermethylation has already predicted in vitro sensitivity for three new experimental PARP1 inhibitors (100, 101). Another recently introduced provocative idea is to render artificially tumor cells "BRCA-less" (or "HR-less") by inhibiting upstream genes, e.g., CDK1 in addition to PARP1 (102).

Expert opinion for genetic analysis algorithm for hereditary breast and/or ovarian cancer

Genetic analysis for hereditary breast and/or ovarian cancer is very expensive and laborious. There exist software programs that may assist in genetic counseling of these patients or people with appropriate indications and can calculate the possibility for a person being a *BRCA* mutation carrier and thus support the value of genetic analysis. Such tools include BRCA Risk calculator from Myriad, BOADICEA (103), BRCAPRO, IBIS, FHAT, Penn, Manchester score, etc. and there exist also references for their comparative assessment (104). Obviously, any future versions of these software programs – pursuant to the updated data mentioned in the pathology section – must include apart from age of onset and number of family members with the disease: i) additional information from histology, IHC routine testing (when available) and observations, such as aneuploidy and proliferation indices, gene expression analysis, etc.; and ii) the possibility of additional testing in

the rest of HR genes (e.g., *PALB2, BRIP1, RAD51C,* etc.) depending on population mutation frequencies and the presence of specific founder or recurrent mutations.

Therefore, after proper genetic counseling and gathering of all data demonstrating the need for genetic analysis, the next logical step would be, as indicated in Figure 3 (outer circle), testing for recurrent population-specific mutations, e.g., *BRCA1* 5382insC [or more appropriately c.5266dupC according to the latest HGVS nomenclature guidelines (105)] for Eastern Europeans and Ashkenazi Jewish populations (85, 106, 107), *BRCA2* 995del5 for Icelanders (108), also *PALB2* c.1592delT for Finnish and *CHEK2* c.1100delC for North European populations (11), etc. Whenever two or more recurrent mutations exist within the same exon, a fast scanning method would be more appropriate for their reliable detection (109).

A second step would be analysis for all *BRCA1* and *BRCA2* coding areas and splicing sites since mutations are scattered throughout the genes with no mutation hot-spots. This is performed by DNA Sequencing in Myriad Labs in the US (the only place allowed for commercial testing in the US after regaining patent rights in July 2011) but more often with a combination of methods elsewhere in the world. As seen in BIC database (http://research.nhgri.nih.gov/bic) a web site where information about BRCA mutations is deposited (should extend to the other HR genes as well), most deleterious mutations are truncating; therefore the protein truncation test (PTT) seems a wise choice, at least in large exons, (85, 110, 111) and nowadays new developments of the method combined with an ELISA format have been published (112). For the small exons, dHPLC (113, 114) or newly emerged High Resolution Melting Curve Analysis (HRMA) (115–118) are excellent and inexpensive choices. With the application of these last two techniques or with DNA sequencing, many missense and/or unclassified variants are usually revealed and have to be interpreted carefully, as pointed earlier in the section of *PALB2* mutations. If not performed simultaneously, in some populations it might be wise to start with one gene, e.g., *BRCA1* if its mutations are overrepresented in the specific population, and then proceed with *BRCA2*.

Third step, would be the evaluation of large genomic rearrangements, mostly for *BRCA1,* (119) with methodologies, such as MLPA (120) or real time quantitative PCR, e.g., QMPSF (121), long-range PCR, etc. For some populations, there is probably need to upgrade them in the first step, e.g., duplication of *BRCA1* exon 13 for North Europeans, specific mutations for the Dutch people, etc.

The next step, if all previous analysis turns out negative, should be to extend the search for germline mutations to other HR genes (*PALB2,* etc.) if there is sufficient ethnic population data to support it (e.g., testing about 1000 *BRCA*(-) samples) (44, 45).

Finally, if everything else was negative, epigenetic study of the tumor DNA might help significantly as an adjunct tool in the selection of targeted therapy, as CpG island promoter methylation is a frequent event in the *BRCA1* gene (122) and at a lower percentage (about 8% of tumors) in few studies that examined *PALB2* (123, 124).

Outlook

With the advent of Next Generation Sequencing methods and the simplicity and automation they provide, it is very likely that all 100% of families with hereditary breast and/or ovarian cancer will have their mutations identified within the whole spectrum of DNA repair genes; even in these loci where mutations have been detected very rarely so far. A research group in the US is extending coverage for 21 cancer genes in Illumina GA IIx with exon capture of not only a significant fraction of the aforementioned genes but also the MMR colorectal cancer genes: *MLH1, MSH2, MSH6, PMS1, PMS2, MUTYH* with

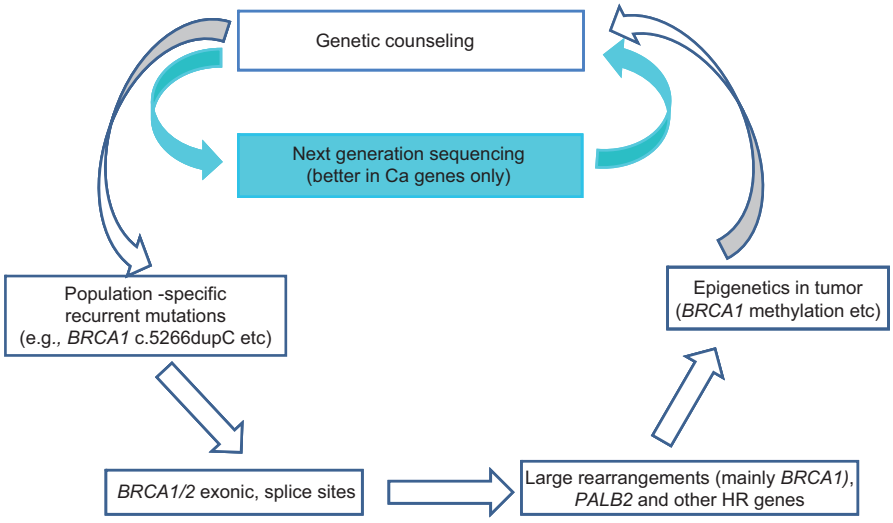

Figure 3 Algorithm for hereditary breast cancer genetic analysis for most countries (outer circle). Those health systems or individuals who can afford the costs of Next Generation Sequencing could follow the inner circle in the future.

100% accuracy (125). Use of other platforms for specialized Next Generation *BRCA* diagnostics is also expected to follow soon (e.g., with pyrosequencing methodology in the Roche 454 GS-FLX etc.).

Increased demand by informed physicians and patients and improvements in the technology will eventually reduce costs and increase prevalence of the method. In this context, more carriers that look "sporadic" will be detected and will benefit from the new therapeutic options, thus making the whole diagnostic approach more cost-effective for the Health systems (Figure 3, inner circle). The detected mutations might be deleterious but there will be also cases with a combination of hypomorphic mutations and polymorphisms in a plethora of genes, with the final result being always the same: an overall HR deficit. Epigenome analysis of bisulfite-converted DNA will certainly become more available and will fill the remaining gap in hereditary breast cancer genetics.

Points to remember

- Genetic counseling is an absolute necessity both before and after laboratory testing and a signed informed consent from the patient is obligatory. Since the genetic basis of cancer will be continually updated with information about germline and somatic mutations, there will be increased needs for training of laboratory professionals in cancer genetics and appropriate certification for genetic counseling in this field. This training will be also very useful in the interaction with oncologists and in the effort for the interpretation of theranostics (diagnostic tests with therapeutic implications, e.g., *HER2, k-ras, EGFR,* etc.).
- No direct-to-consumer (DTC) testing should be advised: false negatives results that might arise due to test limitations should be adequately explained and positive results should be always provided within a frame of medical service with proper psychological support and medical advice about the therapeutic options.
- Testing laboratories must comply with ISO15189 accreditation requirements: special guidelines are available for increasing analytical and diagnostic sensitivity and specificity and performing method validation with appropriate reference materials (126, 127). Laboratories should perform internal quality control and participate regularly in external quality assessment schemes (EQAs) (128).
- For any mutation detected, nomenclature should be assigned according to the latest HGVS guidelines (105). Any mutation detected with Next Generation methods should be always verified by classical DNA Sanger Sequencing (129).

Highlights

- *BRCA1* and *BRCA2* are still the main hereditary breast cancer susceptibility genes.
- *PALB2* emerges as the 3rd important gene and although rarely mutated, its inclusion in genetic analysis is justifiable, at least in some populations.
- All of the above genes play a central role in the terminal pathway of DNA repair through error-free HR mechanism.
- Biallelic mutations of two of the genes (*BRCA2* and *PALB2*) have been detected in patients of the complementation groups D1 and N in Fanconi anemia.
- Tumor pathology and biological characteristics (histological features, e.g., grade, medullary histotype, triple negativity in routine IHC data, aneuploidy and high proliferation, gene expression, etc.) can provide valuable hints for the presence of mutations in one of the above genes.
- The detection of such pathogenic mutations can guide therapy and offer additional options, e.g., synthetic lethality with novel PARP inhibitors.
- Genetic analysis should always begin with population-specific mutations (particularly in any of the three genes), then continue with *BRCA* genetic analysis in coding areas and then proceed with either large *BRCA1* rearrangements or *PALB2* analysis depending on the population and pathology data.
- Next Generation Sequencing for coding/intronic areas of all genes involved in DNA repair in peripheral blood and also their epigenome/somatic analysis in the tumor will certainly be more available in the future and will provide cost-effective diagnostics.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research funding played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
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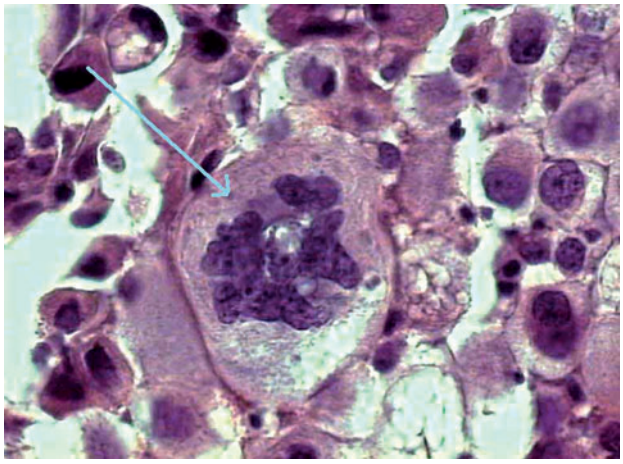
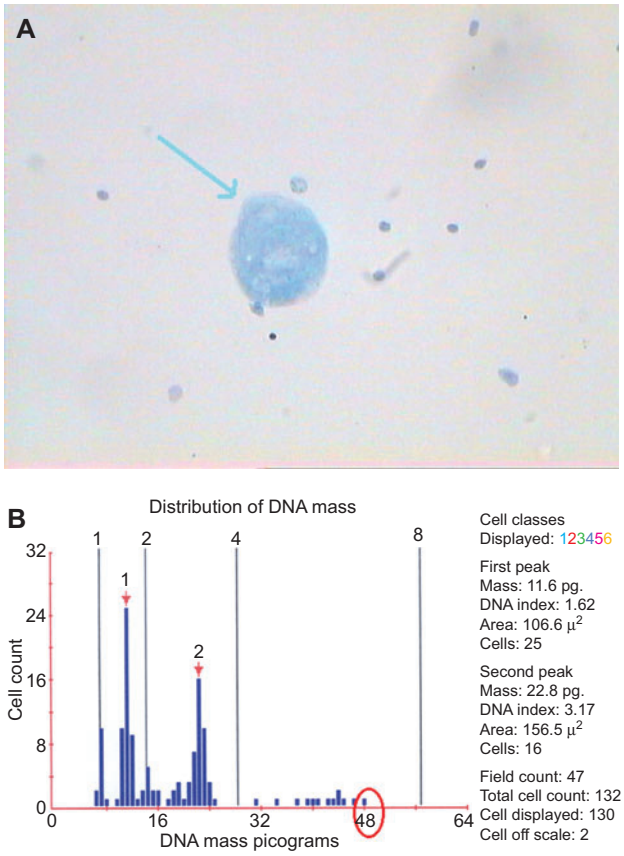
Supplemental Data



Supplementary Figure S1 Giant cell with nuclear pleomorphism (H&E stain, ×400) from a 40-year-old woman with triple-negative medullary breast cancer but with no family history (adopted). PTT analysis revealed a *BRCA1* R1203X mutation (82).



Supplementary Figure S2 (A) Giant cell next to normal cells (Feulgen-stained nuclei) and (B) ploidy analysis by image cytometry from imprints from a TNBC tumor with high Ki-67 proliferation (47-year-old woman with no family history who also developed ovarian cancer at the age of 48).
The arrow-indicated giant cell has a DNA Index result of 6.3 (or else 48 pg DNA/nucleus) and is circled in the cell-cycle histogram. PTT analysis revealed a *BRCA1* 3896delT mutation (85).