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"Decoding hereditary breast cancer" benefits and questions from multigene panel testing

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ABSTRACT

Multigene panel testing for breast and ovarian cancer predisposition diagnosis is a useful tool as it makes possible to sequence a considerable number of genes in a large number of individuals. More than 200 different multigene panels in which the two major *BRCA1* and *BRCA2* breast cancer predisposing genes are included are proposed by public or commercial laboratories. We review the clinical validity and clinical utility of the 26 genes most oftenly included in these panels. Because clinical validity and utility are not established for all genes and due to the heterogeneity of tumour risk levels, there is a substantial difficulty in the routine use of multigene panels if management guidelines and recommendations for testing relatives are not previously defined for each gene. Besides, the classification of variant of unknown significance (VUS) is a particular limitation and challenge. Efforts to classify VUSs and also to identify factors that modify cancer risks are now needed to produce personalised risk estimates. The complexity of information, the capacity to come back to patients when VUS are re-classified as pathogenic, and the expected large increase in the number of individuals to be tested especially when the aim of multigene panel testing is not only prevention but also treatment are challenging both for physicians and patients. Quality of tests, interpretation of results, information and accompaniment of patients must be at the heart of the guidelines of multigene panel testing.

1. Introduction

Since its emergence around 2010, next generation sequencing (NGS) has been a breakthrough for medical genetic laboratories. As with whole exome and whole genome sequencing, NGS through multigene panels makes it possible to sequence a considerable number of genes in a large number of individuals. Multigene panel testing is useful for diagnosis of diseases with genetic heterogeneity and for disease risk estimates in predictive medicine.

In the field of cancer genetics and particularly breast cancer predisposition, panel testing has spread extensively and rapidly over academic and commercial laboratories. This is linked to the decision of the US Supreme Court in June 2013 to invalidate the patents that restricted *BRCA1/BRCA2* testing [1]. Indeed, these two genes are the "core" of multigene panels exploring breast (and ovarian) cancer risks or more largely cancer risks. Invalidation of the patents resulted in decreased costs for genetic testing and more widespread availability to companies. In addition, the demand for breast cancer genetic testing worldwide has doubled since May 2013, when Angelina Jolie revealed that she is a carrier of a pathogenic *BRCA1* variant and that she had chosen to undergo prophylactic mastectomy [2]. Another reason for the rise in multigene breast cancer panels is the development of targeted therapies for cancers with homologous recombination (HR) deficiency, which makes identification of pathogenic variants in genes involved in HR, particularly *BRCA1* and *BRCA2*, a treatment prerequisite [3].

Multigene panel testing is time- and cost-efficient because it avoids sequential tests. It also provides extensive data, however, given the high number of genetic variants identified, transforming the data into information useful for each individual tested can prove difficult. We will review the main characteristics of the breast (and ovarian) cancer predisposing gene panels available, the

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required parameters of their analytical validity, clinical validity and clinical utility. The enormous capacity of gene sequencing has led "panel designers" to include genes whose clinical validity and utility are not established, thus producing "bulky" knowledge. What recommendations can be made for the use of the available breast (and ovarian) cancer multigene panels and for improving their clinical utility?

2. Available breast (and ovarian) cancer multigene panel testing

The latest release of the NCBI Genetic Test Registry lists more than 200 multigene panels including *BRCA1* and *BRCA2* that are proposed by academic or commercial laboratories [4]. Some but not all are specific to breast and ovarian cancer predisposing genes. The 26 genes most frequently included in breast (and ovarian) cancer panels are shown in Table 1. As previously stated, *BRCA1* and *BRCA2* are the core genes. The other genes are either associated with specific phenotypes and breast cancer risks (*TP53* and Li-Fraumeni syndrome; *CDH1* and lobular breast carcinoma and diffuse gastric carcinoma; *PTEN* and Hamartoma Tumour Syndrome; *STK11* and Peutz-Jeghers syndrome; *NF1* and neurofibromatosis type 1), or with ovarian cancer risks (*MLH1, MSH2/EPCAM, MSH6, PMS2*). They also include genes reportedly associated with a risk of breast cancer in case studies or case-control studies. Most are involved in DNA repair, some of which are reportedly partners of BRCA1 or BRCA2 (*ATM, BARD1, BRIP1, GEN1, MCPH1, NBN, MRE11A, PALB2, RAD50, RAD51C, RAD51D, RECQL, RINT1, SLX4, XRCC2*), or in cell-cycle control or mitotic signal transduction (*CHEK1, CHEK2, PI3KCA*). The list is open and grows with each new study. The level of the associated breast/ovarian cancer risk differs depending on the genes: some genes, or rather their pathogenic variants, are associated with high risk, others with moderate risk and for others the reality of an increased risk has not yet been clearly established. The heterogeneity of the risk level of the sequenced genes and thus the diversity of management guidelines are a substantial difficulty in routine use of multigene panels.

3. Analytical validity

In analytical validity, we include the sensitivity and specificity of variant detection and their clinical interpretation or classification.

3.1. Sensitivity and specificity of variant detection

Lincoln et al. examined, in a series of 1105 individuals, the analytical concordance of 750 different variants of 29 genes using NGS versus Sanger sequencing, the gold standard of sequencing methods. There was 99.8% net report concordance [5]. Moreover, NGS technologies showed a higher sensitivity than Sanger

Table 1
Genes most frequently included in breast (and ovarian) cancer panels and their validity and clinical utility.

Gene	Clinical Validity/Risk Level		Clinical Utility				Main References
	Level of breast cancer risk	Level of ovarian cancer risk	Specific Guidelines			Genetic Test for Relatives	
			Breast	Ovary	Related syndrome		
BRCA1	High CLTR(80) 72% [51]	High CLTR(80) 44%	Yes[b]	Yes[c]		Yes	[51]
BRCA2	High CLTR(80) 69%	High CLTR(80) 17%	Yes[b]	Yes[c]		Yes	[51]
PALB2	High OR 6.56–9.47	Conflicted	Yes[b]	No		Yes	[52–57]
TP53	High RR 3.76; CLTR(70) 85%	Conflicted	Yes[b]	No	Li Fraumeni	Yes	[55,58]
PTEN	High CLTR(70): 67–85.2%	Low	Yes[b]	No	Cowden	Yes	[59–61]
CDH1	High RR 6.6–7.7; CLTR(80) 39–42%	Unknown	Yes[b]	No	HDGC	Yes	[62–64]
STK11	High, RR 15; CLTR(70) 77%	High RR 2; CLTR(70) 45%	Yes[b]	Yes[d]	Peutz Jeghers	Yes	[65,66]
MLH1	Conflicted	High CLTR(70): 20%	No	Yes[d]	Lynch	Yes	[55,67]
MSH2/EPCAM	Conflicted	High CLTR(70): 24%	No	Yes[d]	Lynch	Yes	[55,67]
MSH6	Conflicted	Conflicted	No	Yes[d]	Lynch	Yes	[55,67]
PMS2	Conflicted	Conflicted	No	Yes[d]	Lynch	Yes	[55,68]
NF1	Insufficient data	Insufficient data	Discussed	No	NF1	Yes[d]	[69]
RAD51C	No evidence of association	High RR 5.2–6.31; CLTR(80): 9%	No	Yes		Yes	[70–74]
RAD51D	No evidence of association	High OR 6.3; CLTR(80) 13.56%	No	Yes		Yes	[72,75,76]
ATM	Low to moderate RR 1.5–3 [a]	No evidence of association	Discussed[a]	No		Discussed	[57,77–79]
CHEK2	Low to moderate OR 1.58–3 [a]	Insufficient data	Discussed[a]	No		Discussed	[77,80,81]
BRIP1	No evidence of association	Moderate RR 3.41; CLTR(80) 4–12.7%	No	Discussed		Discussed	[77,82]
BARD1	Insufficient data	Insufficient data	No	No		No	[82,83]
GEN1	No evidence of association	No evidence of association	No	No		No	[84,85]
RAD50	Insufficient data	Insufficient data	No	No		No	[54,55]
RINT1	Conflicted	Insufficient data	No	No		No	[86,87]
MRE11A	Insufficient data	Insufficient data	No	No		No	[54]
NBN	Conflicted OR 1.4–2.66 [a]	No evidence of association	No	No		No	[55,82,88]
XRCC2	No excess risk Insufficient data	Insufficient data	No	No		No	[77,89]
MCPH1	Insufficient data	Insufficient data	No	No		No	[77]
SLX4	Insufficient data	Insufficient data	No	No		No	[77]

OR: odds ratio; RR relative risk; CLTR () cumulative lifetime risk (at age); HDGC hereditary diffuse gastric cancer; NF1 neurofibromatosis type I.
In the absence of specific guidelines, surveillance should be based on family history of breast and/or ovarian cancer, regardless of genetic result.
The clinical utility of genetic testing in relatives means that specific guidelines will be applied to carriers of the pathogenic variant and that surveillance will be stopped in non-carriers.

[a] According to type of mutation.
[b] Including breast MRI and prophylactic mastectomy.
[c] Including prophylactic oophorectomy.
[d] According to guidelines on related syndrome.

sequencing for mosaic variants, i.e. *de novo* variants that occur during embryonic development and are present in some but not all of an individual's cells. Conventional NGS can detect variants at low levels, up to 1%, while the limit of detection of Sanger sequencing is about 15–20% [6,7]. In breast cancer multigene panel testing, a low limit of detection is particularly important for *TP53* gene, which has an estimated *de novo* variant rate of at least 14% of *TP53* pathogenic variants identified, and one-fifth of these *de novo* variants are mosaics [8].

However, Sanger sequencing has better specificity than conventional NGS. Using Sanger sequencing, Mu et al. verified 7845 non-polymorphic variants identified through 20,000 hereditary-cancer panels spanning 47 genes. They found 98.7% concordance of the variants between NGS and Sanger sequencing, 1.3% were NGS false-positives. They were located in A/T- and G/C-rich regions, homopolymer stretches and pseudogenes. Simulation of a false-positive rate of zero by adjusting the variant-calling quality-score thresholds decreased the sensitivity from 100% to 97.8% and resulted in missed detection of 176 variants. These data illustrate the need for Sanger confirmation of NGS variants to maintain the highest possible sensitivity [9].

A great benefit of NGS is that it can most often detect both point variants and copy number variations (CNV) in one experiment. A recent survey examined worldwide genetic testing practices for *BRCA1/BRCA2*. Among the 86 laboratories that participated in the survey, 80 (93%) had moved towards NGS. Regarding detection of CNVs, 88% of laboratories in the US and 40% in Europe used NGS. It is expected that in the near future, NGS will also be adopted worldwide for CNV detection [10]. The same survey found that over half of laboratories confirmed variants (except neutral or likely neutral, see below) using another method, in most cases Sanger sequencing. The authors recommend that the results should mention such technical details as minimal read depths, analytical sensitivity and variant confirmation by an independent method. In order to assist clinical laboratories with analytical validation of NGS, the American College of Medical Genetics and Genomics (ACMG) has proposed comprehensive professional standards and guidelines concerning all steps in NGS, from DNA preparation and bioinformatic analysis to data reporting [11].

3.2. Clinical classification of variants

BRCA1 and *BRCA2,* along with other dominantly transmitted cancer predisposing genes, are associated with cancer risks through protein inactivating variants and therefore often through variants introducing a stop codon (nonsense, frameshift, splice defect) and more rarely missense variants. The classification of genetic variants in coding sequences or their intron-exon junctions as pathogenic or neutral is not a new challenge, nor is it NGS specific, however, because NGS is identifying an increasing number of variants, and the proportion of annotated variants is not increasing at the same rate, there is, according to Cutting, an ever-increasing "interpretive gap" that reopens and broadens the challenge [12].

Classification of variants is a particular limitation in *BRCA1/BRCA2* testing. More than 1000 pathogenic variants in each gene have been reported but the number of unclassified variants, variants of unknown significance (VUS), is double [13]. Plon et al. have proposed a classification of variants of cancer predisposing genes based on 5 levels: definitely pathogenic, likely pathogenic, unknown significance, likely not pathogenic and not pathogenic or of no clinical significance or neutral [14]. The classification of variants as pathogenic is of the utmost important for the management of carriers. Guidelines for variant classification have also been proposed by the ACMG [15]. The classification of *BRCA1/BRCA2* and more recently *PALB2* genes is challenging and, in 2009, led to the creation of the international consortium "Evidence-based Network for the Interpretation of Germline Mutant Alleles", ENIGMA [16]. ENIGMA is the main actor in the BRCA Challenge, proof of concept of the Global Alliance for Genomics and Health, GA4GH, whose aim is to facilitate sharing of international data in medical genetics to improve the quality of genetic diagnoses [17,18]. Classification of each known *BRCA1/BRCA2* VUS based on pathogenicity relies on multiple approaches: higher frequency in cases than in controls, phylogenetic conservation of the nucleotide/amino-acid modified, Grantham score for missense variants, co-segregation of the VUS with the disease in multiplex families, abnormal transcripts, functional assays, etc. ENIGMA promotes sharing of efforts at classification by laboratories, clinical cancer geneticists and national variant database curators [19–22]. It should be noted that currently the classification of VUSs identified in non-Caucasian patients and in smaller ethnic groups is more difficult due to the lack of epidemiological data. Special effort should be made in these populations.

Overall, while it is of the utmost importance to classify VUSs, it is equally important to update the test results. In other words, laboratories need to have the ability to come back to patients and their physicians when a VUS has been classified. Indeed, it is expected that some VUSs, hopefully most of them, will be classified as pathogenic or neutral as more data are accrued. Conversely, some pathogenic variants have been "declassified" to non-pathogenic due to a residual functional protein derived from physiological alternative transcripts that were not previously taken into account [23]. Reporting of variants and their clinical classification has to be written clearly so that the numerous physicians who manage women at risk understand the results and use them appropriately. In a survey of 3672 women who had *BRCA1/BRCA2* testing in 2014–2015, Kurian et al. reported that 51% of women carrying a VUS underwent bilateral prophylactic mastectomy even in the absence of a severe family history [24]. In the Prospective Registry of Multiplex Testing (PROMPT), 26% of variants reported by at least two commercial laboratories were of conflicting interpretation [25]. In March 2016, the journalist Jeremy Lange reported in *The New York Times* the case of a woman whose doctors had conflicting information on her *BRCA1/BRCA2* test result. "The situation is ripe for overinterpretation and misinterpretation," said a geneticist interviewed by Jeremy Lange. In summary, laboratories must collaborate with physicians in order to take into account patient clinical and familial data, contribute to and track the classification of variants and be able to pass on the updated variant classification to patients and their physicians. If these objectives are in the DNA of academic laboratories, they are not in that of commercial laboratories. It is however of the utmost importance to organise and sustain variant databases and to pursue all the efforts with all the actors of breast cancer genetic testing.

4. Clinical validity and clinical utility

The aim of predictive medicine is to limit the risk of an expected disease or to limit its impact on quality of life (including psychosocial outcomes) and to limit its mortality. We do not include here specific treatments such as PARP inhibitors in tumours with bi-allelic HR gene inactivation. The main aim of testing for breast (and ovarian) cancer genes is prevention in carriers of pathogenic variants and reassurance of non-carriers. If clinical validity refers to the accuracy with which a condition is identified and clinical utility to the benefits of management guidelines based on genetic testing, clinical validity and utility should be demonstrated before being put into clinical practice [26,27]. Because clinical validity and utility must be demonstrated for each gene in the panel, and because our knowledge of genes and tumour risk levels differ, the clinical use of breast (and ovarian) cancer multigene panel testing is challenging.

4.1. Clinical validity

Strande et al. for the NIH-funded Clinical Genome Resource (ClinGen) created a framework that provides evidence for the strength of the association between a gene and a disease or a disease risk through semi-quantitative classification: definitive, strong, moderate, limited, no reported evidence, or conflicting evidence [28]. The classification is based on genetics, i.e. cosegregation data, case and case-control studies, as well as experimental data, i.e. functional data, cell and animal models.

Some genes, such as *MEN1 and CTNNB1*, included in some breast (and ovarian) multigene panels based on case reports seem to be "lost in translation". Classification of clinical validity is a dynamic process, subject to new information. For example, until recently no studies reported an increased risk of breast cancer associated with *RAD51C* and *RAD51D* pathogenic variants, which were initially thought to be associated only with ovarian cancer. A first study performed in 65,000 women affected with breast cancer reported an increased risk of breast cancer with *RAD51D* [29]. A second and more recent study performed in 10,900 triple negative carcinoma (TN) cases, a subset of 140,000 breast cancer cases, confirmed the risk of breast cancer with *RAD51D* and identified an increased risk in carriers of *RAD51C* pathogenic variants, another paralogue of *RAD51* [30]. Due to the very low frequency of *RAD51C* and *RAD51D* carriers in the general population and the heterogeneity of breast cancer, with TN tumours representing 15% of breast cancers, the increased risk and therefore clinical validity were demonstrated with difficulty.

4.2. Clinical utility

Cancer risk estimates are the key point in defining management guidelines and thus clinical utility. Cancer risks are classified as high, moderate or low, each being associated with different management guidelines (Table 1). Low risk means less than two times as high as the risk in the general population, moderate risk 2 to 4 times as high, and high risk more than four times higher [27]. National guidelines on prophylactic mastectomy and breast MRI are well established and homogenous regarding carriers of high-risk genes (*BRCA1, BRCA2, PALB2*), while guidelines on moderate-risk genes (*ATM, CHEK2*) are not homogeneous. For the same gene, some recommend breast MRI when a test result is positive, while others take family history into account more so than the test result [31–34]. The heterogeneity of guidelines and practices regarding moderate-risk genes illustrates the extent to which clinical utility is not yet firmly established.

Another difficulty related to moderate-risk genes is the use of identified pathogenic variants for testing relatives. When a pathogenic *BRCA1* variant is identified in a woman, variant-targeted tests are offered to her relatives and when negative, relatives are reassured. When a pathogenic variant in a moderate-risk gene, such as *ATM*, is identified, especially in a woman with a severe family history, can a targeted test be offered to her relatives and, if negative, is it reassuring? Probably not, because we consider that the *ATM* variant does not "recap" the family history. The multigene panel test is generally presented as a single test, but it is actually a myriad of tests whose results are as numerous as the number of genes tested. Because the clinical utility of moderate-risk genes has not been firmly established and because the tests available to relatives are not unequivocal, the French Genetic and Cancer Group (GGC)-Unicancer chose not to include *ATM* and *CHEK2* in its breast (and ovarian) cancer multigene panel [31]. However, all multigene panels are welcome provided that the consequences of identifying a pathogenic variant for each gene have been anticipated in terms of follow-up and testing available to relatives.

Specific syndrome genes such as *TP53, CDH1, PTEN, STK11* and *NF1*, are included in most breast (and ovarian) cancer multigene panels. Specific guidelines have been published for carriers whose families have the syndrome. For example, in Li-Fraumeni syndrome, annual "head-to-toe" whole-body MRI is advised in *TP53* pathogenic variant carriers [35]. Does such stringent and stressful follow-up need to be conducted in *TP53* carriers identified through a multigene panel performed because, for example, two relatives developed breast cancer at age 50? A recent study by Rana et al. examined the phenotype of *TP53* carriers identified through multigene panels. Ages at diagnosis of breast cancer were older than reported in the classic Li-Fraumeni syndrome, suggesting that *TP53* pathogenic variants may have a broader phenotypic spectrum than previously reported [36]. The question is similar for *CDH1* carriers [37]. Should prophylactic gastrectomy before the age of 30 be recommended when no relative is affected with gastric carcinoma? Re-assessment of cancer risks by prospective studies is required in individuals whose genetic tests have been conducted outside stringent testing criteria, which is likely to be more frequent with increasing multigene panel use [38].

A similar question is raised regarding *BRCA1* and *BRCA2* carriers. Modifier factors, genetic or otherwise, have long been known to increase or decrease the risk of breast and ovarian cancer in carriers [39,40]. The aims of the international collaborative groups IBCCS (International BRCA1/2 Carrier Cohort Study) and CIMBA (The Consortium of Investigators of Modifiers of *BRCA1*/2) are precisely to identify such modifier factors. Kuchenbaecker et al. have examined a polygenic risk score (PRS) based on the genotypes of 77 single nucleotide polymorphisms (SNP). The PRS modulates breast cancer risks at age 80 between 53% and 78% in *BRCA1* carriers and 35%–55% in *BRCA2* carriers [41] (and Cox et al. this issue). Similar studies are on-going for ovarian cancer risks. It is of particular importance to take into account modifier factors in personalised risk estimates when relatives are not affected with breast or ovarian cancers. The on-going BRIDGES project (Breast cancer RIsk after Diagnostic GEne Sequencing) aims to combine, in addition to *BRCA1, BRCA2, ATM* and *CHEK2* variants, modifier factors including a PRS, [42]. Personalised risk estimates are urgently needed when some groups report that it is time to offer *BRCA1, BRCA2, RAD51C, RAD51D, BRIP1* and *PALB2* tests to all women over the age of 30 [43].

In summary, efforts to identify and combine factors that modify cancer risks linked to the different predisposing genes sequenced in multigene panels are needed to produce personalised risk estimates for women tested, as well as for her relatives, and thus to escape the bias of any testing criteria.

5. Perspectives and conclusion

Multigene panel testing is a promising tool that makes it possible to test a considerable number of genes in a large number of individuals. It is possible that in the future, when the coverage and minimal read depths are high and homogeneous, whole exome sequencing will be performed with a multigene panel reading. Whole exome and later whole genome sequencing associating genome spread SNPs and thus PRS, will be the "Swiss knife" of geneticists. Before this era, more widespread use of multigene panel is also likely to allow us to identify individuals with two predisposing genes (digenism), a situation probably more frequent than hitherto thought. Indeed, *BRCA2* carrier frequency in the Caucasian population may be as high as 1/192 and *ATM* carrier frequency 1/150 [44,45]. Currently, multigene panel testing is already a highly useful tool provided the results are not over-interpreted or misinterpreted. As genetic testing advances, research into the interpretation of VUSs, the accuracy of personalised risk estimates through the identification of risk-modifying factors must

continue. Studies are on-going through international efforts under the auspices of consortia such as ENIGMA, IBCCS, CIMBA and through data-sharing as in the BRCA Challenge. Special research efforts are required in individuals of non-occidental ethnic groups as few data are currently available. Diagnosis and research may be combined in the same multigene panel, however the genes sequenced for research should be defined and patients informed before testing. It is important to remember the bulk of our knowledge in this field was obtained thanks to patients and their families, and this situation has not changed. Information on the issues related to the tests and anticipation of possible results, including VUSs, are key points in genetic counselling.

The complexity of information, the capacity to come back to patients with updated results, such as when VUSs are re-classified as pathogenic, and the expected large increase in the number of individuals to be tested are challenging both for physicians and patients. Indeed, the treatment by PARP inhibitors of women affected with high grade ovarian cancer, TN breast cancer or metastatic breast cancer and whose tumours is inactivated for *BRCA1* or *BRCA2* is going to strongly increase the indications of breast (and ovarian) multigene panel testing and also to modify its process. For example, the recent study SOLO1 has demonstrated the interest of maintenance therapy by PARP inhibitor olaparib in newly diagnosed advanced high grade ovarian carcinoma in women whose tumour is inactivated for *BRCA1* or *BRCA2* through germline or somatic mutation events [46]. It is expected that in the near future multigene panel testing will be performed through tumour DNA with the double aim of treatment and prevention. It is important to keep in mind that *BRCA1/2* tumour testing (ovarian, breast and other locations) is a cancer predisposing test since 80% of pathogenic variants identified are germline [47]. Tumour DNA is the native DNA of the patient! Oncologists and surgeons need to work closely with clinical and molecular geneticists. It is paradoxal that at that time the Food and Drug Administration has authorised direct-to-consumer tests of three *BRCA1-2* pathogenic variants [48]. We can only speculate on what patients understand. ASCO and The European Breast Cancer Council (EBCC) recently issued guidelines for breast cancer predisposition testing. Quality of tests, information, interpretation of results and accompaniment of patients who have no choice when treatment is one of the objectives of genetic testing, are at the heart of the guidelines [49,50].

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