ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) is an international consortium of investigators focused on determining the clinical significance of sequence variants in breast cancer genes. Further information about the consortium purpose, membership criteria and operation can be found at http://www.enigmaconsortium.org/.

Rules for Variant Classification:
Rules describing the 5 class system for classification of BRCA1/2 gene variants were initially devised and documented by ENIGMA Steering committee members, and revised with input from ENIGMA collaborators. These ENIGMA criteria provide a baseline for standardized clinical classification of BRCA1/2 gene sequence variation that may be linked to patient and family management in the genetic counseling arena according to published guidelines (Plon et al., 2008). The proposed classifications are intended to differentiate high risk variants (risk equivalent to classical protein-truncating pathogenic variants) from variants with low or no risk. At the present time, these guidelines are not intended for the evaluation and classification of variants associated with an intermediate or moderate level of risk e.g. BRCA1 c.5096G>A p.Arg1699Gln (Spurdle et al., 2012).

The ENIGMA criteria are based on a combination of the following:
- The 5 class system described for quantitative assessment of variant pathogenicity in Plon et al. (2008) using a multifactorial likelihood model (Goldgar et al., 2004, Easton et al., 2007, Goldgar et al., 2008, Tavtigian et al., 2008) (see Appendix, Table 1);
- The 5 class system for interpretation of possible spliceogenic variants and splicing alterations developed by ENIGMA collaborators (Walker et al., 2013);
- Generic elements of the 5 class quantitative/qualitative scheme for mismatch repair gene variant classification developed by InSiGHT (Thompson et al., 2014);
- Generic elements of the ACMG guidelines for interpretation of sequence variations (Richards et al., 2008)
- Classification criteria developed by individual sites participating in ENIGMA, including established country networks;
- The classification of sequence changes according to standard clinical practice – that is, description of variants generally considered pathogenic (clinically relevant in a genetic counseling setting such that germline variant status is used to inform patient and family management) or non-pathogenic (significant evidence against being a dominant high-risk pathogenic variant).

Appendix, Table 2 summarises the rationale supporting the criteria.
The interpretation of variant clinical significance in relation to functional domains is assisted by definition of clinically important functional domains (Appendix, Tables 3 and 4).

Use of the ENIGMA variant interpretation guidelines is subject to user discretion and responsibility. Guidelines are subject to change with the availability of new information and interpretation processes, and we thus recommend date-stamping for all variant classifications. Interpretation of variants using these criteria does not exclude the very low probability that there is a pathogenic variant in cis undetected by the testing protocol, which may confound interpretation of variant pathogenicity.

For a given class, a bullet point “•” represents an “OR” statement, whereas the symbol “√” represents an “AND” statement. That is, a variant is required to satisfy all the criteria listed for at least one bullet-point that falls within that class.
Class 5 – Pathogenic
There is significant evidence to suggest that this variant is a dominant high-risk pathogenic variant.

- Variant with probability of pathogenicity >0.99 using a multifactorial likelihood model¹.

- Coding sequence variant that encodes a premature termination codon i.e. nonsense or frameshift alteration predicted to disrupt expression of known clinically important functional protein domain(s)² and for which there is low bioinformatic likelihood³ to disrupt normal splicing (e.g. disruption of native donor/acceptor sites, creation of de novo donor, other splicing regulatory motifs). Note: Predicted nonsense or frameshift truncating variants with high bioinformatic likelihood³ to disrupt normal splicing require mRNA assays to assess the nature and possible clinical consequence of aberrant transcripts.

- Variant allele tested for mRNA aberrations using in vitro assays of patient RNA⁴ that assesses allele-specific transcript expression, and is found to produce only transcript(s) carrying a premature termination codon, or an in-frame deletion disrupting known clinically important functional domain(s)² and/or protein conformation.

- Copy number deletion variant that removes one or more exons spanning a known clinically important functional domain² or is proven by laboratory studies to result in a frameshift alteration predicted to disrupt expression of known clinically important functional protein domain(s)².

- Copy number duplication variant of any size that duplicates one or more exons and is proven by laboratory studies to result in a frameshift alteration predicted to disrupt expression of known clinically important functional protein domain(s)².
Class 4 – Likely pathogenic
There is evidence that this variant is a dominant high-risk pathogenic variant.

- Variant with probability of pathogenicity between 0.95-0.99 using a multifactorial likelihood model

- Variant considered extremely likely to alter splicing based on position, namely IVS±1 or IVS±2, or G>non-G at last base of exon if first 6 bases of the intron are not GTRRGT, and
  - is untested for splicing aberrations using in vitro assays of patient RNA that assesses allele-specific transcript expression
  - is not predicted or known to lead to naturally occurring in-frame RNA isoforms that may rescue gene functionality (See Appendix, Table 5 for these exceptions, variants to be considered Class 3 (uncertain) unless proven otherwise).

- A variant demonstrating all these features:
  - encodes the same amino acid change as a previously established Class 5 pathogenic missense variant with a different underlying nucleotide change
  - is located in a known clinically important functional protein domain
  - there is no evidence of mRNA aberration (splicing or expression) from in vitro mRNA assays.
  - the variant is absent from outbred control reference groups

- A small in-frame deletion variant demonstrating these features:
  - removes codon for which a missense substitution Class-5 variant has been described
  - is located in a known clinically important functional protein domain
  - the variant is absent from outbred control reference groups

Class 3 – Uncertain
There is insufficient evidence to place this variant in Class 1, 2, 4 or 5.

- Variant with probability of pathogenicity between 0.05-0.949 using a multifactorial likelihood model

- Variant that has insufficient evidence (molecular or otherwise) to be classified as a high-risk pathogenic variant or as a variant of little clinical significance.

- Variants located at positions listed in Appendix, Table 5, unless proven to fall in another class based on additional evidence.

- Variant considered possibly resistant to classification i.e. where there are multiple apparently conflicting points of evidence regarding variant pathogenicity, and which thus requires further investigation as a possible intermediate risk variant using alternative study design(s).
Class 2 – Likely not pathogenic/little clinical significance
There is evidence against this variant being a dominant high-risk pathogenic variant.

- Variants with probability of pathogenicity between 0.001-0.049 using a multifactorial likelihood model.
- An exonic variant, that encodes the same amino acid change as a previously established Class 1 not pathogenic missense variant with a different underlying nucleotide change, and for which there is no evidence of mRNA aberration (splicing or allelic imbalance) as determined using in vitro laboratory assays.

Class 1 – Not pathogenic/low clinical significance
There is significant evidence against this variant being a dominant high-risk pathogenic variant.

- Variants with probability of pathogenicity <0.001 using a multifactorial likelihood model.
- Variants reported to occur in large outbred control reference groups at an allele frequency ≥1% (MAF ≥ 0.01).
- Variants demonstrating all these features:
  - Exonic variant encoding a missense substitution or resulting in a small in-frame insertion/deletion with prior probability of pathogenicity ≤2% as determined from A-GVGD analysis, OR synonymous substitution, OR intronic variant
  - Increased bioinformatic likelihood to disrupt normal splicing but
  - No associated mRNA aberration (splicing or allelic imbalance) as determined using in vitro laboratory assays.
  - OR
    - Co-occurrence in trans with a known pathogenic sequence variant in the same gene in an individual who has no obvious additional clinical phenotype other than BRCA-associated cancer.

- Variants demonstrating all these features:
  - Exonic variant encoding a missense substitution or resulting in a small in-frame insertion/deletion with prior probability of pathogenicity ≤2% as determined from A-GVGD analysis, OR synonymous substitution, OR intronic variant
  - Low bioinformatic likelihood to disrupt normal splicing
  - Co-occurrence in trans with a known pathogenic sequence variant in the same gene in an individual who has no obvious additional clinical phenotype other than BRCA-associated cancer.
Footnotes

1 To ensure robust variant classification based on multifactorial likelihood analysis results, the following caveats and recommendations should be noted before finalising variant classification. Only independent lines of evidence should be included. Tumour pathology information for a proband cannot be considered if the proband was selected for testing on the basis of breast tumour pathology criteria. Further investigation is necessary for any variant with extreme prior probability and minimal additional evidence, and it is currently recommended that clinical or laboratory evidence should contribute an LR of <0.5 (to reach final class 2 or 1), or >2.0 (to reach final class 4 or 5). A variant which displays an obvious discordance between the predicted prior probability and additional clinical or laboratory evidence should be re-investigated to establish the veracity of results, to assess the possibility that it may be a hypomorph exhibiting intermediate or moderate penetrance relative to high-risk truncating variants, and/or to exclude the very low probability that there is a pathogenic variant in cis. As per published recommendations (Plon et al., 2008), further research segregation testing in family members is recommended for variants in class 2, 3 or 4 to assist variant classification.

2 A known clinically important functional protein domain is a recognized protein functional domain that is reported to harbor sequence variants that introduce deleterious changes to protein function (via missense alteration, protein sequence deletion, or protein truncation in the last exon) AND are also associated with high risk of cancer. Physical boundaries for functional domains, and reported risk-associated variants to establish regions and residues of clinical importance, are described in the Appendix, Table 3 (BRCA1) and Table 4 (BRCA2).

3 Bioinformatic likelihood of altering splicing is likely to consider thresholds set in Houdayer et al (Houdayer et al., 2012) for nucleotide variants at the consensus site, or by Vallee et al (submitted) for exonic variants and variants at positions up to 20bp into the introns. Use of additional bioinformatic tools and prediction of additional motifs relevant for normal splicing will be considered in future iterations of these guidelines.

4 Recommendations for the conduct and interpretation of mRNA assay data for variant classification are drawn from (Walker et al., 2013). Assessment assumes assays on mRNA from patient germline tissue samples (fresh blood, cultured lymphocytes, lymphoblastoid cell lines etc), compared with assays performed in tandem on mRNA from the same tissue type for ≥10 reference controls. Transcripts identified at similar levels in controls are considered to be naturally occurring isoforms and not mRNA aberrations. A variant is considered to be pathogenic due to an effect on mRNA transcription if it produces only transcript(s) carrying a premature stop codon or an in-frame deletion disrupting known functional domain(s), determined by semi-quantitative or quantitative methods. Sequencing of the full length transcript for the variant allele (if exonic), or a common polymorphism in cis (if variant is intronic), is currently considered adequate to assess if variant allele contributes to production of wild-type transcript. A variant may be reported as not associated with an mRNA aberration (splicing or expression) if the variant allele produces transcript patterns comparable to that of controls using assays conducted with nonsense-mediated decay inhibition.

5 Outbred control reference groups currently used for this purpose include datasets from the 1000 Genomes project (http://www.1000genomes.org/) and the Exome Variant Server (http://evs.gs.washington.edu/EVS/).

6 Prior probability derived from calibration of Align-Grantham Variation Grantham Deviation score against BRCA1/2 clinical features of variant pathogenicity (http://priors.hci.utah.edu/PRIORS/).

7 Note: assuming a prior probability of 0.02, the detection rate for class 5 pathogenic variants in that gene is required to be 0.025 in the sample set tested to ensure that a single observation of co-occurrence in trans equates to a co-occurrence LR of 0.04, and consequently class 1 classification. This criterion also requires that the patient is assessed to exclude Fanconi-like or other features (Rodríguez and Henderson, 2000, Chen et al., 1996) that suggest the variant leads to loss of function in vivo. If necessary, consider referral for examination by a clinical geneticist and/or additional in vitro diagnostic tests for molecular features of Fanconi phenotype.
## APPENDIX

### Table 1: IARC 5-tiered classification system with accompanying recommendations for family management

<table>
<thead>
<tr>
<th>Class</th>
<th>Quantitative Measure: Probability of Pathogenicity</th>
<th>Predictive Testing of At-Risk Relatives</th>
<th>Surveillance for At-Risk Relatives</th>
<th>Research Testing of Relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>5: Pathogenic</td>
<td>&gt;0.99</td>
<td>Yes</td>
<td>Full high-risk guidelines for variant carriers</td>
<td>Not indicated</td>
</tr>
<tr>
<td>4: Likely pathogenic</td>
<td>0.95-0.99</td>
<td>Yes&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Full high-risk guidelines for variant carriers</td>
<td>Yes</td>
</tr>
<tr>
<td>3: Uncertain</td>
<td>0.05-0.949</td>
<td>No&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Based on family history &amp; other risk factors</td>
<td>Yes</td>
</tr>
<tr>
<td>2: Likely not pathogenic or of little clinical significance</td>
<td>0.001-0.049</td>
<td>No&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Based on family history &amp; other risk factors - treat as &quot;no BRCA1/2 pathogenic variant detected&quot; for this disorder</td>
<td>Yes</td>
</tr>
<tr>
<td>1: Not pathogenic or of no clinical significance</td>
<td>&lt;0.001</td>
<td>No&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Based on family history &amp; other risk factors - treat as &quot;no BRCA1/2 pathogenic variant detected&quot; for this disorder</td>
<td>Not indicated</td>
</tr>
</tbody>
</table>

<sup>a</sup>Adapted for clarity from original tabular presentation published (Plon et al., 2008)

<sup>b</sup>Recommend continued testing of proband for any additional available testing modalities available for BRCA1/2 e.g. rearrangements.
<table>
<thead>
<tr>
<th>Class</th>
<th>Criterion</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 5: pathogenic</td>
<td>Posterior probability of pathogenicity &gt;0.99 from multifactorial likelihood analysis.</td>
<td>IARC recommendation for Class 5 (Plon et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Coding sequence variant encoding a premature termination codon i.e. nonsense/frameshift predicted to disrupt expression of clinically important functional domain(s).</td>
<td>Treated clinically as pathogenic</td>
</tr>
<tr>
<td></td>
<td>The variant allele produces only transcripts that lead to a premature stop codon, or in-frame deletion predicted to disrupt clinically important domains, as determined by RNA assays on patient germline tissue that assess allele-specific transcript expression.</td>
<td>Treated clinically as pathogenic</td>
</tr>
<tr>
<td></td>
<td>Copy number deletion removing exon(s) spanning clinically important functional domain(s) or proven to result in a frameshift alteration predicted to interrupt expression of clinically important functional domain(s).</td>
<td>Treated clinically as pathogenic</td>
</tr>
<tr>
<td></td>
<td>Copy number duplication proven to result in frameshift alteration predicted to interrupt expression of clinically important functional domain(s).</td>
<td>Treated clinically as pathogenic</td>
</tr>
<tr>
<td>Class 4: likely pathogenic</td>
<td>Posterior probability of pathogenicity 0.95-0.99 from multifactorial likelihood analysis.</td>
<td>Disruption of highly conserved bases at acceptor and donor splice sites is extremely likely to result in a splicing aberration, with suggested prior probability 0.96 (Walker et al., 2013). Conservative classification is warranted since pathogenicity cannot be assumed for all mRNA profiles arising from a variant allele e.g. incomplete effect on splicing, or potential to lead to in-frame (naturally-occurring) transcripts.</td>
</tr>
<tr>
<td></td>
<td>Variant at IVS±1 or IVS±2 or G&gt;non-G at last base of exon when adjacent intrinsic sequence is not GTRRGT but is untested for splicing aberrations using RNA assays on patient blood that assess allele-specific transcript expression, AND is not predicted or known to lead to naturally occurring in-frame RNA isoforms that may rescue gene functionality.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A variant that encodes the same amino acid change as a previously established class 5 pathogenic missense variant with a different underlying nucleotide change, is located in a known clinically important functional protein domain, with no evidence of mRNA aberration (splicing or expression) from in vitro mRNA assays on patient RNA, and the variant is absent from outbred control reference groups.</td>
<td>Having excluded possible mRNA defects caused by a nucleotide change, the clinical consequences of a rare missense variant should be equivalent irrespective of the underlying nucleotide change. Absence in controls and location in a functional domain provides additional support for evidence of pathogenicity.</td>
</tr>
<tr>
<td></td>
<td>A small in-frame deletion variant that removes a codon for which a missense substitution is located in a known clinically important functional protein domain, and is absent from outbred control reference groups.</td>
<td>For a given amino acid, the clinical consequences are equivalent for an in-frame deletion and the most severe missense substitution. Absence in controls and location in a functional domain provides additional support for evidence of pathogenicity.</td>
</tr>
<tr>
<td>Class 3: uncertain</td>
<td>Posterior probability of pathogenicity 0.05-0.949 from multifactorial likelihood analysis.</td>
<td>IARC recommendation for Class 3 (Plon et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Insufficient evidence to classify variant.</td>
<td>Does not fit prescribed criteria for other classes</td>
</tr>
<tr>
<td></td>
<td>Variant located at position listed in Table 5, unless proven to fall in another class based on additional evidence.</td>
<td>Variant has potential to lead to in-frame (naturally-occurring) transcripts that may rescue gene functionality.</td>
</tr>
<tr>
<td></td>
<td>Variant with conflicting evidence for pathogenicity.</td>
<td>Variant with modest effect on gene/protein function and modest/intermediate effect on risk may demonstrate some but not all features of a high-risk pathogenic variant, and should be highlighted for assessment of risk using alternative approaches.</td>
</tr>
<tr>
<td>Class 2: Likely not pathogenic or of little clinical significance</td>
<td>Posterior probability of pathogenicity 0.001-0.049 from multifactorial likelihood analysis.</td>
<td>IARC recommendation for Class 2 (Plon et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Exonic variant that encodes the same amino acid change as a previously established class 1 not pathogenic missense variant with a different underlying nucleotide change, and for which there is no evidence of mRNA aberration from in vitro mRNA assays.</td>
<td>Having excluded possible mRNA defects caused by a nucleotide change, the clinical consequences of a missense variant should be equivalent irrespective of the underlying nucleotide change.</td>
</tr>
</tbody>
</table>
### Class 1: not pathogenic or of no clinical significance

<table>
<thead>
<tr>
<th>Description</th>
<th>IARC recommendation for Class 1 (Plon et al., 2008)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posterior probability of pathogenicity &lt;0.001 from multifactorial likelihood analysis</td>
<td>High-risk variants are not common in the general population, and outbred reference groups exclude the possibility that a selected variant is an undetected founder mutation</td>
</tr>
<tr>
<td>Variant with reported frequency ≥1% in large outbred control reference groups</td>
<td></td>
</tr>
<tr>
<td>Exonic variant encoding missense or small in-frame alteration at a position that is not evolutionary conserved OR Synonymous substitution OR intronic variant; there is bioinformatic prediction of an effect on splicing; AND there is no associated variant-specific mRNA aberration in lab assays OR the variant co-occurs <em>in trans</em> with a known pathogenic sequence variant in the same gene in an individual who has no obvious additional clinical phenotype other than BRCA-associated cancer.</td>
<td>Multiple points of evidence indicate the variant is unlikely to be associated with high risk. Irrespective of bioinformatics predictions the variant is not associated with a splicing aberration, it is predicted to be extremely unlikely to affect protein function; Co-occurrence <em>in trans</em> with a known pathogenic variant in the same gene and with no unusual clinical features provides <em>in vivo</em> evidence for proficient function.</td>
</tr>
<tr>
<td>Exonic variant encoding missense or small in-frame alteration at a position that is not evolutionary conserved OR Synonymous substitution OR intronic variant; the variant co-occurs <em>in trans</em> with a known pathogenic sequence variant in the same gene in an individual who has no obvious additional clinical phenotype other than BRCA-associated cancer.</td>
<td>Multiple points of evidence indicate the variant is unlikely to be associated with high risk: Bioinformatic predictions indicate the variant is unlikely to affect mRNA or protein function; Co-occurrence <em>in trans</em> with a known pathogenic variant in the same gene and with no unusual clinical features provides <em>in vivo</em> evidence for proficient function.</td>
</tr>
</tbody>
</table>
Table 3: BRCA1 functional domains and relevance for interpreting the clinical significance of sequence variants – catalogue of clinically important functional domains and amino acid residues

<table>
<thead>
<tr>
<th>Region</th>
<th>AA start</th>
<th>AA end</th>
<th>AA alterations with Demonstrated Clinical Importance⁸</th>
<th>References and summary interpretation⁸</th>
</tr>
</thead>
</table>
http://hci-exlovd.hci.utah.edu; Multifactorial analysis for H41R (Whiley et al., 2014). |
| NES        | 81       | 99     | None reported                                       | In-frame deletions that remove any of the listed amino acids would be considered clinically important. |
| NLS1       | 503      | 508    | None reported                                       | domain location description (Chen et al., 1996, Thakur et al., 1997). |
| NLS2       | 607      | 614    | None reported                                       | domain location description (Chen et al., 1996, Thakur et al., 1997). |
| NLS3       | 651      | 656    | None reported                                       | domain location description (Chen et al., 1996). |
| COILED-COIL| 1391     | 1424   | None reported                                       | domain location description (Hu et al., 2000). |
Digestion data indicate aa1860-1863 are dispensable based on susceptibility to digestion (Lee et al., 2010), while pathogenic variant data indicate that 1855-1862 are dispensable (Hayes et al., 2000).  
Position 1854 is implicated as clinically important by the observation that Y1853X is a recognized high-risk pathogenic variant.  
Insertion variant 5682delAins6 listed on BIC (single submission; http://research.nhgri.nih.gov/bic/) could support revision of BRCT boundary to aa1855, but the nomenclature is not fully described and there is no clinical evidence to support the assertion as pathogenic.  
These data combined indicate that position 1854 or 1855 is the C-terminal border of the BRCT/BRCA1 relevant to clinical interpretation of sequence variants in exon 24 of BRCA1. That is, a variant predicted to disrupt expression of protein sequence only upstream of position 1855 would not be considered clinically important.  
In-frame deletions that remove any of the listed amino acids would be considered clinically important. |

⁸ Missense substitutions in denoted functional domains that are designated as class 5 pathogenic based on multifactorial likelihood posterior probability of pathogenicity > 0.99 (listed in http://hci-exlovd.hci.utah.edu, or individual references noted), and for which there is no/little effect on mRNA transcript profile.
Note: BRCA1 c.4484G>T R1495M located outside of the denoted functional domains has been classified as class 5 pathogenic (Lindor et al., 2012), but allele-specific assays (Colombo et al., 2013, Houdayer et al., 2012) indicate that this variant leads to complete loss of function of the full length transcript due to a splicing aberration (Δexon 14), and should thus be denoted as mRNA change: r.[4358_4484del], predicted protein change: p.(Ala1453GlyfsX10), stop codon at 1462.
Table 4: BRCA2 functional domains and relevance for interpreting the clinical significance of sequence variants – catalogue of clinically important functional domains and amino acid residues

<table>
<thead>
<tr>
<th>Region</th>
<th>AA start</th>
<th>AA end</th>
<th>AA alterations with demonstrated clinical importance</th>
<th>References and summary interpretation^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>PALB2 Binding</td>
<td>10</td>
<td>40</td>
<td>None reported</td>
<td>Domain location description (Oliver et al., 2009, Xia et al., 2006)</td>
</tr>
<tr>
<td>BRC-1</td>
<td>1002</td>
<td>1035</td>
<td>None reported</td>
<td><a href="http://www.ncbi.nlm.nih.gov/protein/NP_000050.2">http://www.ncbi.nlm.nih.gov/protein/NP_000050.2</a></td>
</tr>
<tr>
<td>BRC-2</td>
<td>1212</td>
<td>1245</td>
<td>None reported</td>
<td><a href="http://www.ncbi.nlm.nih.gov/protein/NP_000050.2">http://www.ncbi.nlm.nih.gov/protein/NP_000050.2</a></td>
</tr>
<tr>
<td>BRC-3</td>
<td>1421</td>
<td>1454</td>
<td>None reported</td>
<td><a href="http://www.ncbi.nlm.nih.gov/protein/NP_000050.2">http://www.ncbi.nlm.nih.gov/protein/NP_000050.2</a></td>
</tr>
<tr>
<td>BRC-4</td>
<td>1517</td>
<td>1550</td>
<td>None reported</td>
<td><a href="http://www.ncbi.nlm.nih.gov/protein/NP_000050.2">http://www.ncbi.nlm.nih.gov/protein/NP_000050.2</a></td>
</tr>
<tr>
<td>BRC-5</td>
<td>1664</td>
<td>1696</td>
<td>None reported</td>
<td><a href="http://www.ncbi.nlm.nih.gov/protein/NP_000050.2">http://www.ncbi.nlm.nih.gov/protein/NP_000050.2</a></td>
</tr>
<tr>
<td>BRC-6</td>
<td>1837</td>
<td>1870</td>
<td>None reported</td>
<td><a href="http://www.ncbi.nlm.nih.gov/protein/NP_000050.2">http://www.ncbi.nlm.nih.gov/protein/NP_000050.2</a></td>
</tr>
<tr>
<td>BRC-8</td>
<td>2051</td>
<td>2084</td>
<td>None reported</td>
<td><a href="http://www.ncbi.nlm.nih.gov/protein/NP_000050.2">http://www.ncbi.nlm.nih.gov/protein/NP_000050.2</a></td>
</tr>
<tr>
<td>NLS1</td>
<td>3263</td>
<td>3269</td>
<td>None reported</td>
<td>Domain location description (Guidugli et al., 2014)</td>
</tr>
<tr>
<td>BRC-9 or TR2</td>
<td>3265</td>
<td>3330</td>
<td>None reported</td>
<td>Note, although this fragment is reported to bind RAD51-DNA filaments, there is no sequence conservation with the BRC repeats located between aa1002 and aa2014. Domain boundaries derived from x-ray crystallography data are aa3265-3330. (Esashi et al., 2005, Esashi et al., 2007).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Case-control and frequency data indicate that K3326X does not confer a high risk of cancer (OR 1.3-1.5, dependent on breast or ovarian cancer subtype, Meeks et al. submitted), demonstrating that residues downstream of 3327 are dispensable. Position 3308 is implicated as clinically important by the observation that a truncating variant c.9924C&gt;G Y3308X is recognized as a high-risk pathogenic variant with known functional relevance ((Kuznetsov et al., 2008); Bayes score 1122:1 from a single large kConFab family,</td>
</tr>
</tbody>
</table>
Truncating variants at position 3309 and 3312 have been reported, but at present there is no clinical evidence to support the assertion of these variants as pathogenic. These data combined suggest that the C-terminal border of the BRC-9 relevant to the clinical interpretation of sequence variants in exon 27 of BRCA2 lies between 3309 and 3325. That is, a variant predicted to disrupt expression only of protein sequence downstream of position 3325 would not be considered clinically important.

<table>
<thead>
<tr>
<th>NLS2</th>
<th>3381</th>
<th>3385</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Domain location description (Guidugli et al., 2014)
This domain is not considered clinically relevant since it lies downstream of position 3325.

Missense substitutions in denoted functional domains that are designated as class 5 pathogenic based on multifactorial likelihood posterior probability of pathogenicity > 0.99, and for which there is no/little effect on mRNA transcript profile (Splicing aberrations are reported for BRCA2 C.7988A>T E2663V and c.8168A>G D2723G (Walker et al., 2010), but these did not lead to complete loss of function of the full length transcript), and missense alterations showed abrogation of functional activity using multiple assays (Walker et al., 2010).

Note: BRCA2 c.7976G>A R2659K has been classified as class 5 pathogenic (Lindor et al., 2012), but is reported to lead to loss of function of the full length transcript due to a splicing aberration (Δ exon 17). Pending allele-specific assays to confirm complete loss of function due to aberrant mRNA splicing, this variant may be denoted as mRNA change: r.[7806_7976del]; predicted protein change p.(Ala2603_Arg2659del).
Table 5: BRCA1 and BRCA2 exon boundary variants predicted or known to lead to naturally occurring in-frame RNA isoforms that may rescue gene functionality. Variants at these positions should be considered class 3 (uncertain) unless proven otherwise.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alternative Splicing Event</th>
<th>Variants Implicated</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δ8p</td>
<td>c.442-1 (IVS7-1)</td>
<td><em>BRCA1</em> exon 8 acceptor site is an experimentally validated tandem acceptor site (NAGNAG) subject to alternative splicing (Colombo et al., 2014). c.442-1,-2 variants are predicted to inactivate the 5' acceptor site, but not the 3' acceptor site, thus producing Δ8p transcripts.</td>
</tr>
<tr>
<td></td>
<td>Δ9,10</td>
<td>c.548-1 (IVS8-1)</td>
<td>Carriers of these variants are predicted to produce normal (or increased) levels of <em>BRCA1</em> Δ(9,10), a major in-frame alternative splicing event (Colombo et al., 2014).</td>
</tr>
<tr>
<td>BRCA1</td>
<td></td>
<td>c.548-2 (IVS8-2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.593 to non-G</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.593+1 (IVS9+1)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>c.593+2 (IVS9+2)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>c.594-1 (IVS9-1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.594-2 (IVS9-2)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.670 to non-G</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.670+1 (IVS10+1)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>c.670+2 (IVS10+2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Δ13p</td>
<td>c.4186-1 (IVS12-1)</td>
<td><em>BRCA1</em> exon 13 acceptor site is an experimentally validated tandem acceptor site (NAGNAG) subject to alternative splicing (Colombo et al., 2014). c.4186-1,-2 variants are predicted to inactivate the 5' acceptor site, but not the 3' acceptor site, thus producing Δ13p transcripts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.4186-2 (IVS12-2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Δ14p</td>
<td>c.4358-1 (IVS13-1)</td>
<td><em>BRCA1</em> exon 14 acceptor site is an experimentally validated tandem acceptor site (NAGNAG) subject to alternative splicing (Colombo et al., 2014). c.4358-1,-2 variants are predicted to inactivate the 5' acceptor site, but not the 3' acceptor site, thus producing Δ14p transcripts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.4358-2 (IVS13-2)</td>
<td></td>
</tr>
<tr>
<td>BRCA2</td>
<td>Δ12</td>
<td>c.6842-1 (IVS11-1)</td>
<td>Carriers of these variants are predicted to produce exon12 skipping. BRCA2 Δ12 is a naturally occurring in-frame splicing event (ENIGMA Splicing Working group, unpublished data). BRCA2 exon12 is functionally redundant (Li et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.6842-2 (IVS11-2)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>6937 to non-G</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6937+1 (IVS12+1)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>6937+2 (IVS12+2)</td>
<td></td>
</tr>
</tbody>
</table>

*BRCA1 c.594-2A>C has recently been reported to demonstrate clinical characteristics inconsistent with a high risk of cancer expected for a pathogenic *BRCA1* variant (Rosenthal et al., 2015), findings that are supported by unpublished genetic and pathology data from ENIGMA.
References:


**Additional Criteria to be circulated to membership for discussion/approval and inclusion in the ENIGMA BRCA classification scheme.**

**Class 2 – Likely not pathogenic/little clinical significance**

- Synonymous substitution variant with low bioinformatic likelihood to disrupt normal splicing, with combined prior probability of pathogenicity of ≤2% from clinically calibrated bioinformatic analyses.

<table>
<thead>
<tr>
<th>Class</th>
<th>Criterion</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 2: Likely not pathogenic or of little clinical significance</td>
<td>Synonymous substitution with low bioinformatic likelihood to disrupt normal splicing, determined to have prior probability of pathogenicity ≤2% from clinically calibrated bioinformatic analyses.</td>
<td>A silent substitution variant that is not bioinformatically predicted to effect mRNA function is extremely unlikely to result in clinical consequences equivalent to a high-risk pathogenic variant, as indicated by prior probability of 0.02 for variants in this stratum from analysis calibrating bioinformatic predictions of variant effect on splicing against clinical information (Vallee et al, submitted)).</td>
</tr>
</tbody>
</table>

- A variant demonstrating all these features:
  - ✓ Exonic variant encoding a missense substitution OR exonic variant resulting in a small in-frame insertion/deletion OR intronic variant,
  - ✓ Low bioinformatic likelihood to disrupt normal splicing and protein structure/function, with combined prior probability of pathogenicity of ≤2% from clinically calibrated bioinformatic analyses.
  - ✓ Allele frequency ≥0.001 and <0.01 in large outbred control reference groups

<table>
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<tbody>
<tr>
<td>Class 2: Likely not pathogenic or of little clinical significance</td>
<td>Exonic variant encoding a missense substitution, or exonic variant resulting in a small in-frame insertion/deletion, or intronic variant AND Low bioinformatic likelihood to disrupt normal splicing and protein structure/function, with combined prior probability of pathogenicity of ≤2% from clinically calibrated bioinformatic analyses. AND Allele frequency ≥0.001 and &lt;0.01 in large outbred control reference groups.</td>
<td>A variant that is not bioinformatically predicted to affect mRNA or protein structure/function is highly unlikely to result in clinical consequences equivalent to a high-risk pathogenic variant, as indicated by prior probability of 0.02 for variants in this stratum from analysis calibrating bioinformatic predictions of variant effect on mRNA splicing or protein structure/function against clinical information (Vallee et al, submitted)). AND Further support for no effect on function is provided by the observation that allele frequency in reference groups is greater than would be expected for a single non-founder variant leading to a dominant disorder.</td>
</tr>
</tbody>
</table>
Class 1 – Not pathogenic/low clinical significance

- *BRCA2* coding sequence variant that encodes a premature termination codon at position Lys3326 or downstream.

<table>
<thead>
<tr>
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<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 1: not pathogenic or of no clinical significance</td>
<td><em>BRCA2</em> truncating variant at position Lys3326 or downstream</td>
<td>Lys3326X is a common polymorphism that is not associated with a high risk of cancer. (OR 1.3-1.5, dependent on breast or ovarian cancer subtype, Meeks et al, submitted) Variants leading to loss of protein residues from position 3326 to the C-terminus will similarly not be associated with high risk of cancer.</td>
</tr>
</tbody>
</table>
Additional points for committee to discuss, for potential development and circulation to membership:

What LR scores for variants for which there is no adequate prior estimated, Eg UTR variants, deep intronic that come out of future sequencing. If we are very conservative and

- if we assume C0 prior 0.02, we need LR 950 to take a variant to class 4, and LR 1000 to take a variant to class 5 (Note LR 1000:1 was the original definition for class 5 pathogenic in Goldgar et al 2004)
- if we assume C65 prior 0.81, then we need LR 0.01 to take a variant to class 2, and LR 0.00002 to take a variant to class 1. That is probably too stringent (much more stringent than the cutpoints in Goldgar et al 2004), and does not take into account the overall low prior for variants outside of functional domains etc.

Copy number deletions/duplications that might be considered class 4 (as opposed to class 3)

Note that initiation codon changes may lead to rescue initiation and functional and clinical studies are required to resolve them (reference Parsons et al).

Define a specific term/class eg 3b/4b for proven intermediate risk variants for which it is possible to determine distinct clinical management protocols.

Document verified regulatory element/regions (using supporting data from Melissa Brown’s group), and develop qualitative criteria to address classification of this type of variant.

Provide advice as to which variants may be easily resolved by gathering additional information as per published recommendations (Plon et al., 2008), for further research segregation testing in family members eg posterior 0.8 or 0.1? Confirm Dutch recommendations.

If necessary, define the “clinical” difference between “little clinical significance (falling under class 2) and “no clinical significance (class 1).