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Design and validation of a next generation sequencing assay for hereditary breast and ovarian cancer

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Hereditary breast and ovarian cancer syndrome, caused by a germline deleterious variant in the *BRCA1* or *BRCA2* genes, is characterized by an increased risk for breast, ovarian, pancreatic and other cancers. Identification of those who have a *BRCA1/2* mutation is important so that they can take advantage of genetic counseling, screening, and potentially life-saving prevention strategies. We describe the design and analytic validation of the Counsyl Inherited Cancer Screen, a next-generation-sequencing-based test to detect pathogenic variation in the *BRCA1* and *BRCA2* genes. We demonstrate that the test is capable of detecting single-nucleotide variants (SNVs), short insertions and deletions (indels), and copy-number variants (CNVs, also known as large rearrangements) with zero errors over a 96-sample validation set consisting of samples from cell lines and deidentified patient samples, including the well-characterized NA12878 sample from HapMap/1000 Genomes.

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2 **ovarian cancer**

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10

11

12 **ABSTRACT**

13

14 Hereditary breast and ovarian cancer syndrome, caused by a germline deleterious variant in the
15 *BRCA1* or *BRCA2* genes, is characterized by an increased risk for breast, ovarian, pancreatic and
16 other cancers. Identification of those who have a *BRCA1/2* mutation is important so that they can
17 take advantage of genetic counseling, screening, and potentially life-saving prevention strategies.
18 We describe the design and analytic validation of the Counsyl Inherited Cancer Screen, a next-
19 generation-sequencing-based test to detect pathogenic variation in the *BRCA1* and *BRCA2* genes.
20 We demonstrate that the test is capable of detecting single-nucleotide variants (SNVs), short
21 insertions and deletions (indels), and copy-number variants (CNVs, also known as large
22 rearrangements) with zero errors over a 96-sample validation set consisting of samples from cell
23 lines and deidentified patient samples, including the well-characterized NA12878 sample from
24 HapMap/1000 Genomes.

25

26

27 **INTRODUCTION**

28

29 **Clinical Scenario and Public Health Importance**

30 Hereditary breast and ovarian cancer syndrome (HBOC) is associated with mutations in tumor
31 suppressor genes *BRCA1* and *BRCA2*. Genetic analysis for individuals who are at risk for HBOC
32 has become widely accepted. Several professional organizations and expert panels, including the
33 National Comprehensive Cancer Network (NCCN) (National Comprehensive Cancer Network,
34 2014), the American Society of Clinical Oncology (ASCO) (Robson et al., 2010), the American
35 Society of Human Genetics (ASHG) (Statement of the American Society of Human Genetics on
36 genetic testing for breast and ovarian cancer predisposition, 1994), the American College of
37 Medical Genetics and Genomics (ACMG) (Hampel et al., 2015), the National Society of Genetic
38 Counselors (NSGC) (Hampel et al., 2015), the U.S. Preventive Services Task Force (USPSTF)
39 (Nelson et al., 2014), the Society of Gynecologic Oncologists (SGO) (Lancaster et al., 2007), and
40 the European Society for Medical Oncology (ESMO) (Balmaña et al., 2011) have developed
41 clinical criteria and practice guidelines for identifying individuals who may benefit from *BRCA1*
42 or *BRCA2* mutation testing. A selection of these is summarized below.

43

44 According to the NCCN guidelines, personalized risk assessment, genetic counseling, and often
45 *BRCA1/2* testing and management are recommended for individuals with a significant personal

46 and/or family history of breast, ovarian, pancreatic and/or prostate cancer (National
47 Comprehensive Cancer Network, 2014).

48
49 ASCO recommends genetic testing when there is personal or family history suggestive of genetic
50 cancer susceptibility, the test can be adequately interpreted, and the results will aid in diagnosis
51 or medical management of the patient or family member at hereditary risk for cancer. It also
52 recommends genetic testing only when pre-test and post-test counseling are included (Robson et
53 al., 2010).

54
55 The USPSTF guidelines recommend that primary care providers prescreen women with a family
56 history of breast or other cancers to identify individuals at an increased risk for germline
57 mutations in the *BRCA1* and *BRCA2* genes. Women with positive screening results should
58 receive genetic counseling and, if indicated after counseling, *BRCA* testing (Grade B) (Nelson
59 et al., 2014). The USPSTF recommends against routine genetic counseling or *BRCA* testing for
60 women whose family history is not associated with an increased risk for mutations in the
61 *BRCA1* or *BRCA2* genes (Grade D) (Nelson et al., 2014).

62
63 SGO recommends genetic risk assessment for individuals with a personal risk of more than
64 approximately 20% to 25% for an inherited predisposition to cancer and states that it may be
65 helpful for patients with more than approximately 5% to 10% risk. Genetic testing for cancer
66 predisposition requires informed consent that should encompass pre-test education and
67 counseling about the risks, benefits, and limitations of testing, including the implications of both
68 positive and negative genetic test results (Lancaster et al., 2007).

69 The ESMO clinical practice guidelines indicate that *BRCA* testing criteria may differ between
70 countries based on mutation prevalence (Balmaña et al., 2011). Widely accepted clinical criteria
71 for referral include: three or more breast and/or ovarian cancer cases, at least one <50 years; two
72 breast cancer cases <40 years; male breast cancer and ovarian cancer or early onset female breast
73 cancer; Ashkenazi Jew with breast cancer of <60 years; young onset bilateral breast cancer; and
74 breast and ovarian cancer in the same patient. In some countries, the criterion for testing is based
75 on an *a priori* 10–20% probability of finding a mutation based on predictive models such as
76 BRCAPRO, BOADICEA or Manchester Score (Fischer et al., 2013; Kast et al., 2014). The
77 performance of the models can vary in specific ethnic groups. For instance, the BRCAPRO
78 model appeared to best fit a series of French Canadian families (Oros et al., 2006).

79
80 As suggested by various guidelines, individuals identified with *BRCA1* or *BRCA2* mutation are
81 at significantly increased risk for breast, ovarian, prostate, pancreatic and possibly other cancers:
82 a 12% general population risk for breast cancer rises to 50-80% for *BRCA1* mutation carriers or
83 40-70% for *BRCA2* mutation carriers (Petrucci, Daly & Feldman, 2015). Recommended risk-
84 reducing options include increased screening, chemoprevention and/or prophylactic surgery
85 (Balmaña et al., 2011; Hampel et al., 2015; Lancaster et al., 2007; National Comprehensive
86 Cancer Network, 2014; Nelson et al., 2014; Robson et al., 2010; Statement of the American
87 Society of Human Genetics on genetic testing for breast and ovarian cancer predisposition,
88 1994). Table 1 summarizes these options and their effect on cancer risks.

89
90 Genetic testing for *BRCA* mutation status has the potential to offer multiple benefits, including:
91 identification of high-risk individuals who will benefit from the initiation of cancer risk

92 management; identification of noncarriers in families with a known mutation, who do not need to
93 have rigorous cancer screening; and perhaps relief of anxiety through increasing the
94 understanding of medical options. However, 20-73% of mutation carriers may not be identified
95 with current guidelines (Alsop et al., 2012; Brozek et al., 2012; Frank et al., 2002; Kang et al.,
96 2014; Norquist et al., 2013) or only meet current guidelines once they are diagnosed with
97 ovarian cancer or early onset breast cancer, resulting in some researchers to call for more
98 inclusive guidelines or even population screening (Finch et al., 2014; Gabai-Kapara et al., 2014;
99 Metcalfe et al., 2013). It is also important to consider limitations and pitfalls of *BRCA* mutation
100 testing, including the possibility of uncertain or uninformative results, potential for psychological
101 distress, and effect on family members.

102

103

104 MATERIALS AND METHODS

105

106 Ethics Statement

107 The study was approved by Western Institutional Review Board (IRB number 1145639) and
108 complied with the Health Insurance Portability and Accountability Act (HIPAA). The
109 information associated with patient samples was de-identified in accordance with the HIPAA
110 Privacy Rule. A waiver of informed consent was requested and approved by the IRB.

111

112 Test Description

113 The reportable range of the test is all coding exons of *BRCA1* and *BRCA2*, 20 bp into the introns
114 from intron/exon junctions, and selected intronic regions where pathogenic variants have been
115 reported in the literature. DNA from a patient's blood or saliva sample is isolated and then
116 fragmented by sonication. The fragmented DNA is converted to an adapter-ligated sequencing
117 library; samples are multiplexed and identified by molecular barcodes. Hybrid capture-based
118 enrichment for *BRCA1/2* targeted regions is performed on these multiplexed samples, after
119 which next generation sequencing of the selected targets is performed with sequencing-by-
120 synthesis on the Illumina HiSeq 2500 instrument. All SNPs, insertions/deletions, and large
121 deletions/duplication within the reportable range are analyzed and classified by the method
122 described in the section "Variant Classification".

123 All target nucleotides are required to be covered with a minimum depth of 50 reads. Sequence
124 reads are aligned to the hg19 human reference genome using the BWA-MEM algorithm (Li,
125 2013), which also trims sequencing adapters. Automated statistical analysis is used to identify
126 and genotype single-nucleotide variants (SNVs) and short insertions and deletions (indels)
127 following methods in GATK and FreeBayes (Garrison & Marth, 2012; McKenna et al., 2010).
128 The calling algorithm for copy number variants (insertions or deletions longer than 100bp) is
129 described below. Ancillary quality-control metrics, including fraction of sample contamination,
130 library complexity, and bias, are computed on the final output and used to exclude and re-run
131 failed samples. All reportable calls are reviewed by licensed clinical laboratory personnel.

132

133 CNV Calling Algorithm

134 Reads are extracted from the Illumina instrument output, and aligned to the human reference
135 genome using BWA.

136 Analysis is performed on a per-lane basis. A matrix of counts of reads for each putative CNV in
137 each sample is created. Reads for all probes targeting the same CNV region are added together.

138 Let $d_{i,j}$ be a matrix representing the number of reads observed from the i th sample for the j th
139 variant.

140

141 This matrix must be normalized. To protect against normalization issues due to individual
142 samples with very large CNVs (such as a whole-gene deletion), we generate a normalization
143 matrix $n_{i,j}$ by removing the highest variance probes from the total data set D via the invariant set
144 method described in (Li & Hung Wong, 2001).

145 The data matrix d is then normalized in two steps:

146

$$147 \quad d'_{i,j} = d_{i,j} / \text{mean}(n_{i,j} \text{ for all } j)$$

$$148 \quad d''_{i,j} = d'_{i,j} / \text{mean}(n_{i,j} \text{ for all } i)$$

149

150 For each putative CNV j in sample i , a hypothetical copy number and corresponding Z-score is
151 computed:

152

$$153 \quad c_{i,j} = 2 * d''_{i,j}$$

$$154 \quad z_{i,j} = (d''_{i,j} - \text{mean}(d''_{i,j} \text{ for all } i)) / \text{stdev}(d''_{i,j} \text{ for all } i)$$

155

156 A CNV call is considered confidently non-reference if $\text{abs}(z) \geq 4$ and the estimate c is <1.2 or
157 >2.8 .

158

159 **Variant Classification**

160 We have designed custom curation software to compile information from a wide range of
161 sources. For each variant, information is collected from the following: entries in public databases
162 such as ClinVar (Landrum et al., 2014), the Human Gene Mutation Database (HGMD) (Stenson
163 et al., 2003), and selected locus-specific databases (e.g., BIC (the Breast Cancer Information
164 Core) (Szabo et al, 2000) and UMD-BRCA1/2 (Caputo et al., 2012)); population-specific
165 frequencies in ESP (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP),2013),
166 1000 Genomes (Abecasis et al., 2012), and internal data; results of computational algorithms
167 based on evolutionary conservation, structural modeling, and splice site predictors. A curation
168 team also reviews articles in the medical literature that mention each variant and collects
169 additional information from them such as numbers and clinical characteristics of cases and
170 controls the variant was seen in and the results of functional assays. All of this information is
171 analyzed and variants are categorized according to ACMG Standards and Guidelines for the
172 Interpretation of Sequence Variants (American College of Medical Genetics and Genomics,
173 2015) to arrive at a final classification of benign, deleterious, or unknown. All variants that are
174 known or predicted to be deleterious are reported; patients and providers have an option to have
175 variants of uncertain significance reported as well. Final variant classifications are regularly
176 uploaded to ClinVar.

177

178 **RESULTS**

179

180 **Evidence Overview**

181 Data to calculate the validation metrics were compiled by testing three classes of samples:
182 deidentified blood samples (N=25), deidentified paired blood and saliva samples (3 pairs),
183 genomic DNA reference materials obtained from Coriell (N=56), and deidentified DNA samples
184 provided by external laboratories (N=15) (Table 2).

185
186 Sequence data of 41 Coriell samples was compared to reference data obtained from the 1000
187 Genomes project and sequence data for NA12878 (a Coriell sample) was compared to high-
188 quality reference data published by Illumina, Inc. (<http://www.illumina.com/platinumgenomes/>).
189 Sequence data of 15 samples from the BIC *BRCA1/BRCA2* Mutation Panel, available from
190 Coriell, was analyzed to confirm the detection of documented variants in *BRCA1/BRCA2*. Data
191 for copy number calls was compared to calls provided by reference labs, when available, and
192 MLPA assays otherwise, on 56 samples: 15 samples from reference labs; 15 samples from the
193 BIC *BRCA1/BRCA2* Mutation Panel available from Coriell; 25 random blood samples; and
194 NA12878. Sequence data and CNV calls for the saliva samples were compared to the respective
195 paired blood sample.

196 197 **Analytic Validity**

198 The results of the Counsyl Inherited Cancer Screen validation are presented below in Tables 3
199 and 4. For SNPs and small insertions/deletions, 536 true positive calls, 12,920 true negative calls
200 and no false positive or false negative calls were observed from the analysis of 57 samples. For
201 copy number variants, 60 true positive calls, 2,736 true negative calls and no false positive or
202 false negative calls were observed from the analysis of 40 analyzed samples. The accuracy,
203 sensitivity and specificity are therefore all 1.0 for SNPs, small insertions/deletions, and copy
204 number variants. The results from paired blood and saliva samples (n=3) were 100% concordant.

205 However, there are some limitations to the study presented here. The validation was limited to
206 mostly blood-derived and Coriell cell line samples and included only three saliva samples.

207 208 **DISCUSSION**

209 210 **Clinical Validity And Utility**

211 Deleterious mutations in the *BRCA* genes are known to be associated with increased risk for
212 breast, ovarian and other cancers. For women, the risk of developing breast cancer by age 70 is
213 approximately 60-70% for *BRCA1* and 45–55% for *BRCA2* mutation carriers. The cumulative
214 ovarian cancer risk by age 70 (including fallopian tube and primary peritoneal carcinomas) is
215 40% for *BRCA1* and 20% for *BRCA2* mutation carriers respectively (Antoniou et al., 2003; Chen
216 & Parmigiani, 2007; King, Marks, & Mandell, 2003). Identification of those who have
217 a *BRCA1/2* mutation is important so that they can take advantage of genetic counseling,
218 screening, and potentially life-saving prevention strategies.

219
220 The optimal cancer risk management approach for *BRCA1/2* mutation carriers continues to
221 evolve. For breast cancer risk management, current options include intensive screening,
222 chemoprevention, and risk-reducing surgery (National Comprehensive Cancer Network, 2014;
223 Nelson et al., 2015; Petrucelli, Daly & Feldman, 2015). Prophylactic bilateral mastectomies
224 (PBM) showed an 85%–100% reduction in breast cancer risk in retrospective and prospective
225 studies (Hartmann et al., 1999; Hartmann et al., 2001; Meijers-Heijboer et al., 2001; Rebbeck et

226 al., 2004). The Prevention and Observation of Surgical Endpoints study is the largest,
227 prospective cohort study performed to estimate the risk reduction benefit of PBM in women
228 with *BRCA* mutations (Rebbeck et al., 2004). Results of this trial supported a 90% reduction in
229 risk with breast cancer being diagnosed in 2% of *BRCA* carriers undergoing PBM compared to
230 49% of carriers who did not. Risk reduction was increased to 95% in women undergoing prior or
231 concurrent prophylactic bilateral oophorectomy (Rebbeck et al., 2004).

232

233 Intensive screening for early detection of breast cancer is an alternative approach for a woman
234 who does not desire surgery. Screening guidelines are available from numerous organizations,
235 including the NCCN (National Comprehensive Cancer Network, 2014) and USPSTF (Nelson et
236 al., 2014). The addition of breast magnetic resonance imaging (MRI) to screening
237 mammography has been shown to significantly increase sensitivity and lead to earlier detection
238 of breast cancers (Hagen et al., 2007; Kriege et al., 2004; Kuhl et al., 2005; Lehman et al., 2005;
239 Rijnsburger et al., 2010; Sardanelli et al., 2011; Warner et al., 2004; Warner et al., 2008).
240 However, the impact of any surveillance strategy (including MRI) on breast cancer mortality has
241 not been established.

242

243 Chemoprevention, specifically prophylactic use of tamoxifen, is recommended
244 for *BRCA1/2* carriers. The randomized, double-blind, Breast Cancer Prevention Trial (BCPT)
245 demonstrated that tamoxifen reduced breast cancer incidence among healthy *BRCA2* carriers by
246 62%. In contrast, tamoxifen use beginning at age 35 years or older did not reduce breast cancer
247 incidence among healthy women with inherited *BRCA1* mutations (King et al., 2001). A
248 differential effect of tamoxifen in *BRCA2* as compared to *BRCA1* mutation carriers may be
249 attributed to estrogen receptor (ER) status of *BRCA1*- and *BRCA2*-associated tumors. Tamoxifen
250 might be expected to have an impact only against ER-positive tumors, and *BRCA2*-associated
251 tumors have a greater likelihood than *BRCA1*-associated tumors of being ER-positive. However,
252 in other settings, tamoxifen has shown benefit for both *BRCA1*- and *BRCA2*-associated tumors,
253 irrespective of ER-status (Foulkes et al., 2002; Gronwald et al., 2006; Narod et al., 2000).

254

255 Ovarian cancer risk management options are more limited, with no proven effective early
256 detection method available. Risk reducing salpingo-oophorectomy (RRSO) has been shown to
257 reduce the risk of developing breast cancer by approximately 50%, with higher benefits
258 associated with earlier age at surgery, and that of ovarian/fallopian tube cancer by approximately
259 80% to 90% (Domchek et al., 2010; Finch et al., 2014; Haber, 2002; Kauff et al., 2002; Rebbeck
260 et al., 2002). In addition, one study showed a 69% reduction in all-cause mortality associated
261 with RRSO among *BRCA1/2* mutation carriers (Domchek et al., 2010).

262

263 For chemoprevention of ovarian cancer, oral contraceptive use has been associated with a
264 decrease in ovarian cancer risk. A meta-analysis of 18 studies, which were either case-control or
265 retrospective cohort studies, of oral contraceptive use in *BRCA1* and *BRCA2* mutation carriers
266 and included 2855 breast cancer cases and 1503 ovarian cancer cases, demonstrated a
267 significantly reduced risk of ovarian cancer [summary relative risk (SRR), 0.50, 95% CI 0.33–
268 0.75]. For each additional 10 years of oral contraceptive use, there was a significantly reduced
269 ovarian cancer risk (SRR 0.64, 95% CI 0.53–0.78) (Iodice et al., 2010).

270

271 In conclusion, *BRCA1* and *BRCA2* are the most prevalent high-penetrance breast/ovarian cancer
272 susceptibility genes identified to date. It is important to identify individuals who have mutations
273 in these genes so that they can benefit from surveillance and preventative options, primarily for
274 breast and ovarian cancers.

275

276 **Competing Interests**

277 All authors are employees and shareholders of Counsyl, Inc.

278

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542 **Tables**

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545 **Table 1.** *BRCA1* and *BRCA2* cancer risk management options and effectiveness

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Risk Management Options	Effectiveness
Prophylactic mastectomy	Up to 90% reduction in breast cancer risk (Hartmann et al., 1999; Meijers-Heijboer et al., 2001)
Prophylactic oophorectomy	~50% reduction in breast cancer risk when performed premenopausally (more pronounced effect for <i>BRCA2</i> mutation carriers compared to <i>BRCA1</i>) (Kauff et al., 2002; Kauff et al., 2008) Up to 96% reduction in ovarian cancer risk (Olopade & Artioli, 2004; Rebbeck et al., 2002; Rutter et al., 2003)
Tamoxifen	Up to 62% reduction in breast cancer risk among <i>BRCA2</i> mutation carriers Up to 50% contralateral breast cancer risk reduction in both <i>BRCA1</i> and <i>BRCA2</i> Limited data but appears to be more effective in <i>BRCA2</i> mutation carriers compared to <i>BRCA1</i> (King et al., 2001; Metcalfe et al., 2005; Narod et al., 2000)
Oral contraceptives	Up to 50% reduction in ovarian cancer risk (Iodice et al., 2010)
Breast MRI/mammogram	No risk reduction, but earlier detection (Kuhl et al., 2010; Sardanelli et al., 2011; Warner et al., 2011)
Ovarian cancer screening (transvaginal ultrasound and serum cancer antigen 125 (CA-125))	No risk reduction and no effect on cancer mortality (Buys et al., 2011; Clarke-Pearson, 2009)

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553 **Table 2.** Source of samples and reference data used in validation.

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Mutation Type	Test Samples	Reference Data
SNP/Indel	41 Coriell Cell Line Samples	1000 Genomes Project Exomes
	NA12878	Illumina Platinum Genome
	15 BIC samples	BIC reference data
CNV	15 reference lab samples	Reference lab results
	25 random anonymized samples	Orthogonal confirmation by MLPA

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562 **Table 3.** Performance of Counsyl Inherited Cancer Screen for SNPs and small
563 insertions/deletions.

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	Term or Formula	Value (95% Confidence Interval)
True positive calls	TP	536
True negative calls	TN	12920
False positive calls	FP	0
False negative calls	FN	0
Accuracy	$(TP + TN) / (TP + FP + TN + FN)$	1.0 (0.9997146 – 1.0)
Sensitivity	$TP / (TP + FN)$	1.0 (0.9928841 – 1.0)
Specificity	$TN / (TN + FP)$	1.0 (0.9997028 – 1.0)

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Table 4. Performance of Counsyl Inherited Cancer Screen for Copy Number Variants.

	Term or Formula	Value (95% Confidence Interval)
True positive calls	TP	60
True negative calls	TN	2736
False positive calls	FP	0
False negative calls	FN	0
Accuracy	$(TP + TN) / (TP + FP + TN + FN)$	1.0 (0.998628 – 1.0)
Sensitivity	$TP / (TP + FN)$	1.0 (0.9398281 – 1.0)
Specificity	$TN / (TN + FP)$	1.0 (0.9985979 – 1.0)

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