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BRUNO DA SILVEIRA CORRÊA

RECLASSIFICAÇÃO DE VARIANTES DE SENTIDO TROCADO NO GENE *BRCA1* ASSOCIADAS AO CÂNCER DE MAMA E OVÁRIO HEREDITÁRIOS

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RECLASSIFICAÇÃO DE VARIANTES DE SENTIDO TROCADO NO GENE *BRCA1* ASSOCIADAS AO CÂNCER DE MAMA E OVÁRIO HEREDITÁRIOS

Trabalho de Conclusão de Curso apresentado como requisito parcial para obtenção do título de Bacharel em Ciências Biológicas na Universidade Federal do Rio Grande do Sul

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SUMÁRIO

Lista de Abreviaturas

ACMG-AMP - American College of Medical Genetics and Genomics and Association for Molecular Pathology

CM - Câncer de mama

CO - Câncer de ovário

DNA DSB - Quebra de DNA de fita dupla

HBOC - Síndrome de predisposição ao câncer de mama e ovário hereditários

HDR- Reparo direcionado por homologia

HR - Recombinação homóloga

INCA - Instituto Nacional de Câncer José Alencar Gomes da Silva

MMEJ - Junção terminal mediada por microhomologia

NCCN - National Comprehensive Cancer Network

NGS - Sequenciamento de nova geração

NHEJ - Junção de extremidades não-homólogas

PARP - Poli (ADP-ribose) polimerase

SNV – Variante de nucleotídeo único

SSA - Anelamento de fita simples

VUS - Variante de significado incerto

Introdução Estendida:

Nos últimos anos, o número de mortes por câncer e sua incidência global aumentaram significativamente, com estimativas globais para o ano de 2020 indicando cerca de 19 milhões de novos casos diagnosticados e 10 milhões de mortes por câncer (BRAY et al., 2018). No Brasil, o Instituto Nacional de Câncer José Alencar Gomes da Silva (INCA) estima que nos anos de 2018 e 2019 sejam diagnosticados 1,2 milhões de novos casos. Além disso, previsões recentes sinalizam que o câncer se tornará a principal causa de morte entre adultos nos próximos anos (DAGENAIS et al., 2019).

As neoplasias resultam do acúmulo de alterações genéticas em células individuais, essencialmente causadas por mutações em genes "condutores" do processo de carcinogênese (*driver gene mutations*), bem como da atuação da seleção natural sobre o fenótipo resultante (STRATTON; CAMPBELL; FUTREAL, 2009; TOMASETTI; LI; VOGELSTEIN, 2017). Entretanto, a carcinogênese é multifatorial, sendo decorrente da interação complexa de fatores etiológicos genéticos e ambientais (WU et al., 2015)

A importância da hereditariedade na carcinogênese foi inicialmente demonstrada através de estudos familiares (e.g. estudos de gêmeos) e da identificação de genes relacionados à predisposição hereditária ao câncer (HARRIS et al., 2019; SUD; KINNERSLEY; HOULSTON, 2017; ESTEBAN-JURADO et al., 2014). Ademais, a herdabilidade geral do câncer é estimada em 33%, variando de acordo com o tipo tumoral e a população avaliada, com estudos de gêmeos na população fino-escandinava apresentando estimativas de herdabilidade variando desde 9%, para o câncer de cabeça e pescoço, até 57% para o câncer de próstata (MUCCI et al., 2016; HJELMBORG et al., 2014; POLDERMAN et al., 2015).

As síndromes de predisposição hereditária ao câncer representam, na maior parte dos casos, doenças mendelianas de herança autossômica dominante, ocasionadas principalmente por alterações germinativas de perda de função em genes supressores de tumor (RAHNER; STEINKE, 2008). Estima-se que cerca de 5-10% dos casos de câncer estejam estritamente ligados a estas síndromes (NAGY; SWEET; ENG, 2004). Além disso, neoplasias malignas resultantes costumam ocorrer em idade precoce, e são frequentemente multifocais. (VOGT et al., 2017). Presença de história familiar de câncer em múltiplas gerações e recorrência de determinados tipos tumorais são também indicativos de formas hereditárias de câncer (RILEY et al., 2011; SOUBA; WILMORE, 2001). As Síndromes de Li-Fraumeni, de Lynch e de

Predisposição Hereditária ao Câncer de Mama e Ovário (HBOC, do inglês, *Hereditary Breast and Ovarian Cancer Syndrome*) estão entre as mais estudadas (VALDEZ; NICHOLS; KESSERWAN, 2016; SEHGAL et al., 2014; KOBAYASHI et al, 2013).

Síndrome de Predisposição Hereditária ao Câncer de Mama e Ovário - HBOC

O câncer de mama (CM) é a forma mais frequente de câncer entre as mulheres, contando com cerca de 2,08 milhões de novos casos diagnosticados no ano de 2018 no mundo. Já para o câncer de ovário (CO) no mesmo ano foram registrados aproximadamente 295 mil novos casos (BRAY et al., 2018). Muitos são os fatores de risco para que estes tumores se desenvolvam, e entre estes estão: idade, sexo, etnia, densidade do tecido mamário, fatores hormonais, estilo de vida. No entanto, o maior fator preditivo para o seu desenvolvimento é a história familiar, relacionando-se a variantes germinativas patogênicas que são transmitidas ao longo das gerações (MCPHERSON; STEEL; DIXON, 2000; KAMIŃSKA et al., 2015).

HBOC é uma síndrome autossômica dominante com alta penetrância, caracterizando-se pelo aumento do risco de desenvolvimento de alguns cânceres, principalmente CM, em mulheres e homens, e CO em mulheres. Além destes tumores, indivíduos com esta síndrome apresentam risco aumentado para o desenvolvimento de câncer de próstata, pâncreas e melanoma, entre outros tumores (CASTRO et al., 2013; GINSBURG et al., 2010; IQBAL et al., 2012). Os portadores da síndrome tendem a apresentar as neoplasias mais cedo na vida, mais de um tumor sincrônico ou metacrônico e história familiar de tumores relacionados ao espectro de HBOC (KOBAYASHI et al, 2013).

A síndrome HBOC é responsável por uma porção significativa dos casos de CM e CO epitelial, representando entre 5-7% e 8-13% dos casos, respectivamente. A sua principal causa é a presença de variantes germinativas patogênicas ou provavelmente patogênicas nos genes *BRCA1* e *BRCA2* (LEVY-LAHAD; FRIEDMAN, 2007; ROY; CHUN; POWELL, 2011; LIU et al., 2012). Estima-se que mulheres portadoras de variantes patogênicas em *BRCA1* tenham cerca de 72% de chance de desenvolver CM até os 80 anos. Já para o CO, o risco cumulativo vital até os 80 anos é de 44% (KUCHENBAECKER et al., 2017).

Cerca de 5% de todos os casos de CM e 15% dos casos de CO podem ser atribuídos a mutações germinativas em *BRCA1* e *BRCA2* (ALSOP et al., 2012; CLAUS et al., 1996). Atualmente, mais de 25 outros genes já foram associados à predisposição hereditária ao CM e CO (NIELSEN; HANSEN; SØRENSEN, 2016). Dentre estes, destacam-se: *ATM*, *BARD1*,

BRIP1, *CDH1*, *CHEK2*, *MLH1*, *MSH2*, *NBN*, *NF1*, *PALB2*, *PTEN*, *RAD51C*, *RAD51D*, *STK11* e *TP53* (APOSTOLOU; FOSTIRA, 2013; NIELSEN; HANSEN; SØRENSEN, 2016; KOBAYASHI et al., 2013). No entanto, para uma parcela importante destes genes que emergem como associados ao fenótipo, ainda não é possível determinar riscos precisos de desenvolvimento de câncer em portadores de mutações, sendo este um grande desafio a ser superado no manejo clínico.

O diagnóstico da síndrome de HBOC é complexo devido à sua extensa heterogeneidade fenotípica e genotípica e sobreposição fenotípica com outras síndromes, sendo necessário diferenciar o quadro clínico do paciente em questão dentre as diferentes síndromes que se associam ao CM e CO hereditários (LYNCH et al., 2008). Existem diversos critérios clínicos que podem auxiliar o clínico nesta tarefa, mas a definição diagnóstica será dada pela análise molecular. Os critérios de indicação de análise molecular mais conhecidos são os critérios da Rede Nacional de Câncer dos Estados Unidos (*National Comprehensive Cancer Network* - NCCN), o mais recente deles publicado no ano de 2019 (NCCN guidelines 2019.3). Quando uma mutação germinativa em *BRCA1 ou BRCA2* é identificada em um indivíduo, outros familiares podem ser encaminhados para aconselhamento genético. Esse processo pode auxiliar na redução de riscos de câncer e diagnóstico precoces. Alternativas de manejo clínico como a salpingo-ooforectomia (remoção de ovários e trompas de falópio) bilateral podem reduzir os riscos de desenvolver CO, trompas de falópio e peritônio em até 80% e também impactar sobrevida global. Já a adenomastectomia bilateral (remoção das mamas) reduz o risco de desenvolver CM em até 90% (HARTMANN; LINDOR, 2016). As opções de tratamento dos tumores incluem cirurgia, radioterapia, quimioterapia e mais recentemente uso de inibidores da família de enzimas Poli Adenosina Difosfato Ribose Polimerase (PARP), dependendo de inúmeras variáveis histopatológicas e clínicas (NIELSEN; HANSEN; SØRENSEN, 2016) (Figura 1).

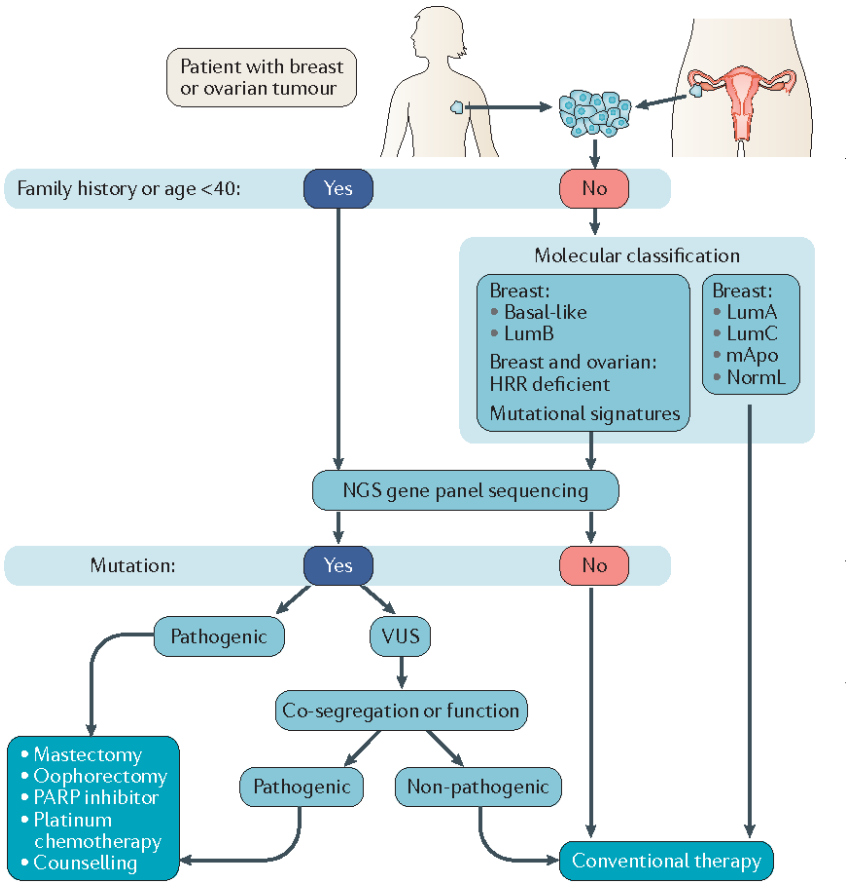

Figura 1 - Manejo clínico proposto para pacientes com CM e CO. Fonte: Nielsen, Hansen e Sørensen (2016).

Pacientes *BRCA* mutados, por apresentarem deficiência no reparo de DNA de fita dupla são sensíveis a agentes de ligação cruzada do DNA (*crosslinking),* que se ligam aos nucleotídeos e bloqueiam a síntese de DNA (BHATTACHARYYA et al., 2000). No contexto da medicina personalizada, a quimioterapia baseada em platina é uma alternativa eficaz no tratamento destes pacientes (TELLI et al., 2016; DANN et al., 2012). Além disso, tumores com BRCA1 e/ou BRCA2 não funcionais apresentam sensibilidade à inibição de PARP, à medida que esta família de enzimas também auxilia na recuperação de dano ao DNA e regula processos de reparo. Quando a via é inibida por diferentes drogas, intensifica a deficiência na recombinação homóloga, levando a morte celular (MACEDO; ALEMAR; ASHTON-PROLLA, 2019 (FARMER et al., 2005).

Desta forma, a identificação de variantes patogênicas em *BRCA* gradualmente se tornou essencial na rotina clínica, auxiliando não só em diversas etapas do manejo do paciente, mas como também de sua família (ALEMAR et al., 2017).

O gene *BRCA1*

BRCA1 é um gene supressor tumoral, localizado no braço longo do cromossomo 17, que possui 23 éxons e codifica a proteína BRCA1 (*breast cancer type 1 susceptibility protein*). Esta contém 1.863 aminoácidos dispostos em múltiplos domínios funcionais. Destacam-se o domínio proteico *Zinc RING-finger*, que interage com proteínas associadas, como BARD1, e os domínios BRCT, na porção C-terminal. Além disso, também são conhecidos domínios denominados *serine cluster* e *coiled-coil*. Embora não seja considerado altamente conservado ao longo da evolução em mamíferos, possui algumas regiões mais conservadas, pertencentes a seus domínios funcionais críticos (ROY; CHUN; POWELL, 2011; LOU et al., 2014). Quando combinado com outros supressores tumorais sensores de dano ao DNA e transdutores de sinais, BRCA1 forma o complexo BASC (*BRCA1-associated genome surveillance complex*), envolvido no reconhecimento e reparo de estruturas de DNA anômalas (Wang et al., 2000). O papel de *BRCA1* não é bem definido nas vias de junção de extremidades não homólogas clássica e alternativa (SAHA; DAVIS, 2016).

Por meio de suas interações complexas, *BRCA1* possui um papel central no reparo do DNA por recombinação homóloga, principalmente durante a replicação. Esta via é considerada a mais precisa no reparo às quebras bifilamentares de DNA (DSBR, do inglês *Double-Strand Break Repair*), pois geralmente utiliza a cromátide irmã como modelo para a reparação (Figura 2). Por conseguinte, defeitos no funcionamento da via levam a uma maior predisposição a erros no reparo de DSB, consequentemente gerando instabilidade genômica, mutações adicionais e alterações cromossomais (MOYNAHAN; JASIN, 2010; CHEN et al., 2018).

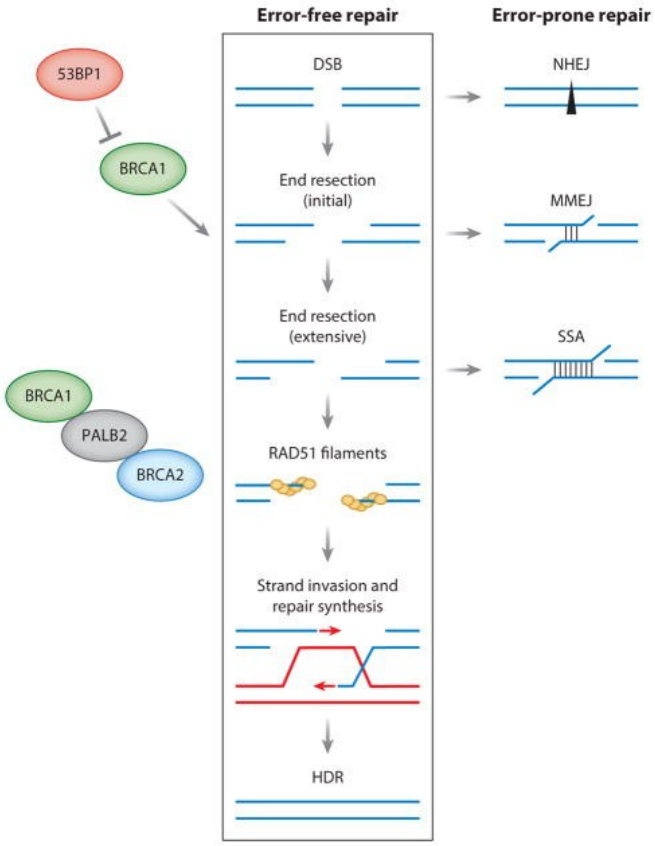

Figura 2 - Vias de reparo de quebras bifilamentares de DNA: A via de reparo direcionado por homologia (HDR) através da recombinação homóloga ocorre principalmente durante as fases S/G2 do ciclo celular. A proteína BRCA1 organiza o processo de clivagem em contraste com a proteína 53BP1. BRCA1 também interage com PALB2, que por sua vez recruta BRCA2. A BRCA2 então controla a formação de filamentos de RAD51 na extremidade de fita simples 3', então uma das extremidades 3' livre invade uma região homóloga do outro DNA, geralmente a cromátide irmã, preparando a síntese e reparo do DNA. Vias como o anelamento de fita simples (SSA) e a junção de extremidades não homólogas clássica (NHEJ) e alternativa (MMEJ) ocorrem, no entanto, podem gerar erros no processo, como pequenas inserções e deleções. A clivagem é determinante na definição de qual via de reparo ocorrerá. Fonte: Chen et al. (2018).

Recentemente, descobriu-se que BRCA1, BRCA2 e outras proteínas relacionadas ao CM e CO hereditários têm uma função importante nas vias de *checkpoint* de dano do DNA, atuando também na proteção da forquilha de replicação (SANTOS-PEREIRA; AGUILERA, 2015; KOLINJIVADI et al., 2017). Durante a replicação, estas proteínas previnem a degradação das fitas de DNA recém-sintetizadas por parte da enzima MRE11 nuclease, reparando o dano ao DNA e prevenindo que maiores danos ocorram (SCHLACHER et al., 2011).

As células com perda de função de *BRCA1* possuem grande prejuízo em suas funções de reparo, apresentando duplicação anormal do centrossomo, rearranjos cromossomais, defeitos na transcrição e outras anormalidades no ciclo celular. Além disso, variantes

patogênicas nos dois alelos do gene podem ser letais no período embrionário ou estarem relacionadas a displasias nas trompas de falópio, dependendo da localização da mutação (FRIEDENSON, 2007).

Classificação de variantes

A evolução das tecnologias de sequenciamento de DNA proporcionou avanços importantes no que diz respeito ao diagnóstico molecular de doenças genéticas e compreensão da variabilidade genética normal e patológica humana. Não apenas a quantidade de dados gerados aumentou significativamente, como também os desafios associados ao processo de classificação e interpretação clínica de variantes genéticas (STENSON et al., 2017). Ao longo do tempo, diversos genes associados a doenças genéticas foram descobertos, assim como diversas variantes genéticas passaram a ser encontradas, com uma porção significativa destas possuindo significado incerto (LANDRUM et al., 2017).

Neste contexto, fez-se necessária a elaboração de diretrizes internacionais para classificação correta de variantes identificadas pelos métodos de sequenciamento de DNA. Essa padronização nos processos de classificação e interpretação clínica de variantes genéticas é fundamental para a tomada de decisões clínicas efetivas. Este processo envolve aspectos como o tipo de variante, frequência populacional e impacto funcional, permitindo a classificação de uma determinada alteração em cinco categorias, sendo elas: benigna, provavelmente benigna, variante de significado incerto (VUS, do inglês *Variant of Uncertain Significance*), provavelmente patogênica ou patogênica (RICHARDS et al., 2015; NYKAMP et al., 2017).

Ressalta-se a importância de uma classificação acurada dessas variantes, impactando não apenas a nível familiar e individual como também identificando variantes mais frequentes em determinadas populações, subsidiando conceitualmente ações de saúde pública. Como parte das evidências utilizadas, os estudos funcionais que avaliam o impacto das alterações na estrutura e função da proteína tornam-se um diferencial na classificação de variantes, especialmente naquelas classificadas como VUS.

Recentemente, Findlay et al. (2018) realizaram um estudo baseado em edição genômica como uma maneira de compreender os efeitos funcionais das variantes de sequência em *BRCA1*, caracterizando 96,5% de todas as variantes possíveis em 13 éxons que codificam os domínios funcionais críticos para seu funcionamento como supressor tumoral, RING, BRCT 1 e BRCT 2. Os resultados obtidos neste estudo funcional auxiliam na classificação de

variantes ainda não observadas em pacientes e também na reclassificação de variantes já conhecidas.

Dessa forma, um maior conhecimento do perfil mutacional de *BRCA1*, através da correta classificação de suas variantes, seria de imediata relevância para a prática da genética clínica e oncologia, impactando no prognóstico, diagnóstico, tratamento e acompanhamento de pacientes. Neste contexto, o presente estudo se justifica pela necessidade de aprimorar a classificação de diferentes variantes identificadas em ensaios de análise de toda a região codificadora de *BRCA1*, em especial em uma população como a brasileira que ainda é pouco estudada e onde se espera encontrar variantes ainda não descritas na literatura e bases de dados internacionais. Portanto, esse estudo tem como objetivo refinar a classificação das variantes de sentido trocado do gene *BRCA1* já conhecidas e classificar novas variantes identificadas em pacientes brasileiras usando como base dados de estudos funcionais publicados recentemente e comparando-as com classificações já depositadas no banco de dados ClinVar, um arquivo público que armazena os relatos fornecidos por laboratórios moleculares do mundo todo acerca das variantes genéticas de relevância clínica (LANDRUM et al., 2017).

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Reclassification of missense *BRCA1* variants associated with hereditary breast and ovarian cancer using Sherloc and ACMG-AMP classification criteria

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Abstract

Germline pathogenic variants in *BRCA1* and *BRCA2* are the main cause of hereditary breast and ovarian cancer syndrome (HBOC). Affected individuals tend to develop cancer at an earlier age, more frequently breast and/or ovarian tumors before the age of 50. Therefore, an accurate classification of *BRCA1/2* variants is critical in molecular diagnosis, leading to a precise clinical management and genetic counseling. Although some advances were made in the functional characterization of *BRCA1* single nucleotide variants, there are still many variants classified as variants of unknown significance (VUS). Here we set out to reclassify *BRCA1* missense variants by using functional data and comparing the resulting classification to the one encountered in the ClinVar database. As a result, 318 *BRCA1* missense variants in critical functional domains of the protein were analyzed according to ACMG-AMP and Sherloc variant classification guidelines. A total of 85 (27%) variants using ACMG-AMP and 32 (10%) based on Sherloc were reclassified either as likely pathogenic or pathogenic. Moreover, ACMG-AMP and Sherloc classifications had a rate of concordance of 64%. ACMG-AMP guidelines presented a higher rate of shift between different variant classes (P=2.3E-13), reflecting the use of more strict evidence criteria in the Sherloc classification framework. Our results show that comprehensive re-analysis of variant classification directly impacts clinical management, highlighting the importance of consistently using new available tools to reinterpret the significance of sequence variants.

Abbreviation List

ACMG-AMP - American College of Medical Genetics and Genomics and the Association for Molecular Pathology

B - benign

BC - breast cancer

DSB - double-strand break

EOC - epithelial ovarian cancer

HBOC - hereditary breast and ovarian cancer

HR - homologous recombination

LB - likely benign

LOF - non-functional

LP - likely pathogenic

MMEJ - microhomology-mediated end-joining

NCCN - National Comprehensive Cancer Network

NGS - next-generation-sequencing

NHEJ - non-homologous end-joining

P - pathogenic

PARP - Poly ADP-ribose polymerase

SNV – single nucleotide variant

SSA - single strand annealing

VUS - variant of unknown significance

Introduction

Germline pathogenic variants in *BRCA1* and *BRCA2* genes are associated with hereditary breast and ovarian cancer syndrome (HBOC)[1], an autosomal dominant disorder which accounts for 5-7% and 8-13% cases of breast (BC) and epithelial ovarian cancer (EOC), respectively.[2,3] Women carrying pathogenic variants in *BRCA1* have an estimated cumulative lifetime risk of BC of 72% at the age of 80 years, while the cumulative ovarian cancer risk is 44%.[4]

Through complex interactions, BRCA proteins have an important role in DNA double-strand break (DSB) repair, mainly by homologous recombination (HR). HR is considered the most precise pathway of DSB repair, since it uses the undamaged sister chromatid as a template.[5] Thus, defects in HR ultimately lead to more errors in DSB repair, hence leading to genomic instability.[6] Cells with non-functioning BRCA1 or BRCA2 proteins tend to lack DSB DNA repair through HR, since they use an error prone pathway such as nonhomologous end-joining (NHEJ) to repair DSB.[7]

Although genetic testing of *BRCA1* and *BRCA2* genes began in the last century, the recent increased access to comprehensive genetic testing in clinical routine has resulted in production of information not readily transposable to clinical practice, such as identification of variants of uncertain significance. The changes in diagnostic practices in the field are in some extent due to changes in patent laws, higher knowledge background and newly incorporated methods like high throughput sequencing.[8]

In the last two decades several target therapies have been developed based on the genetic abnormalities of tumors. For instance, targeting HR deficiency with PARP inhibitors in HBOC patients is one of the most promising therapeutic approaches.[9] Tumors with complete BRCA1 or BRCA2 deficiency are highly sensitive to therapies targeting PARP enzymes, since they also act in DNA damage repair and regulation, its inhibition along with HR deficiency ends up leading to cell death, a phenomenon called synthetic.[10,11] Some PARP inhibitors (e.g Olaparib[12], Niraparib[13] and Rucaparib[14]) are in clinical trials or have already been approved as effective treatments for HBOC related cancers, either in monotherapy regimens or in combined therapy protocols. In this context, identification and accurate classification of *BRCA* sequence variants is critical not only for HBOC diagnosis, but also to determine eligibility to treatment with PARP inhibitors.

Advancements in next-generation-sequencing (NGS) technologies allowed the detection and discovery of numerous novel DNA sequence variants. However, the increasing

amount of data also increased the challenges in variant classification, with a large number of variants being classified as variants of unknown significance (VUS). In this context, in 2015 the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG– AMP) developed guidelines to interpret sequence variants in any gene, including computational, functional and segregational data.[15] More recently, based on ACMG-AMP (2015) experience, the clinical genomics group Invitae proposed a refined semiquantitative and hierarchical method to interpret sequence variants, named Sherloc (2017)[16], which is intently open to future updates.

A significant number of *BRCA1* single nucleotide variants (SNVs) are still classified as VUS, and several have conflicting interpretations of pathogenicity, which differ among laboratories.[17] The incomplete penetrance of *BRCA1* in addition to the rarity of some variants make the classification process more challenging.[18] Since clinical information alone is not sufficient to classify *BRCA1* SNVs, *in-silico* prediction tools or *in-vitro* and *in-vivo* functional assays are alternative methods to predict and/or confirm the impact of a genetic alteration. In this sense, a remarkable effort was done by Findlay et al. (2018)[19], which developed the first *in-vitro* assay based on saturation genome editing to evaluate all functional domains of a gene. Functional scores for 96,5% of possible SNVs in 13 exons of *BRCA1* that encode RING, BRCT 1 and BRCT 2, all critical domains to its functioning as a tumor suppressor. The results achieved in Findlay's study largely support the interpretation of novel *BRCA1* variants and, more importantly, offers an opportunity to reclassify SNVs previously reported as VUS or with conflicting interpretations. Thus, proper classification of *BRCA1* variants would be of immediate clinical relevance, impacting prognosis, diagnosis, treatment and genetic counseling. Therefore, considering the new functional data on *BRCA1* variant consequences and its clinical relevance, this study aims to reclassify *BRCA1* missense variants using data from published functional studies and comparing it to classifications presented in ClinVar, a public archive that aggregates information about clinically relevant variants submitted by multiple molecular diagnostic laboratories worldwide. We also aimed to compare ACMG-2015 and Sherloc guidelines in classifying rare or dubious *BRCA1* variants.

Materials and Methods:

Variant selection

BRCA1 variants functionally studied by Findlay et al. (2018) were assembled and compared to the classification/interpretation found in ClinVar database. All conflicting classifications were selected for reclassification (last review in 08/25/2019).

Evidence gathering

The canonical transcript of *BRCA1* used was NM_007294.3 (REFSEQ), ENST00000357654.8 (ENSEMBL) or LRG_292t1 (LRG).

The conflicting missense variants previously selected were then assessed according to their frequencies in general population genetic databases. Here, we used population information from Exome Aggregation Consortium (ExAC)[20], Genome Aggregation Database (GnomAD)[21], NHLBI Trans-Omics for Precision Medicine (TOPMed)[22], The 1000 Genomes Project (1000G)[23], Online Archive of Brazilian Mutations (AbraOM)[24] and Fabulous Ladies Over Seventy (FLOSSIES)[25].

Furthermore, functional and structural impact of *BRCA1* amino acid substitutions were evaluated through part of the most widely used in-silico prediction tools: SIFT[26], Polyphen-2[27] and Align-GVGD[28] (*BRCA1* from human to frog).

In Findlay et al. (2018), *BRCA1* variants were addressed to functional classes accordingly to their functional scores, fitting a two-component Gaussian mixture model that estimated the probability of each SNV to be non-functional. Then, SNVs were classified as functional (FUNC), non-functional (LOF) or intermediate (INT).

Reclassification of variants

All selected variants were reclassified using ACMG-AMP and Sherloc classification criteria, considering evidences showed above (Supplementary Table S1) and literature review.

ACMG-AMP framework is based on pathogenic evidences (divided in very strong, strong, moderate and supporting) and benign evidences (divided in stand-alone, strong and supporting). The classification outcome is provided when criteria are combined, with a stated classification to each combination. Variants are then categorized as: pathogenic (P), likely pathogenic (LP), VUS, likely benign (LB) and benign (B). In contrast, the Sherloc framework is based on 108 scored evidences, divided as either pathogenic or benign evidences. The classification outcome is calculated weighting all criteria and fitting into the five categories of ACMG-AMP. Since Sherloc specifies exclusive use of Align-GVGD, SIFT and Polyphen-2 as its computational evidences (EV0122, EV0126 and EV0109), those predictors were also used in ACMG-AMP guidelines. The maximum expected allele frequency value for a *BRCA1* variant to be considered pathogenic was settled at 0.0010005.[29]

Both ACMG-AMP (PM5) and Sherloc (EV0172) have evidence criteria approaching presence of mutational hotspots. However, there is not a stated threshold for when to consider a mutational hotspot region other than well-established pathogenic variants in nearby amino

acid residues. Addressing it, we have established a threshold of at least two variants confidently classified as P or LP in a range of two upstream and downstream residues, without any B or LB variant.

When reviewing literature case reports of affected individuals with BC or EOC, we have determined that the patients had to fulfill *BRCA* testing criteria according to the National Comprehensive Cancer Network (NCCN) guidelines (version 3.2019). Variants with intermediate functional consequences were considered as weak functional evidence of protein disruption (EV0024) in Sherloc. The remaining ACMG and Sherloc evidences were interpreted according to the original manuscript and are not detailed here. Lastly, every variant reclassification was independently reviewed by at least two independent evaluators.

Statistical Analysis:

Pearson's chi-square test was used to compare the rate of altered classifications between Sherloc and ACMG-AMP. The results were considered statistically significant when the P was < .05.

Results

Functional scores of a total of 3,893 *BRCA1* SNVs were retrieved from Findlay et al (2018), while interpretations on 4,904 *BRCA1* germline SNVs were obtained from ClinVar database (last access in 25 August, 2019). Functional classes were then compared with ClinVar interpretations for 890 SNVs present on both. Of all 890 SNVs, 416 (47%) had conflicting interpretations and 474 were in agreement (functional score versus ClinVar classification). Only missense variants were selected from the conflicting variants list, resulting in 318 *BRCA1* missense variants (Figure 1a). The composition of ClinVar *BRCA1* variant classifications was: VUS (n=292), P (n=5), LP (n=1), B (n=1) and with conflicting interpretations (n=19). Additionally, the number of variants in each functional class was: 81 variants classified as LOF, 31 variants as INT and 206 as FUNC (Figure 1b).

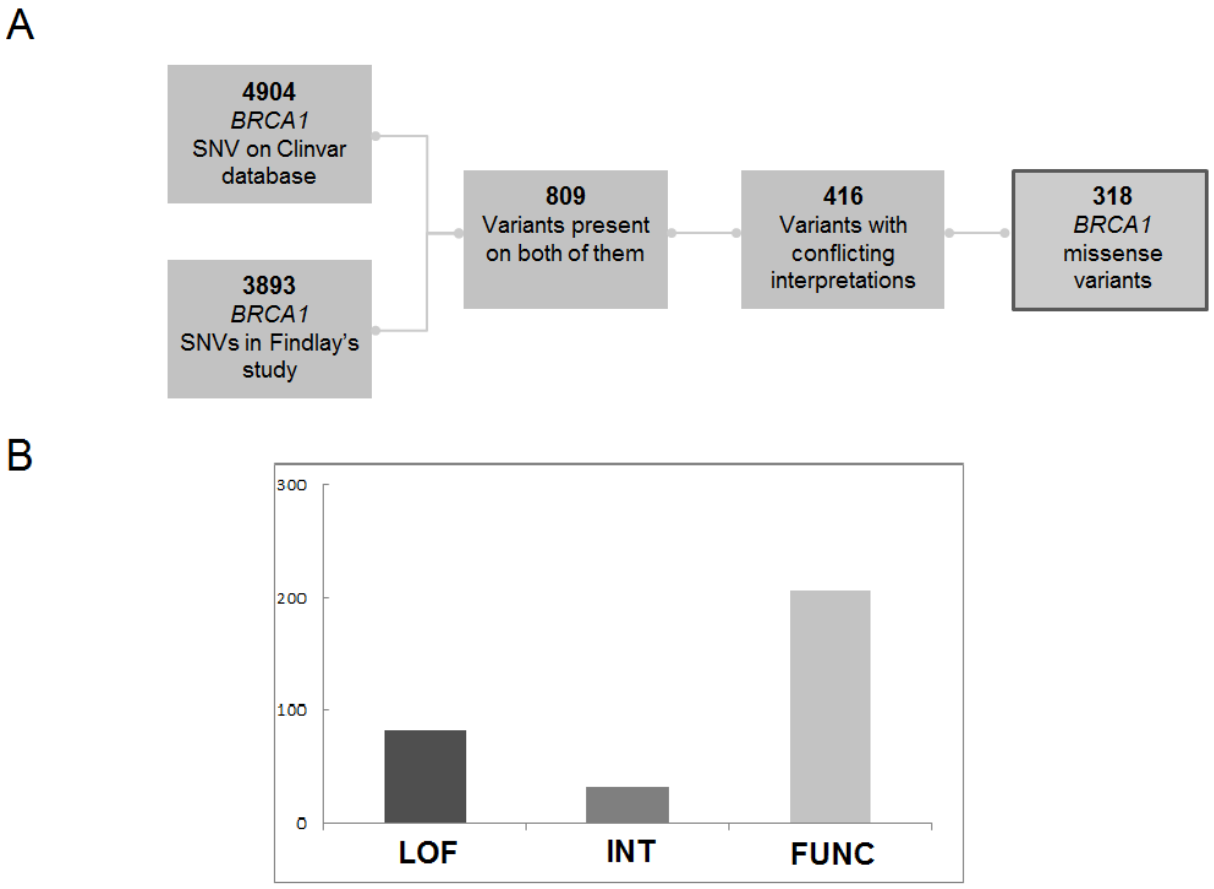

Figure 1: A: Filtering process of single nucleotide variants (SNVs), ultimately selecting only conflicting missense variants between functional assay and ClinVar. **B:** Functional classes composition of the selected *BRCA1* missense variants in Findlay's study: LOF, non-functional; INT, intermediate; FUNC, functional.

Reclassification process:

When we considered variants previously classified as VUS in ClinVar, ACMG-AMP guidelines presented a higher rate of shift variant class (P=2.3E-13), which accounted for 37% (n=109) of all variants the classification of 63% (n=183) of the variants remained unchanged). While using Sherloc 11% (n=33) were reclassified and 89% (n=259) remained unchanged (Figure 2a). Furthermore, the rate of concordance between the two guidelines in terms of variant reclassification was 8% (n=10) (Figure 2b).

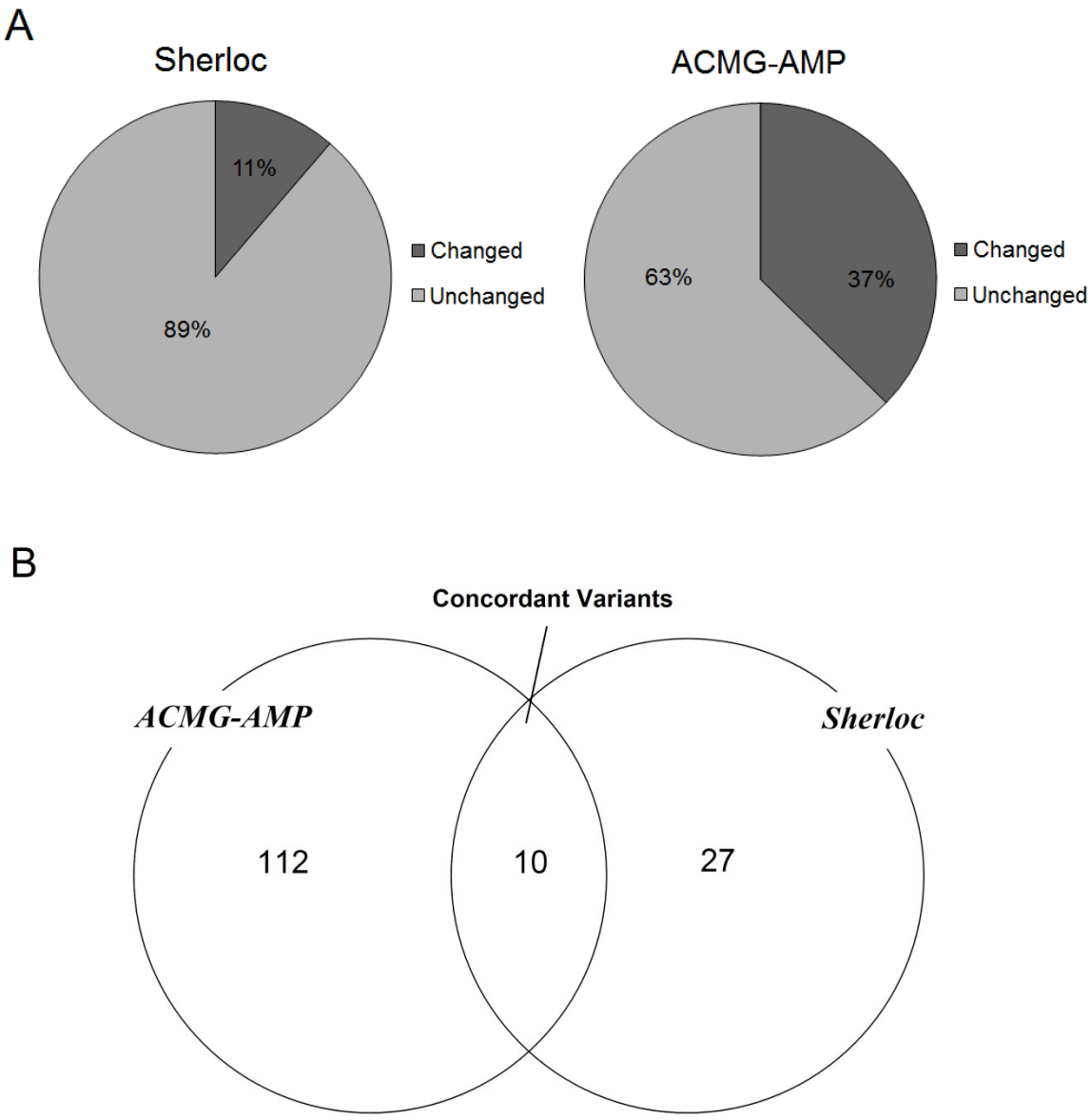

Figure 2: Reclassification using ACMG-AMP and Sherloc criteria. **A:** Proportion of altered variant classifications that were previously classified as variants of unknown significance. **B:** Rate of agreement between Sherloc and ACMG-AMP reclassified variants with altered classifications.

Detailed results of variant reclassification using both guidelines are described in details in Supplementary Table S2.

First, when we used ACMP-AMP criteria for reclassifications, the 292 variants previously reported as VUS in ClinVar were reclassified as follows: P (n=4), LP (n=77), LB (n=28) and VUS (n=183). Variants with conflicting interpretations between two or more laboratories in ClinVar (n= 19) were reclassified as: LP (n=1), LB (n=6), B (n=3) and VUS

(n=9) (Figure 3). Second, when we considered Sherloc criteria, variants previously classified as VUS in ClinVar were reclassified as: P (n=25), LP (n=6), LB (n=2) and VUS (n=259). Variants with conflicting interpretations in Clinvar were reclassified as: P (n=1), LB (n=3) and VUS (n=5) (Figure 3).

Seven variants (5 P, 1 LP, 1 B) were neither classified as VUS nor as conflicting. According to Sherloc, they were all reclassified as VUS, while in ACMG-AMP they were reclassified as: P to LP (n=2), P to VUS (n=3), LP to VUS (n=1) and B to LP (1).

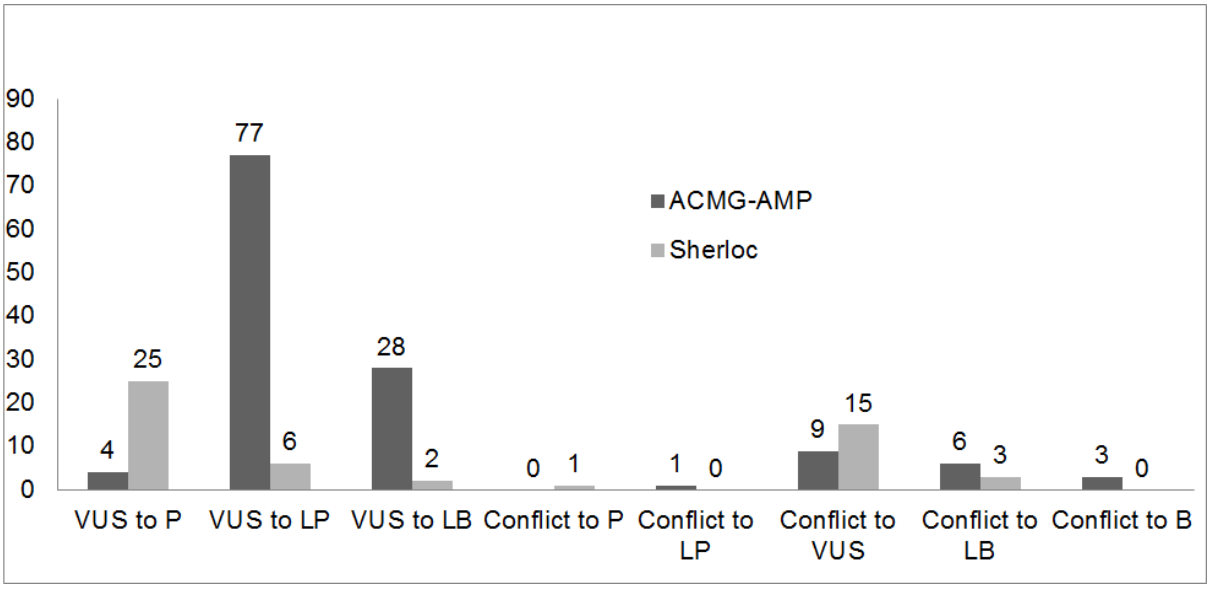

Figure 3: Observed types of reclassification: B, benign; LB, likely benign; VUS, variant of unknown significance; LP, likely pathogenic; P, pathogenic; Conflict, Conflicting interpretations.

Discussion:

Germline pathogenic variants in *BRCA*1 and *BRCA2* are associated with HBOC syndrome, a cancer predisposition syndrome associated with increased risk of developing BC and EOC.[1] In this context, one of the main challenges in clinical practice is the accurate interpretation of sequence variants in *BRCA1*. In the last decade, the advancements in NGS platforms increased the diagnostic and therapeutic fields.[17]

Variant classification guidelines are designed to limit false-positive and false-negative results, hence presenting stringent criteria. ACMG-AMP is intently broad in its rules, leading to adapted interpretations in each specific disease or gene.[15] Different interpretations of the same rules might lead to different classification outcomes, as demonstrated by studies in ClinVar database, in which high rates of discordant interpretations between different

laboratories were observed ranging from 11% to 70%.[30–32] Sherloc guidelines refines the ACMG-AMP classification framework, introducing 108 detailed criteria that provide a more robust approach to variant classification.[16] However, it is considerably more conservative in its approach, what could result in under-calling pathogenic variants, as demonstrated by Walsh et al. (2016)[33] when stringent criteria were used. Here, we observed that Sherloc classification criteria were more strict than ACMG-AMP, as its rate of altered classifications was significantly lower (Figure 2a). One of the reasons for these discrepancies is that Sherloc considers either computational/predictive evidence or functional experiment evidence, while ACMG-AMP is supported by both criteria. Since both types of evidence access the disruption of protein function, functional evidence overlays computational/predictive evidence as they are considered redundant. Hence, part of ACMG-AMP altered classifications could be linked to the use of both *in silico* and functional evidences in the classification process.

The Sherloc framework also highlights the value of clinical data and unrelated case reports, but due to the approach used in our study attaining such evidence requirements was challenging. Literature observations of cancer-affected individuals are often unavailable or incomplete, making it difficult to determine a causal effect for a variant, even when patients are said to be referred to HBOC molecular testing or to have family history of BC/EOC. Therefore, it was unlikely for us to have enough clinical data available to fulfill NCCN guidelines.

Surprisingly, Sherloc had more variants reclassified as P than ACMG-AMP. All variants reclassified as P on Sherloc fulfilled at least one of the evidences related to a well-established P or LP missense variant on the same residue (EV0044) or nucleotide (EV0139). Each of these criteria is powerful enough to classify a variant as P when considered with strong functional evidence and absence in large public databases. However, in ACMG-AMP criteria there is just one evidence (PM5) addressing it, being less powerful and not enough to change a LP variant to a P one.[15,16] For instance, *BRCA1 (*NM_007294.3) c.191G>C, p.(Cys64Ser) is a variant previously classified as VUS. Due to its LOF class, absence in general population databases, computational predictions of structural/functional impact and the presence of another P variant (c.191G>A) in the same residue, this variant was reclassified as LP (PS3, PP3, PM2, PM5) in ACMG-AMP while in Sherloc it was classified as P (EV0135, EV0044, EV0023). A total of 85 (27%) variants based on ACMG-AMP and 32 (10%) using Sherloc were reclassified as LP or P. These findings may represent a remarkable impact on

clinical practice, since dozens of patients could be benefited through proper identification and classification of a P *BRCA1* variant.

Another example is c.5044G>A p.(Glu1682Lys), previously classified as B in ClinVar, but reported as LOF on the functional assay. Findlay et al (2018) discuss about this variant and its low function score, reaffirming their interpretation of pathogenicity. In our study, c.5044G>A was classified as LP (ACMG-AMP) and VUS (Sherloc). These findings underscore the need of carefully considering which pathogenicity algorithm is used in variant classification.

Macklin et al. (2017)[34] published a study addressing the challenges and frequency of variant reclassification in a hereditary cancer clinic between 2013 and 2017. Their conclusion was that the majority of variant reclassifications do not impact medical management. The variant reclassification pattern presented was dissimilar than ours, as the vast majority of their variants were reclassified as likely benign, hence only 1 of 40 reclassifications was an upgrade of a VUS to a LP. In contrast, the great majority of our reclassifications were of VUS to LP (Figure 3). This is likely due to the different types of evidence used, while in their work a great amount of their own clinical information was available, in our study most of the evidences were of function, frequency and predictive evidence. In addition, the sample composition itself was remarkably different, as our study focused on dubious/rare *BRCA1* variants.

Since new ClinVar interpretations depend on a molecular laboratory to find a variant and submit their interpretations in the dataset, rarely with detailed evidence, our study emerges as an important attempt to accelerate the reclassification of missense *BRCA1* variants. Likewise, laboratories and/or health professionals may benefit from our results, either to help on their decisions or as a starting point to perform an independent evaluation. In summary, the findings reported here illustrate some of the complexities involved in variant classification located in the *BRCA1* gene and the need of a constant review in the classification process of previously reported germline variants.

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