UNIVERSITY OF CALIFORNIA,
IRVINE

Investigating Variants of Uncertain Significance: Reclassification Triggers and Drivers in Breast Cancer Predisposition Genes

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Genetic Counseling

by

Kirsten Anne Kelly

Thesis Committee:
Professor Emerita Moyra Smith, MD, PhD, Chair
Associate Clinical Professor Kathryn Singh, MPH, MS, LCGC
Assistant Adjunct Professor Elizabeth Chao, MD, FACMG

2019

DEDICATION

To

My family and Grizz.

"Our loved ones are the compass that guide us. They are the inspiration to reach great heights, and our comfort when we occasionally falter."

- Brad Henry

TABLE OF CONTENTS

	Page
LIST OF FIGURES	vi
LIST OF TABLES	vii
ACKNOWLEDGMENTS	viii
ABSTRACT OF THE THESIS	ix
I. INTRODUCTION	1
1.1 Background and Significance of the Research	1
1.1.1 Hereditary cancer susceptibility multigene panel testing	1
1.1.2 Differentiating between high and moderate cancer risk genes	4
1.1.3 Current practices of multigene panel testing	5
1.1.4 Genetic testing results	6
1.1.5 Multigene panel testing leads to increased identification of variants of uncertain significance (VUSs)	9
1.1.6 Identification of VUSs cause anxiety and distress for providers and patients	9
1.1.7 Historical and current published standards and guidelines for the interpretation of sequence variants	11
1.1.8 Commercial laboratory variant assessment and classification scheme	13
1.1.9 Importance of accurate variant classification	14
1.1.10 Prevalence of variant reclassification following hereditary cancer genetic testing	15
1.2 Purpose of Study and Specific Aims	17
II. METHODS	18
2.1 IRB approval	18

2.2 Collection of VUS reclassifications over study period 18
2.3 Collection of VUS reclassification drivers and triggers 19
2.4 Data Analysis 21
2.4.1 VUS reclassification direction over study period 21
2.4.2 Frequency of discrepant classification 22
2.4.3 Differences in time to VUS reclassification 22

III. RESULTS 24
3.1 Direction of VUS reclassification over study period 24
3.2 Criteria used in VUS reclassification 30
3.3 Triggers that precede VUS reclassification 37
3.4 Frequency of discrepant classification in public variant database 38
3.5 Average time to VUS reclassification 41

IV. DISCUSSION 42
4.1 VUS reclassification direction over study period 42
4.2 Significant differences in criteria used for VUS reclassifications 46
4.3 Significant differences in triggers that precede VUS reclassification 50
4.4 Differences in time to VUS reclassification 51
4.5 Events following VUS reclassification 52
4.6 Limitations 53
4.7 Future Directions 55
4.8 Conclusions 56

REFERENCES 58

APPENDIX A ACMG Standards and Guidelines for the Classification of Sequence Variants 67
APPENDIX B Ambry Scheme for Autosomal Dominant and X-linked Mendelian Diseases 69
APPENDIX C IRB Approval 70

APPENDIX D Table of "Other" Criteria Used for Reclassification 72
APPENDIX E Categories of triggers that precede reclassification 73
APPENDIX F RStudio logistic regression model code 74
APPENDIX G Average time to reclassification per gene 75

LIST OF FIGURES

Page

Figure 1 Overall distribution of VUS reclassifications 24

Figure 2 Gene specific distributions of VUS reclassifications 25

Figure 3.1 Upgrade and downgrade frequency of VUSs in HBRGs and MBRGs 26

Figure 3.2 Frequency of VUS reclassification upgrade or downgrade per gene 27

Figure 4.1 Frequency of VUS upgrade and downgrade in HBRGs per year 28

Figure 4.2 Frequency of VUS upgrade and downgrade in MBRGs per year 29

Figure 5 Frequency of "strong to moderate evidence of pathogenicity" used for VUS reclassification in HBRGs and MBRGs 34

Figure 6 Frequency of "strong to moderate evidence for benign" used for VUS reclassification in HBRGs and MBRGs 35

Figure 7 Frequency of "supporting evidence for benign" used for VUS reclassification in HBRGs and MBRGs 36

Figure 8 Frequency of VUS reclassification triggers in HBRGs and MBRGs 37

Figure 9 Overall frequency of discrepant variant classification in HBRGs and MBRGs 38

Figure 10 Level of discrepancy for 506 (total number) variants with a discrepant variant classification as of February 2019 39

Figure 11 Frequency of discrepant variant classification by year of reclassification 40

Figure 12 Average time to VUS reclassification in HBRGs and MBRGs 41

LIST OF TABLES

		Page
Table 1.1	Logistic regression analysis for VUS reclassification in HBRGs	30
Table 1.2	Logistic regression analysis for VUS reclassification in MBRGs	30
Table 2	Distribution of criteria used for VUS reclassification	31
Table 3	Distribution of "Other" evidence used in VUS reclassifications	33

ACKNOWLEDGMENTS

Throughout the writing of this thesis I have received a great deal of support and assistance. I would first like to thank my entire committee, Dr. Moyra Smith, Kathryn Singh, and Dr. Elizabeth Chao. Thank you for your guidance from the very beginning. Without your support and help this dissertation would not have been possible.

I would also like to thank my program director, Pamela Flodman, and all of the program faculty members. Thank you for your support and encouragement the last two years. You have provided me with the tools I need to be a great genetic counselor.

I would like to thank my classmates. Thank you for encouraging me, and being both classmates and friends. I look forward to being your colleague.

My sincere thanks also go to Kelly McGoldrick, Tina Pesaran, and Ambry Genetics who provided me an opportunity to join their team as a Clinical Assistant, and who gave me access to the dataset. Without their precious support it would not be possible to conduct this research.

Finally, I must express my very profound gratitude to my parents, sister and partner for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

ABSTRACT OF THE THESIS

Investigating Variants of Uncertain Significance:
Reclassification Triggers and Drivers in Breast Cancer Predisposition Genes

By

Kirsten Anne Kelly

Master of Science in Genetic Counseling

University of California, Irvine, 2019

Professor Emerita Moyra Smith, MD, PhD, Chair

This study investigated the factors that trigger and drive reclassification of DNA variants of uncertain significance (VUS) in genes associated with high and moderate breast cancer risk (HBRGs and MBRGs). We examined 617 VUS reclassifications in 15 genes associated with hereditary breast cancer predisposition performed by a single commercial laboratory from January 2014 to January 2018. Almost 81% of the VUS reclassifications were downgrades, i.e. a VUS reclassified to likely benign (VLB) or benign. However, for VUSs that were reclassified in HBRGs over the study period it was determined that every year the odds of upgrade, i.e. a VUS reclassified to likely pathogenic (VLP) or pathogenic, increased by 68%. These results are significant considering VUS reclassification to pathogenic or VLP can lead to significant medical management changes for patients. Additionally, reclassified variants in HBRGs were more likely to use several lines of evidence (*de novo* occurrences, computational data, and protein functional assays) more frequently than reclassified variants in MBRGs. There were significant differences seen in evidence used depending on the reclassification direction, i.e. upgrade or downgrade. Availability of new research or data triggered reclassification of VUSs more frequently in

HBRGs, but periodic variant review triggered reclassification more frequently in MBRGs. On average variants in HBRGs were reclassified five months sooner than variants in MBRGS. The results of this study support previously published data regarding frequency and direction of VUS reclassification and shed new light on the triggers of and evidence used for variant reclassification in HBRGs and MBRGs.

I. INTRODUCTION

1.1 Background and Significance of the Research

1.1.1 Hereditary cancer susceptibility multigene panel testing

The funding of the Human Genome Project (HGP) was the catalyst of the genetics resurgence in the 20th century. The HGP was an international, collaborative research program whose goal was the complete mapping and understanding of the human genome. The 15-year-long project formally launched in April of 1990 and was declared complete in April of 2003 and cost a total of $3 billion. The complete mapping of the human genome allowed us to better understand the gene-disease relationship by identifying about 20,500 human genes (National Human Genome Research Institute 2016). Since the completion of the project numerous applications of the research have been discovered including but not limited to: identification of pathogenic variants linked to human disease, advancement in forensic applied sciences, animal husbandry, anthropology, and evolution. One of the most notable applications of the project was the commercial development of genomics research and products, which is now a multibillion-dollar industry.

Amidst the launch of the HGP a group of researchers at UC Berkeley discovered evidence for the existence of a gene involved in breast cancer susceptibility. By comparing genetic data from multiple families in which several individuals had a history of breast cancer the researchers mapped the gene to a region on the long arm of human chromosome 17, specifically 17q21 (Hall *et al.* 1990). This provided strong evidence of the potential existence of genes for hereditary forms of breast cancer. Following this discovery an international race ensued to identify and clone the breast cancer susceptibility gene,

which would eventually be named *BRCA1* (for BReast CAncer gene 1). Three years after the research at UC Berkeley, the *BRCA1* gene was identified using positional cloning techniques in samples from affected families (Bowcock 1993). Ultimately, this research allowed for the identification of individuals with inherited germline mutations in *BRCA1*. About a year after the *BRCA1* gene discovery a second breast cancer susceptibility gene called *BRCA2* (for BReast CAncer gene 2) was identified on chromosome 13q12.313. While these genes are often referred to as cancer susceptibility genes, these genes are present in all individuals and in most cases the normal products of the genes play roles in preventing cancers from developing. When pathogenic variants are present in these genes they can disrupt the normal function and thus increase risk for cancer, which is why they are called cancer susceptibility genes. There are two types of DNA changes: those that are inherited and are present in every cell of a person's body (germline) and those that happen after conception and can be contained to a specific cell line or location in a person's body, such as a tumor (somatic). Inherited DNA changes can be passed down through generations because they are present in the cell lines of the ovary or sperm (gonads) and are referred to as germline alterations. DNA changes that occur after conception in any cell(s) of a person's body, excluding the gonadal cells, are referred to as somatic variants. These somatic variants, in combination with other factors, can, but do not always, result in cancer or other disease.

Commercially available *BRCA1* and *BRCA2* gene sequencing was first marketed by a diagnostic laboratory named Myriad Genetic Laboratories, Inc in 1996. At the time Myriad held patents on the *BRCA1* and *BRCA2* genes which granted them sole control over the use of diagnostic sequencing tests based on those genes. Myriad began marketing three

principal diagnostic tests: (1) the Comprehensive BRACAnalysis, which involved full sequencing of the *BRCA1* and *BRCA2* genes, (2) the Single Site BRACAnalysis test, and (3) the Multisite three BRACAnalysis, three mutation *BRCA1*/*BRCA2* analysis, which identified three specific pathogenic variants that are prominent in the Ashkenazi Jewish population (Gold *et al.* 2008). However, in 2013 the United States Supreme Court ruled that "A naturally occurring DNA segment is a product of nature and not patent eligible merely because it has been isolated […]" (Association for Molecular Pathology v. Myriad Genetics, Inc. [June 13, 2013]). This ruling prevented any future infringement complaints that Myriad would file against competing clinical and research laboratories.

Following the Supreme Court ruling, numerous genetic testing laboratories began offering *BRCA1* and *BRCA2* gene sequencing. Since the discoveries of the *BRCA1* and *BRCA2* genes, researchers have identified additional genes in which pathogenic alterations occur and are linked to breast and ovarian cancer susceptibility. Pathogenic variants in these genes do not increase the risk of breast cancer as much as pathogenic variants in the *BRCA1/2* genes. However, pathogenic variants in these genes are linked to an increased risk for breast cancer in some families.

The development of next-generation sequencing (NGS) allowed for more rapid and cost-effective gene sequencing, which facilitated the implementation of multigene panel testing in the clinical setting Multigene panel testing refers to sequencing multiple pre-selected genes, not limited to *BRCA1* and *BRCA2*, using an NGS sequencing platform.

Hereditary breast cancer susceptibility multigene panel testing became commercially available in 2012 and has since expanded to include anywhere from 5 to > 100 genes.

1.1.2 Differentiating between high and moderate cancer risk genes

Until recently, genetic testing for cancer susceptibility focused on classic syndromes, such as hereditary breast and ovarian cancer (HBOC) and Lynch syndrome, which is associated with an increased risk of colorectal and endometrial cancer among other types of cancer. In the clinical setting multigene panel testing has helped identify patients and families who carry pathogenic variants in the *ATM*, *BARD1*, *BRIP1*, *CHEK2*, *NBN*, *RAD50*, *RAD51C* and *RAD51D* genes. The presence of a pathogenic variant in one of these genes can confer breast cancer risks that are not as high *BRCA* pathogenic variants but still above the breast cancer risk in the general population, so the genes are referred to as moderate breast cancer risk genes (MBRGs). Pathogenic variants in these genes usually confer a modest increase in breast cancer risk (relative risk 2-4) as compared to the general population (Couch, F.J. *et al.* 2017, Hollestelle, A *et al.* 2010, Tung *et al.* 2016). These individuals are in stark contrast to carriers of pathogenic variants in genes associated with a high risk for breast cancer (HBRGs). Pathogenic variants in these genes can confer a lifetime breast cancer risk of up to 87 percent (relative risk 5-9) (Brose MS *et al.* 2002, Kuchenbaecker KB *et al.* 2017, Levy-Lahad E *et al.* 2007, Suarez-Kelly *et al.* 2019). Numerous studies have shown that variants in MBRGs are identified between 1.1-9.4% of the time in individuals referred for genetic testing (Couch *et al.* 2015, Cragun *et al.* 2014, Kurian *et al.* 2014, LaDuca *et al.* 2014, Lincoln *et al.* 2015, Maxwell *et al.* 2015, Minion *et al.* 2015, Tung *et al.* 2015, Walsh *et al.* 2011, Yurgelun *et al.* 2015). Management guidelines

exist for carriers of pathogenic variants in HBRGs, (such as *BRCA1*, *BRCA2*, *CDH1*, *NF1*, *PTEN* and *TP53*) although these guidelines may not apply to all patients depending on family history (NCCN Guidelines for Genetic/Familial High-Risk Assessment: Breast and Ovarian 2019). There are also management guidelines available for patients with pathogenic variants in MBRGs such as *ATM*, *CHEK2*, *NBN*, and *PALB2 (*NCCN Guidelines for Genetic/Familial High-Risk Assessment: Breast and Ovarian 2019). However, to date, there are no consensus guidelines on how to manage breast cancer risk in patients who carry pathogenic variants in genes such as *BARD1*, *BRIP1*, *RAD50*, *RAD51C*, and *RAD51D*. It is important to note that the threshold for distinguishing a HBRG from a MBRG is sometimes left to the discretion of individual providers and genetic testing laboratories. For instance, in recent studies *PALB2* has been suggested to be re-categorized as a HBRG but some clinicians and laboratories still consider it to be a MBRG (Antonis C *et al.* 2014, Rahman *et al.* 2007). This is because some pathogenic variants in *PALB2* confer a higher risk for breast cancer only in certain families (Antoniou *et al.* 2014). Additionally, pathogenic variants in *NF1* can be considered to cause high risk in younger women and moderate risk in older women (Madanikia *et al.* 2012, Seminog *et al.* 2015). For the purpose of this study, *NF1* was considered a HBRG and *PALB2* a MBRG.

1.1.3 Current practices of multigene panel testing

The use of multigene panels for the assessment of hereditary cancer susceptibility is expanding rapidly in clinical practice. Multigene panel testing in the cancer genetics setting refers to sequencing with or without deletion/duplication analysis of a curated group of genes associated with cancer susceptibility. One survey found that 94 percent (143/152) of

genetic counselors had begun implementing multigene panel testing into their practice (Hooker *et al.* 2017). However, the value of multigene-panel testing remains debatable due to the uncertainty regarding the strength of association between pathogenic variants in some genes and the development of cancer (clinical validity), and a lack of evidence demonstrating improved outcomes for the individuals tested (clinical utility) (Easton *et al.* 2015, Domchek *et al.* 2013, Kurian *et al.* 2017). Despite this controversy, it is clear that multigene panel testing has become entrenched in clinics and as one oncologist put it "there seems little chance of forcing the genie back into the bottle" (Kurian *et al.* 2015).

1.1.4 Genetic testing results

It is important for patients and providers to consider the risks and benefits associated with genetic testing. While there are no risks of physical harm, other than a blood draw, genetic testing can have unintended emotional effects, particularly when a pathogenic variant is found. However, there are many possible benefits of genetic testing. For instance, identifying a pathogenic variant can give a patient and their family members a better understanding of their cancer risks which can lead to increased cancer screening and/or risk-reducing surgeries or preventative treatments. When genetic testing is performed there are three possible test results: positive (pathogenic or likely pathogenic variant detected), negative (no clinically significant variants detected), and uninformative (variant of uncertain significance detected).

A positive test result means that a pathogenic variant was identified which confers an increased risk for certain types of cancer. A person's individual risk for cancer depends

on the gene and often the specific variant that was identified. For some variants, the risks of specific cancers may be very high. For other variants, the risks may be lower. Even with a positive test result a patient's risk for developing cancer is not 100 percent; it is possible to carry a pathogenic variant associated with increased cancer risk and never develop cancer. A positive test result may affect treatment decisions for patients with cancer and can affect screening and risk-reducing options for individuals who have not developed cancer. A positive test result also has serious implications for family members. Variants in genes associated with hereditary cancer susceptibility can be inherited. For the majority of these genes, there is a 50% chance that a person who carries a variant will pass it to each of their children. Therefore, it is important for patients to discuss their positive test results with their genetic counselor or health care team.

A negative test result means that no pathogenic variant was identified in the gene(s) analyzed. There are several reasons why someone might receive a negative result including: (1) an individual could have a variant in an analyzed gene that cannot be identified by the current testing method, (2) an individual could have a variant in a different gene that was not included in the testing, (3) an individual's cancer is not due to an inherited gene variant. In many cases sequencing alone cannot detect large deletions or duplications in the genome and therefore an additional testing methodology must be performed to identify these types of variants. Historically this additional methodology was not routinely performed so some individuals who had "negative" testing may actually carry a pathogenic variant. In addition to improved *BRCA1/2* pathogenic variant detection with deletion and duplication analysis, current hereditary cancer susceptibility testing includes

non-*BRCA1/2* genes associated with an increased risk of developing cancer. Patients with previous negative results from genetic testing that only included *BRCA1/2* may still be at risk to carry a pathogenic variant and may want to consider multigene panel testing. Finally, patients with a negative test result may not have an inherited genetic cause for their personal and/or family history of cancer. It is important to note that inherited genetic variants play a role in only five to ten percent of all breast cancers (American Cancer Society. Breast Cancer Facts & Figures 2015-2016). Therefore, a majority of patients who undergo genetic testing will not have an inherited pathogenic variant identified.

The final, and at times most disconcerting, type of test result is an uninformative test result, e.g., a variant of uncertain significance. In other words, there is a change in the sequence of a gene, but there is insufficient evidence to conclude whether it is disease causing or part of the spectrum of benign variation in the human population. Genetic variants can be classified as variants of uncertain significance for a myriad of reasons including: lack of phenotypic evidence associated with the variant, conflicting evidence, or rarity in the general population. A variant of uncertain significance is deemed uninformative because it will not add any additional information to the clinical assessment of cancer risk and therefore risk estimates must be made based on personal and family medical history. It is important for individuals who are identified as carriers of a variant of uncertain significance to stay in contact with their genetics specialist in the event that their variant is reclassified to pathogenic, likely pathogenic, likely benign or benign variant.

1.1.5 Multigene panel testing leads to increased identification of variants of uncertain significance (VUSs)

Clinical utility remains the fundamental issue with multigene panel testing, particularly for variants identified in MBRGs. One claim against clinical utility is the increase in identification of variants of uncertain significance (VUS) concomitant with an increase in the number of genes on a panel. It has been well established that multigene panel testing, compared with traditional single gene testing, increases the VUS rate, mainly as a result of sequencing non-*BRCA1/2* genes (Chong *et al.* 2014, Kapoor *et al.* 2015, Kurian *et al.* 2014, LaDuca *et al.* 2014, Slavin *et el.* 2015, Tung *et al.* 2015). The likelihood of a VUS result is even higher for individuals from non-Caucasian populations due to insufficient information on the common benign genetic variation in those populations (Hall *et al.* 2009). This lack of information is due in part to the disproportionate overrepresentation of people of European ancestry in reference genetic databases. Additionally, one study found that *BRCA1/2* reclassification rates varied by ancestry and increased over time; the rates increased more steeply for people of African, Ashkenazi, and Chinese ancestry (Slavin *et al.* 2018).

1.1.6 Identification of VUSs cause anxiety and distress for providers and patients

Due to the uninformative nature of variants of uncertain significance, both patients and clinicians can experience confusion and anxiety regarding test result interpretation and associated health recommendations. One study found that even with genetic counseling, patients with a VUS result reported higher cancer distress as compared to patients with a negative test result (Culver *et al.* 2013). In addition to increased stress, it

has been shown that patients with a VUS result fail to recall the clinical significance of their result approximately one-third of the time (Richter *et al.* 2013). The confusion surrounding VUSs is not limited to patients; numerous studies have shown inappropriate interpretation of uninformative results among non-genetics providers (Macklin *et al.* 2018, Keating *et al.* 2008, Welsh *et al.* 2017, Kurian *et al.* 2017). Macklin *et al.* found that, when surveyed about how comfortable providers feel discussing genetics in their practice, 25% of respondents reported feeling uncomfortable, 24% somewhat uncomfortable, 34% somewhat comfortable, and only 15% comfortable (Macklin *et al.* 2018). Additionally, the study found that nearly 60% (54/91 respondents) did not feel comfortable explaining a VUS to their patient.

The misinterpretation of a VUS result could lead to unnecessary treatment, surgery, anxiety and/or false reassurance. The genetics community is in agreement that testing affected relatives for a VUS, unless it is in the context of a research study, is not recommended (Richards *et al.* 2015). Testing relatives can lead to unnecessary care or a false sense of reassurance if the VUS is not detected. VUS-related anxiety is due in part to rapid advances in genetic testing and integration that many non-genetics providers feel unequipped to keep up with (Najafzadeh *et al.* 2012). While comfort is likely to increase with clinical exposure, it is also important to actively increase non-genetics providers' knowledge of genetics.

1.1.7 Historical and current published standards and guidelines for the interpretation of sequence variants

The widespread implementation of next-generation sequencing in clinical care has highlighted the importance of standardizing the interpretation and reporting of genetic test results across laboratories. As previously discussed genetic testing may have positive, negative, or uninformative results. The correct interpretation of variants is crucial to the clinical validity and utility of genetic testing. Simultaneous multigene sequencing in the clinical setting did not become widely available until the advent of high-throughput sequencing technologies which allowed for faster turn-around times and less cost. A majority of DNA sequence changes that result in an increased risk of developing cancer are pathogenic variants which disrupt the function of the gene. For example, *BRCA1/2* are tumor suppressor genes, meaning these genes encode for proteins that help control and regulate cell growth. Inactivation of tumor suppressor genes, via pathogenic variants, leads to uncontrolled cell growth which precipitates tumor development. Alternatively, activating pathogenic variants in proto-oncogenes, such as *MET*, which promote cell growth cause the gene to become permanently activated. This permanent activation causes cells to divide out of control, which leads to tumor development.

In 2007 practice guidelines were proposed for classification of variants as a joint action of the Dutch and British societies for clinical molecular genetics (Bell *et al.* 2007). These guidelines helped establish quality standards and lines of evidence which could be utilized when assessing whether or not a variant might be pathogenic. These practice guidelines conclude that it is essential to report all sequence variants and proposed

reporting uncertain variants in three classes: a) certainly not pathogenic; b) unlikely to be pathogenic; and c) likely to be pathogenic. However, this classification scheme is not currently used by commercial laboratories in the United States. Around the same time, the American College of Medical Genetics (ACMG) published recommendations in 2008 which included 6 interpretative categories of sequence variations for the purposes of clinical reporting (Richards *et al.* 2008). More recently, ACMG published updated standards and guidelines for the classification of sequence variants using criteria informed by expert opinion and empirical data (Richards *et al.* 2015).

The current joint guidelines published by ACMG and AMP include five classification categories in order of decreasing clinical significance: pathogenic, likely pathogenic, VUS, likely benign, benign. The guidelines defined 28 criteria that address evidence such as population data, case-control analyses, functional data, computational predictions, allelic data, segregation studies, and *de novo* observations. Each pathogenic criterion is weighted as very strong (PVS1), strong (PS1–4); moderate (PM1–6), or supporting (PP1–5), and each benign criterion is weighted as stand-alone (BA1), strong (BS1–4), or supporting (BP1-6). It is important to note that the numbering within each evidence category does not convey any differences of weight and is merely assigned to help refer to the different criteria. The criteria weight and direction are combined to arrive at one of the five classifications. For example, a variant that causes a nonsense or frameshift mutation in a gene where loss of function (LOF) is a known mechanism of disease is very strong evidence towards pathogenic classification (PVS1). A complete list of the ACMG-AMP criteria for classifying pathogenic and benign variants can be found in Appendix A. ACMG-AMP guidelines also

introduced a uniform nomenclature of variants to enable effective sharing and future use of genomic information. A standard gene variant nomenclature (http://www.hgvs.org/mutnomen) is maintained and versioned by the Human Genome Variation Society (HGVS). Due to the complex and evolving nature of variant analysis and interpretation ACMG-AMP strongly recommend that clinical molecular genetic testing be performed at a Clinical Laboratory Improvement Amendments (CLIA) certified laboratory with results being interpreted by a board-certified clinical molecular geneticist or equivalent.

1.1.8 Commercial laboratory variant assessment and classification scheme

Once a genetic variant is detected in the laboratory, its clinical significance must be determined. Although there are published standards and guidelines for the interpretation of sequence variants, adherence to these standards and guidelines is entirely voluntary and does not necessarily assure an accurate classification. Clinical laboratories have created, and in some instances published, their own classification schemes. Individual laboratory classification schemes often have significant overlap with the ACMG-AMP criteria. The variant classification scheme used for this study is detailed in Appendix B and published online by Ambry Genetics (https://www.ambrygen.com/file/material/view/272/Variant_ClassificationScheme_0617_Final.pdf). The scheme is an adapted version of the previously published ACMG-AMP standards and guidelines for the interpretation of sequence variants (Richards S *et al.* 2015). Genetic testing laboratories will often share the variant classification information if an inquiry is made. In addition to having a documented classification scheme, each

laboratory will have a documented variant review process. The variant review process generally involves collaboration between statisticians, bioinformaticians, variant scientists, structural biologists, genetic counselors and lab directors. Each of these individuals offers a unique perspective on variant analysis and interpretation. Most commercial laboratories offer varied approaches to VUS resolution including but not limited to proprietary variant databases, segregation studies, and collaboration with variant experts. Although the sequencing technology used across laboratories is similar, laboratories have individual VUS and reclassification rates. If a discrepant variant classification exists between laboratories, there is a working group hosted by an organization named ClinGen called "Sequence Variant Inter-Laboratory Discrepancy Resolution" which aims to resolve the discrepant variant classification. As of this year there are 19 commercial genetic testing laboratories that participate in this process (https://clinicalgenome.org/working-groups/sequence-variant-inter-laboratory-discrepancy-resolution/). One study found that nine laboratories who were asked to classify variants using either the ACMG-AMP system or their own criteria were in agreement on calls about one-third of the time (33/99) (Amendola *et al.* 2016). However, after either emails or conference calls among the reporting laboratories they were able to agree on 71% of calls (70/99). While this data is encouraging, this level of collaboration is not feasible for every variant call.

1.1.9 Importance of accurate variant classification

To facilitate accurate and uniform classification, laboratories are encouraged to share evidence and internal data used for classification when applicable. Similar to misinterpretation of a VUS result the inaccurate classification of a VUS can lead to

unnecessary treatment or surgery, anxiety and/or false reassurance (Makhnoon *et al.* 2019, O'Neill C. *et al.* 2009). Previous studies have shown a majority of VUS reclassifications are downgrades to less severe classifications, but VUS reclassification to pathogenic variant or likely pathogenic variant can lead to drastic medical management changes that accompany serious implications for patients (Macklin *et al.* 2018, Mersch *et al.* 2018). For example, management for women with a pathogenic variant in *BRCA1/2* can include a variety of options from high-risk surveillance to prophylactic removal of the ovaries and/or breasts. With the implementation of genetic testing into the medical management paradigm there is a risk for inaccurate variant calling and/or variant reclassification.

1.1.10 Prevalence of variant reclassification following hereditary cancer genetic testing

As previously discussed, the clinical implications of accurate variant interpretation are substantial. Therefore, it is crucial that new data and research related to variant interpretation for cancer-risk genes is reviewed by genetic testing laboratories on a periodic and consistent basis. One study analyzed variant reclassifications for individuals who underwent single-syndrome or multigene panel cancer genetic testing for hereditary cancer predisposition at a single laboratory over a 10-year period (Mersch J *et al.* 2018). They found that of the 26,670 unique VUSs reported during that time period, 2,048 (7.7%) were reclassified, which lead to 24.9% of the cohort receiving an amended clinical report. Importantly, the overwhelming majority (91.2%) of the VUSs were downgraded to benign variant or likely benign variant. Another study analyzed test reports of patients tested at the Mayo Clinic in Florida between September 2013 and February 2017 and found that

11.3% (30/266) of the VUSs were reclassified (Macklin *et al.* 2018). Again, a majority (around 75%) of the reclassified VUSs were downgraded to likely benign.

Overall, the evidence to date suggests that most VUS reclassifications are downgrades to likely benign or benign. However, in the event of a VUS being upgraded to pathogenic or likely pathogenic, a patient's individual cancer risks and management strategies should be discussed with their care team. Sharing of data and variant classifications between laboratories can increase knowledge on how specific genetic variants impact a patient's health.

Given the possibility of a VUS being reported in a gene for which cancer risks are not well-defined, several registries have been created to catalog and curate these variants with the goal of advancing our knowledge about their clinical utility. The Prospective Registry of Multiplex Testing (PROMPT) is a multi-institutional online registry that encourages patients to self-enter information about their genetic testing results and to complete questionnaires about their personal medical and family histories (http://promptstudy.info/). Other resources related to variant classification include ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) Consortium and ClinVar, a peer-reviewed database funded by the National Institutes of Health, which is a freely available archive of reports of relationships among medically important variants and phenotypes. Multiple professional societies have adopted positions in favor of data sharing (American Medical Association 2013, National Institutes of Health 2014, National Society of Genetic Counselors 2015, ACMG Board of Directors 2017).

1.2 Purpose of Study and Specific Aims

Using a retrospective database review of variant reclassifications in 15 breast cancer predisposition genes from January 2014 to January 2018, this study aims to identify and compare the criteria used for reclassification of VUSs in HBRGs and MBRGs. Additionally, this study aims to compare the triggers that lead to reclassification of VUSs in HBRGs and MBRGs. I hypothesize that there will be a significant difference in the criteria used for VUS reclassification when comparing HBRGs and MBRGs. I hypothesize that these differences are likely due to the availability of specific criteria, such as functional data, as well as the prevalence of certain variants. I also hypothesize that there will be a significant difference seen when looking at the triggers that lead to VUS reclassification when comparing HBRGs and MBRGs; because implications of a pathogenic variant in a HBRG are more likely to significantly impact clinical management than are pathogenic variants in a MBRG, clinicians may be more motivated to push for reclassification of variants in HBRGs.

This study will also examine and quantify the time between original VUS classification and reclassification. I hypothesize that there will be a significant difference in time to reclassification for variants in HBRGs versus MBRGs. I predict time to reclassification for VUSs in HBRGs will be significantly shorter than that in MBRGs. An additional aim of this study will be to highlight the importance of specific criteria such as functional studies, family studies and clinical phenotypes towards VUS reclassification. Finally, the overall purpose of this study is to provide a better understanding of the variant reclassification process for patients and healthcare providers.

II. METHODS

2.1 IRB approval

This study was determined to be non-human subject research by the University of California, Irvine (UCI) Institutional Review Board (IRB) under application HS#: 2018-4835 (Appendix C).

2.2 Collection of VUS reclassifications over study period

Individual variants were queried and collected by the lead author using a proprietary DNA sequence variant database from a single commercial genetic testing laboratory, Ambry Genetics, from January 2014 to January 2018. The variants analyzed are stored in a secure proprietary database that is accessible only by password-protected log-in. Each variant is annotated with relevant meta-data, including information on variant genomic location, evidence supporting classification, nucleotide alignment, disease information, population frequency, etc.

Variants were included if they were identified in one of 15 breast cancer predisposition genes and were reclassified from VUS to either pathogenic, VLP, VLB, or benign during the aforementioned time period. The genes included were separated into two breast cancer risk categories; high (HBRGs) and moderate (MBRGs). HBRGs included *BRCA1*, *BRCA2*, *CDH1*, *NF1*, *PTEN*, *TP53*; associated cancer risks range from 5-9 times that of the general population (Brose MS *et al.* 2002, Kuchenbaecker KB *et al.* 2017, Levy-Lahad E *et al.* 2007, Suarez-Kelly *et al.* 2019). MBRGs included *ATM*, *BARD1*, *BRIP1*, *CHEK2*, *NBN*, *PALB2*, *RAD50*, *RAD51C*, and *RAD51D;* associated cancer risks range from 2-

4 times that of the general population (Couch, F.J. *et al.* 2017, Hollestelle, A *et al.* 2010, Tung *et al.* 2016). The variants included were identified in patients in whom clinical genetic testing was ordered by a health care provider and performed between 2012 and 2017. These variants were then further queried to identify those with a non-VUS classification as of February 2019. A complete list of all VUSs that were reclassified between January 2014 and January 2018 was generated and each variant was determined to have an outcome of either upgrade or downgrade. An upgrade was defined as a VUS being reclassified to either a likely pathogenic or pathogenic. A downgrade was defined as a VUS being reclassified to a likely benign or benign. In total 900 variants met these inclusion criteria and were available for review. This number reflects all of the VUSs that were reclassified from January 2014 to January 2018. Any variant that was reclassified outside of the specified time period or was reclassified for less than 24 hours was disqualified and not included in the data set. A total of 283 variants were excluded on the basis of these criteria, leaving 617 variants.

2.3 Collection of VUS reclassification drivers and triggers

A data collection sheet was generated to review each reclassified variant, and only this data collection sheet was used for subsequent analyses. No protected health information (PHI) was documented on the data collection sheet. For each variant the following information was collected: gene name, coding DNA sequence change(c.), predicted protein sequence change (p.), variant classification as of February 2019, original VUS classification date, reclassification date, reclassification trigger,

reclassification criteria (driver), and discrepant classification in a public variant database (ClinVar)(https://www.ncbi.nlm.nih.gov/clinvar/).

Using ACMG-AMP guidelines and documented internal laboratory procedures for adaption to hereditary cancer predisposition testing, the category of evidence and criteria used for reclassification was documented (Appendix A). The evidence categories consisted of very strong evidence for pathogenicity (PVS1), strong to moderate evidence for pathogenicity (PS1-4, PM1-6), supporting evidence for pathogenicity (PP1-5), very strong evidence for benign (BA1), strong to moderate evidence for benign (BS1-4), and supporting evidence for benign (BP1-6). There are specific criteria that are assigned to each evidence category, for example, in very strong evidence for pathogenicity the following criteria is listed: Null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where loss of function is a known mechanism of disease. In each evidence category there is a criterion that states "Other […] data supporting […] classification." If this criterion was cited more details were collected regarding the "other data". For example, a number of variants were reclassified due to updates in classification evidence and/or weighting which was documented as "other data" criteria. A complete list of "other" criteria is listed in Appendix D.

The event that triggered the reclassification process was also documented for each variant. There are several events that can trigger the reclassification process including but not limited to: availability of new research or data, periodic re-review of evidence, or

provider request. A complete list of all the reclassification trigger categories investigated is available in Appendix E.

The error rate of collected data was determined by randomly selecting 11.2% of the variants for re-collection. All data on 69 variants (randomly distributed across all 15 genes) were re-collected for all variables. Initially collected variant data was compared to re-collected data to determine the data collection error rate.

2.4 Data Analysis

Data was analyzed using qualitative and quantitative analyses techniques. Frequencies of variant information were calculated for categorical and binomial variables using Microsoft® Office Excel. Chi-square values were calculated using IBM SPSS®. Logistic regression analysis was performed using RStudio. For all statistical analyses $p<0.05$ were considered statistically significant.

2.4.1 VUS reclassification direction over study period

A chi-square test was performed to determine if there was a significant association between gene category (HBRG or MBRG) and VUS reclassification direction (upgrade or downgrade). The number of upgrades and downgrades seen each year was calculated and was further stratified by category of gene, HBRG or MBRG. A logistic regression model was used to quantify the strength of the association between reclassification outcome (upgrade or downgrade) and the independent variate of reclassification year (2014, 2015, 2016 or 2017), separately for reclassified variants in HBRGs and MBRGs. It was assumed that every

variant in each year had an equal opportunity for reclassification. Downgrade was coded as the reference level and upgrade was the outcome of interest. For each group of cancer risk genes, HBRG and MBRG, the odds ratio of upgrade was reported along with a 95% confidence interval and corresponding p-value. The odds ratio was considered to be statistically significant if the confidence interval excluded the value 1, or equivalently if $p<0.05$. The year 2018 was excluded from the regression analysis due to low counts (n=7). A summary of the logistic regression model is provided in Appendix F.

2.4.2 Frequency of discrepant classification

Each reclassified variant was queried in a public variant database, ClinVar, to determine if there was a discrepancy between the variant call in the dataset and the call made by at least one other laboratory that was present in ClinVar as of February 2019. The presence of at least one discrepant classification from another laboratory was counted as discrepant, regardless of the number of other concordant classifications. The frequency of discrepant classification was quantified as well as the level of discrepancy. If the commercial laboratory that provided the variant information for this study called a variant likely benign and another laboratory in ClinVar called the variant a VUS or benign, this was counted as one level of discrepancy. The levels of discrepancy collected included 1, 2, and 3.

2.4.3 Differences in time to VUS reclassification

Time to reclassification was calculated from date of initial classification as a VUS to date of variant reclassification. For reclassified variants in HBRGs and MBRGs minimum value, maximum value, and mean were calculated to generate a box and whisker plot.

Additionally, an independent t-test was performed to analyze and compare average time to VUS reclassification in HBRGs versus MBRGs.

III. RESULTS

3.1 Direction of VUS reclassification over study period

A retrospective variant database review of VUSs that were reclassified from January 2014 to January 2018 was conducted to quantify if there was a difference in the reclassification direction as well as if there were any differences in the criteria used for reclassification depending upon the risk category of the gene (HBRG or MBRG). A total of 617 variants in 15 genes that were reclassified between January 2014 and January 2018 were reviewed.

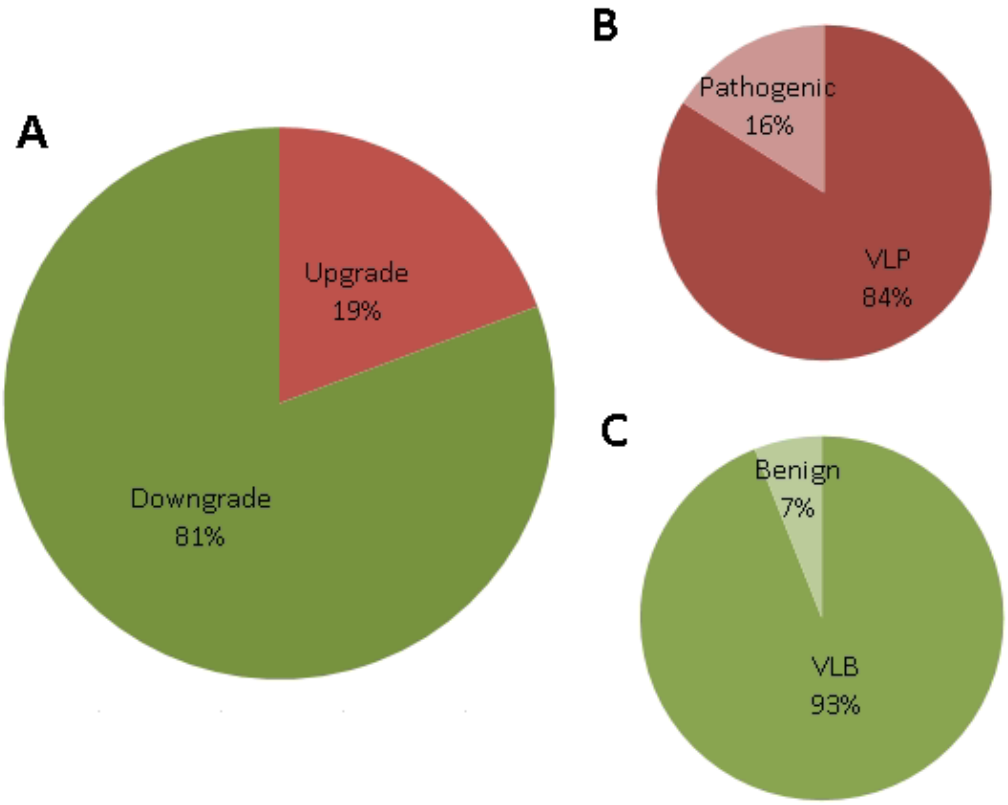


Figure 1. **Overall distribution of VUS reclassifications.** A) Green colors indicate variant downgrades; red colors indicate variant upgrades. Percentage reflects percentage of total reclassified variants. B) Shades of red reflect variants that were upgraded to either likely pathogenic (dark red) or pathogenic (light red). Percentage reflects percentage of total variants that were upgraded. C) Shades of green indicate variants that were downgraded to either likely benign (dark green) or benign (light green). Percentage reflects percentage of total variants that were downgraded.

The majority of reclassifications were downgrades, 81% (498/617) (Figure 1). Within the downgrades almost all of the variants were downgraded to VLB, 94% (468/498). Within the upgrades the majority of the variant reclassifications were to VLP, 84% (100/119).

BRCA2 had the greatest number of reclassified variants (195/640), followed by *BRCA1* (100/640) (Figure 2). Reclassified variants in MBRGs made up about 28% (220/617) of the total reclassifications. Reclassified variants in HBRGs accounted for the rest of the reclassifications, about 71% (397/617).

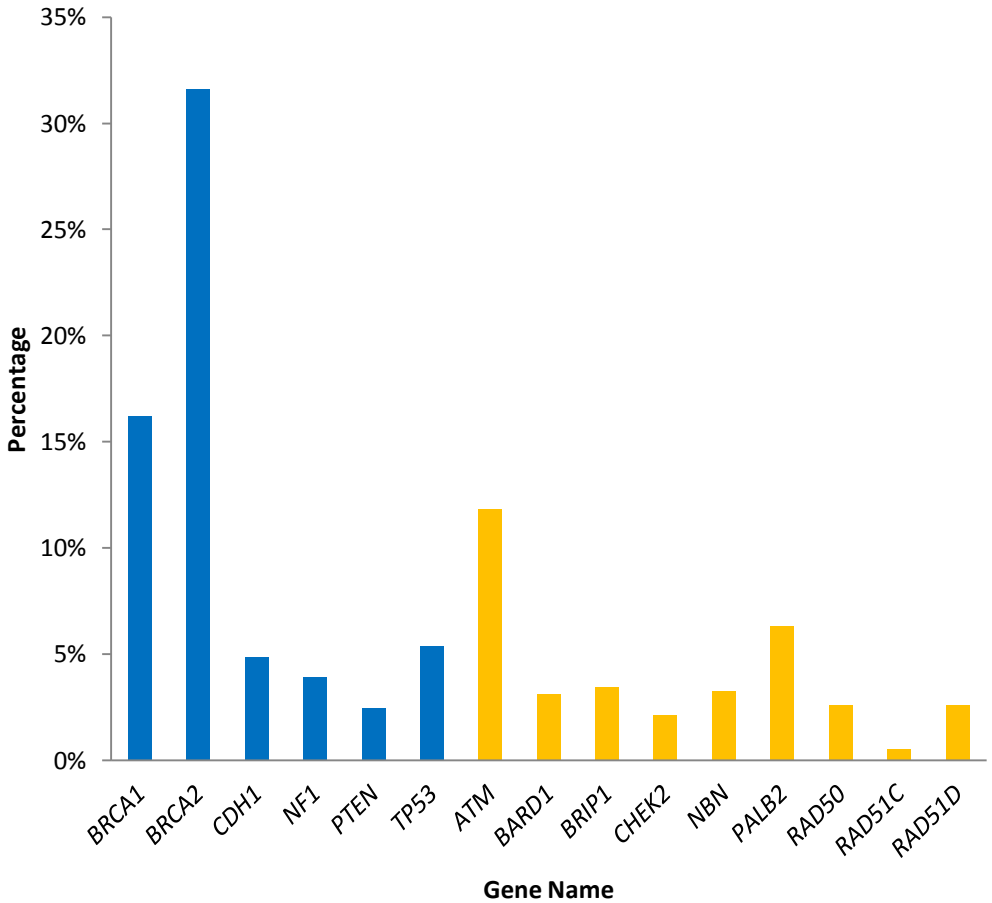


Figure 2. **Gene specific distribution of VUS reclassifications.** Blue colors indicate HBRGs; yellow colors represent MBRGs. Percentage reflects percentage of total reclassified variants.

The number of upgrades and downgrades of reclassified variants in HBRGs and MBRGs were compared (Figure 3.1). For reclassified variants in both HBRGs and MBRGs a majority of reclassifications resulted in downgrades (79% and 83%, respectively). There was no statistically significant difference in the number of variants upgraded or downgraded when comparing reclassified variants in HBRGs and MBRGs.

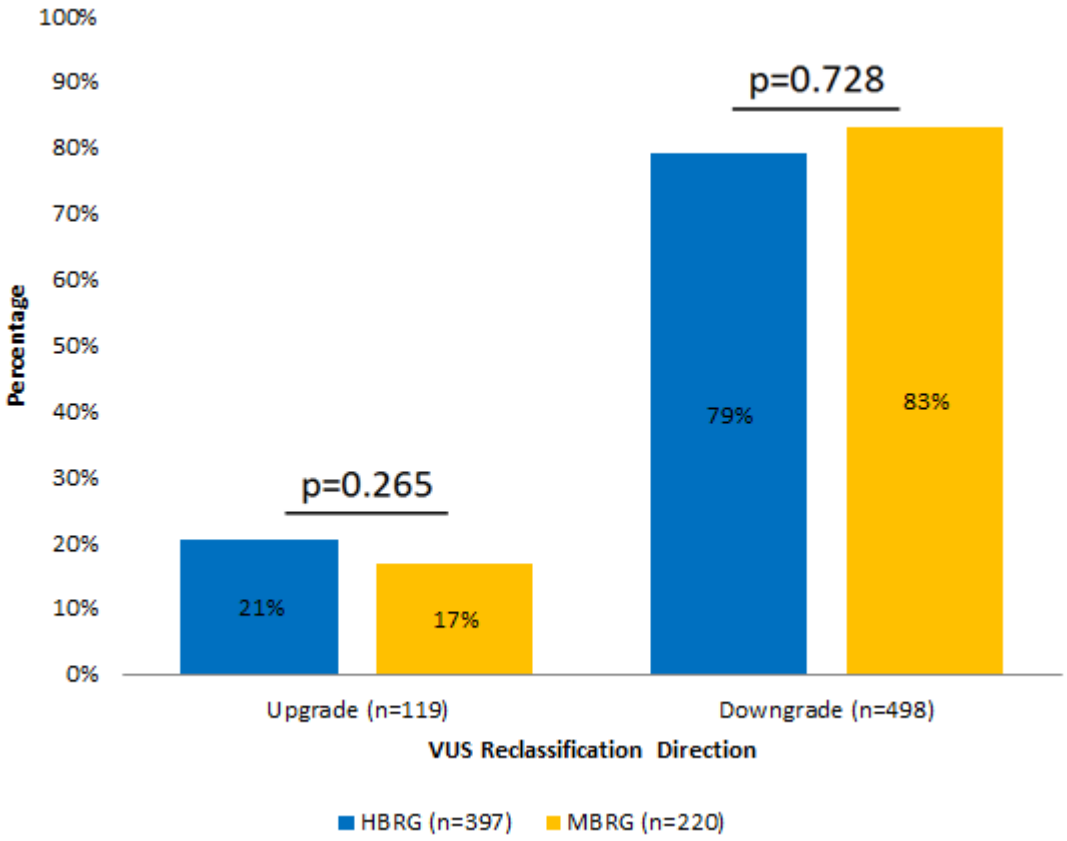


Figure 3.1 **Upgrade and downgrade frequency of VUSs in HBRGs and MBRGs.** N equals the number of variants in each group. Percentages reflect percentage of HBRGs and MBRGs respectively.

Additionally, the frequency of upgrades and downgrades was further calculated for each gene. Nearly all (14) of the genes included had a higher frequency of variant downgrades (Figure 3.2). Only one gene, *PTEN,* was shown to have a higher frequency of variant upgrades, 53% (8/15). In particular, the genes with the highest reclassified variant

counts, *BRCA1* (100/617) and *BRCA2* (195/617) showed variant downgrade frequencies of 75% or higher. Two genes, *BARD1* and *NBN,* exclusively had variant downgrades.

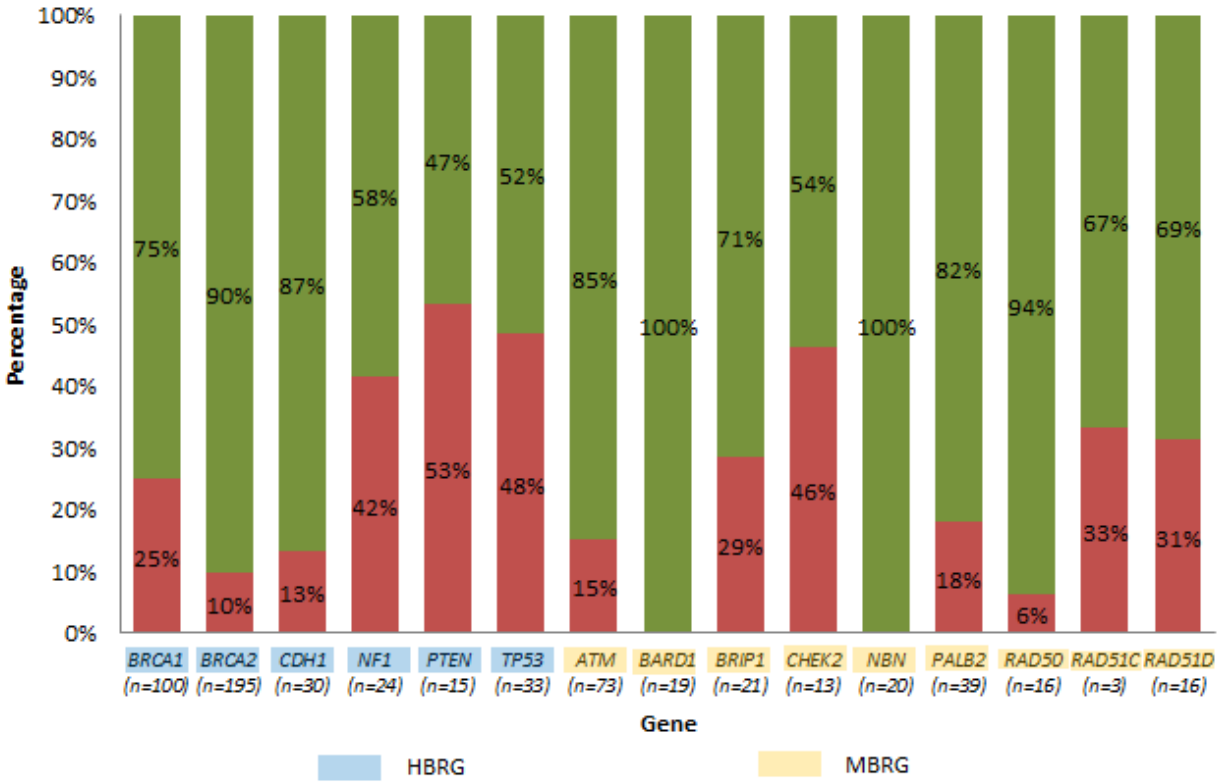


Figure 3.2 **Frequency of VUS reclassification upgrade or downgrade per gene.** Green colors indicate downgrades; red colors represent upgrades. N equals the number of variants in specified gene. Percentages reflect percentage of upgrades and downgrades of variants in specified gene.

The upgrade and downgrade frequency for reclassified VUSs in HBRGs and MBRGs was further analyzed by year of reclassification (Figure 4.1 and Figure 4.2). For reclassified VUSs in HBRGs the highest incidence of upgrades was in the year 2017, with 33% of reclassifications that year being upgrades (42/129). Alternatively, the year 2014 had the lowest incidence of upgrade for reclassified variants in HBRGs (8%, 7/86). The largest difference in frequency of upgrades between consecutive years was seen between 2016 and 2017 with a difference of 13%.

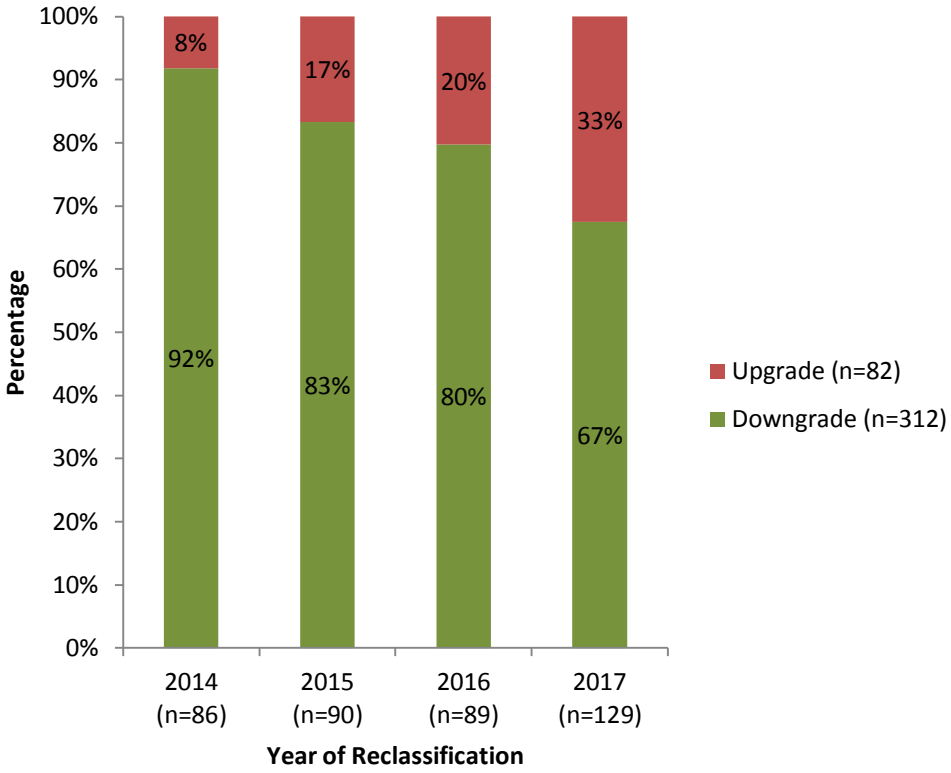


Figure 4.1 **Frequency of VUS upgrade and downgrade in HBRGs per year.** N equals the number of reclassified variants in each year. Percentages reflect percentage of all reclassification upgrades and downgrades in specified year.

For reclassified variants in MBRGs, the highest incidence of upgrades was seen in the year 2017 at 21% (17/82), with the year 2015 following closely behind at 20% (8/40). The years 2014 and 2016 had the lowest incidence of upgrades at 11% each (2/18 and 8/76 respectively).

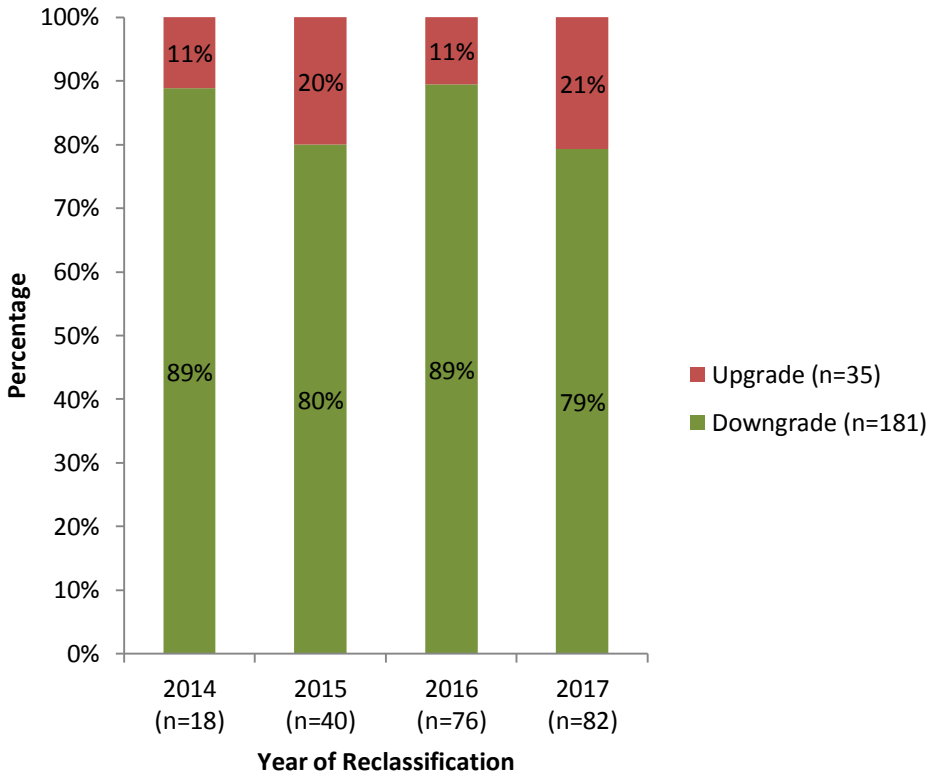


Figure 4.2 **Frequency of VUS upgrade and downgrade in MBRGs per year.** N equals the number of reclassified variants in each year. Percentages reflect percentage of all reclassification upgrades and downgrades in specified year.

Logistic regression was used to model the odds of variant upgrade based on the year of variant reclassification, with separate models generated for HBRGs and MBRGs. The regression models showed an increase in the odds of upgrade for VUSs that were reclassified over the specified period (January 2014 to December 2017) (Table 1.1 and Table 1.2). The odds of upgrade for reclassified VUSs in HBRGs were 1.68 times higher each year of the study (CI 1.33 - 2.13). For reclassified VUSs in MBRGs, the odds of upgrade were 1.17 times higher each year but this finding was not statistically significant (CI 0.79 - 1.74).

Table 1.1 **Logistic regression analysis for VUS reclassification in HBRGs.**

Independent variable	*Z value*	*p*	*OR (upgrade)*	*95% CI*
Year of Reclassification	4.28	**<0.001**	1.68	1.33 – 2.13

Table 1.2 **Logistic regression analysis for VUS reclassification in MBRGs.**

Independent variable	*Z value*	*p*	*OR (upgrade)*	*95% CI*
Year of Reclassification	0.783	0.433	1.17	0.79 – 1.74

3.2 Criteria used in VUS reclassification

One aim of this study was to quantify the criteria used for VUS reclassification. Table 2 details the criteria cited most frequently for VUS reclassification. The most common criteria cited were in the "strong to moderate evidence" for benign category, 43.6% (269/617). Within this evidence category, the criteria cited most often was "*BP4_strong/BS1* Other strong data supporting benign classification" (222/617) with "*BP2_strong* Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern" being cited second most frequently (28/617). "Supporting evidence for benign" was the second most frequent evidence category cited at 36.1% (223/617). Within this evidence category, "*BP6/BP4_strong/BS4/BS4_supporting* Other data supporting benign classification" was cited most frequently at 10.5% (65/617). The third evidence category cited most often was "strong to moderate evidence" of pathogenicity at 17.2% (106/617). "*PM1/5* Other moderate data supporting pathogenic classification" was the criteria cited most frequently within this evidence category at 5.3% (33/617). Criteria referred to as

"[…] Other […]" was frequently cited for VUS reclassification. Table 3 shows the frequency and further details of "other" data cited.

Table 2. **Distribution of criteria used for VUS reclassification.** N equals the number of variants included in the study. Percentages reflect percentage of total evidence used. This chart has been modified from La Duca *et al.* 2015

	Evidence Category & Criteria	2014 to 2018 (n=617) n (%)
Pathogenic	**Very Strong Evidence**	**1 (0.2%)**
	PVS1 In-frame gross deletion with loss of an important functional domain	1 (100%)
	Strong to Moderate Evidence	**106 (17.2%)**
	PS2/PM6 De novo (both maternity and paternity confirmed) in a patient with the disease and no family history or assumed *de novo*, but without confirmation of paternity and maternity	10 (1.6%)
	PS3 Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product (splicing alteration)	15 (2.4%)
	PS4 The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls	0 (0%)
	PP4 Patient's phenotype or family history is highly specific for a disease with a single genetic etiology	17 (2.8%)
	PVS1_strong/ PVS_ moderate Last nucleotide of exon	0 (0%)
	PP1_strong Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease	3 (0.5%)
	PS3_moderate Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product	25 (4.1%)
	PM5 Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before	2 (0.3%)
	PM1/5 Other moderate data supporting pathogenic classification	33 (5.3%)
	PVS1_strong/ PVS1_moderate Alterations at the canonical donor/acceptor sites without other strong evidence supporting pathogenicity	1 (0.2%)
Likely Pathogenic (VLP)	**Supporting Evidence**	**11 (1.8%)**
	PM2 Absent from controls in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium	0 (0.0%)
	PP3 Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)	0 (0.0%)
	PP1_moderate Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease	0 (0.0%)
	PM1_supporting Other data supporting pathogenic classification	11 (1.8%)

Likely Benign (VLB)	**Supporting Evidence**	**223 (36.1%)**
	BS3 Intact protein function observed in appropriate functional assay(s)	19 (3.1%)
	BP2 Co-occurrence with mutations in the same gene (phase unknown)	52 (8.4%)
	BP5 Variant found in a case with an alternate molecular basis for disease	17 (2.8%)
	BS1_supporting Allele frequency is greater than expected for disorder, but not statistically significant	52 (8.4%)
	BP4 Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.)	1 (0.2%)
	BS4_supporting Lack of segregation in affected members of a family in gene with incomplete penetrance	11 (1.8%)
	No disease association in a small case-control study	6 (1.0%)
	BP6/BP4_strong/BS4/BS4_supporting Other data supporting benign classification	65 (10.5%)
	Strong to Moderate Evidence	**269 (43.6%)**
	BS3 Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing	19 (3.1%)
	BP2_strong Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern	28 (4.5%)
	BP4_strong/BS1 Other strong data supporting benign classification	222 (36.0%)
Benign	**Very Strong Evidence**	**7 (1.1%)**
	BA1 Allele frequency is >5% in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium	2 (0.3%)
	BS4 Lack of segregation in affected members of a family in gene with complete penetrance	0 (0.00%)
	BA1/BS1 Allele frequency is greater than expected for disorder and is statistically significant	3 (0.5%)
	No disease association in appropriately sized case-control study(ies)	2 (0.3%)

"[…] Other […]" was the criteria cited most often. Parsing this data further, we see that "*BP4_strong* Amino acid is reference allele in ≥3 primates OR ≥15 mammals" was the criteria cited most often at 63.1% (209/331). "*BP6* Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation" was cited second most often at 17.2% (57/331). The third criteria cited most often was "*PM1/5* Computational data, including structural analysis and/or modeling" at 7.6% (25/331).

Table 3. **Distribution of "Other" evidence used in VUS reclassifications.** Bolded percentages, rounded to the nearest tenth, reflect percent of the total variants. Non-bolded percentages, rounded to the nearest tenth, reflect percent of each bolded category. N equals the number of variants.

Criteria Cited	2014 to 2018 (n=331) n (%)
Other moderate data supporting pathogenic classification	**33 (10.0%)**
PM1/5 Mutational hotspot, computational data, including structural analysis and/or modeling	25 (75.8%)
Updated classification evidence and/or weighting	8 (24.2%)
Other data supporting pathogenic classification	**11 (3.3%)**
PM1_supporting Computational data, including structural analysis and/or modeling	11 (100.0%)
Other strong data supporting benign classification	**222 (67.1%)**
BP4_strong Amino acid is reference allele in ≥3 primates OR ≥15 mammals	209 (94.1%)
BS1 Internal allele frequency greater than expected based on disease prevalence	3 (1.4%)
Updated classification evidence and/or weighting	10 (4.5%)
Other data supporting benign classification	**65 (19.6%)**
BP6 Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation	57 (87.7%)
BP4_strong Amino acid is reference allele in ≥3 primates OR ≥15 mammals	3 (4.6%)
BP4 Computational data, including structural analysis and/or modeling	3 (4.6%)
BS4_supporting Multifactorial models using likelihood ratio of pathogenicity for segregation*	2 (3.1%)

*International Agency for Research on Cancer (IARC) and Open Variation Database (LOVD) (https://www.lovd.nl/)

Within each evidence category, i.e. very strong evidence, moderate evidence, supporting evidence, etc., a comparison between HBRGs and MBRGs was performed. There was no significant difference in the frequency of criteria used in the evidence category "Very strong evidence for pathogenicity" between reclassified variants in HBRGs and MBRGs. A statistically significant difference was seen for two criteria cited in the "Strong to moderate evidence of pathogenicity" evidence category "*PS2/PM6*" and "Updated classification evidence and/or weighting" (p=0.033 and p=0.042 respectively). "*PS2/PM6 De novo* in a patient with the disease and no family history", was never cited as criteria for reclassification for variants in MBRGs (0%).

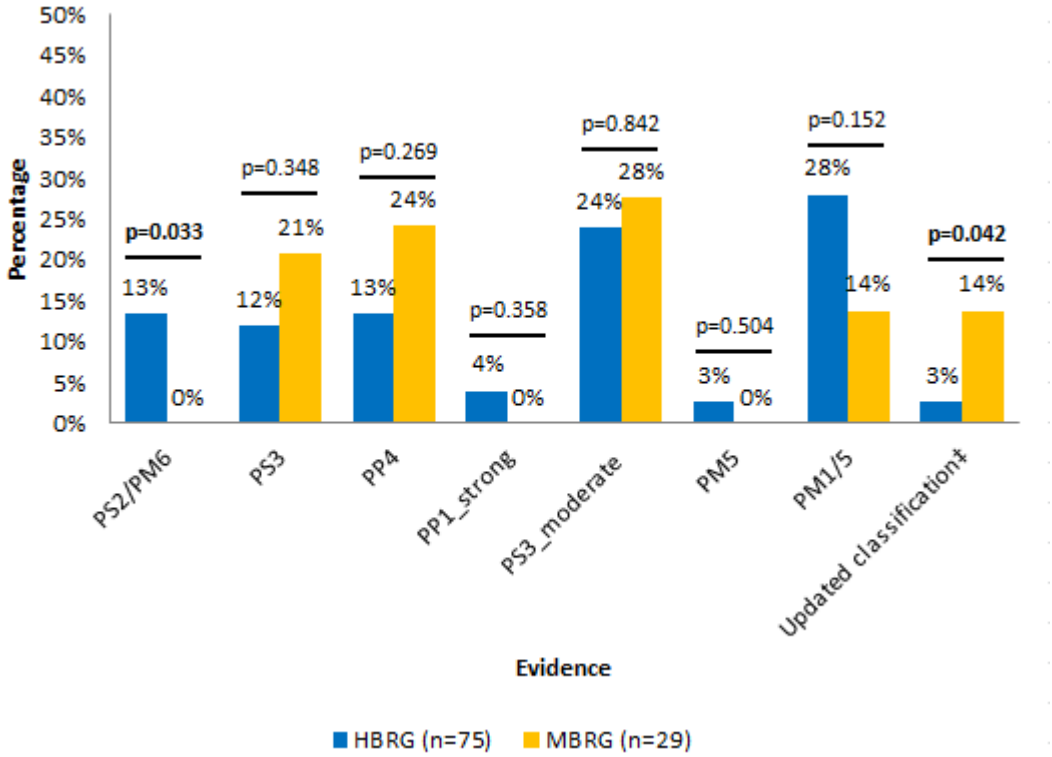


Figure 5. **Frequency of "strong to moderate evidence of pathogenicity" used for VUS reclassification in HBRGs and MBRGs.** N equals the number of variants. Percentages reflect frequency of criteria used for each category of genes. P-values in bold indicate statistical significance.

‡: Updated classification evidence and/or weighting
*See Table 2 for a full description of ACMG evidence criteria codes

A chi-square test was performed for evidence category "Strong to moderate evidence for benign" to analyze differences between reclassified variants in HBRGs and MBRGs. "Updated classification evidence and/or weighting" was cited significantly more often for reclassified variants in HBRGs (6%) as compared to reclassified variants in MBRGs (1%) (p=0.040). Of note, "*BP4_strong* Amino acid is reference allele in ≥3 primates OR ≥15 mammals" was cited frequently for reclassified variants in both HBRGs and MBRGs. All other criteria cited in this evidence category were not found to have a statistically significant difference in frequency.

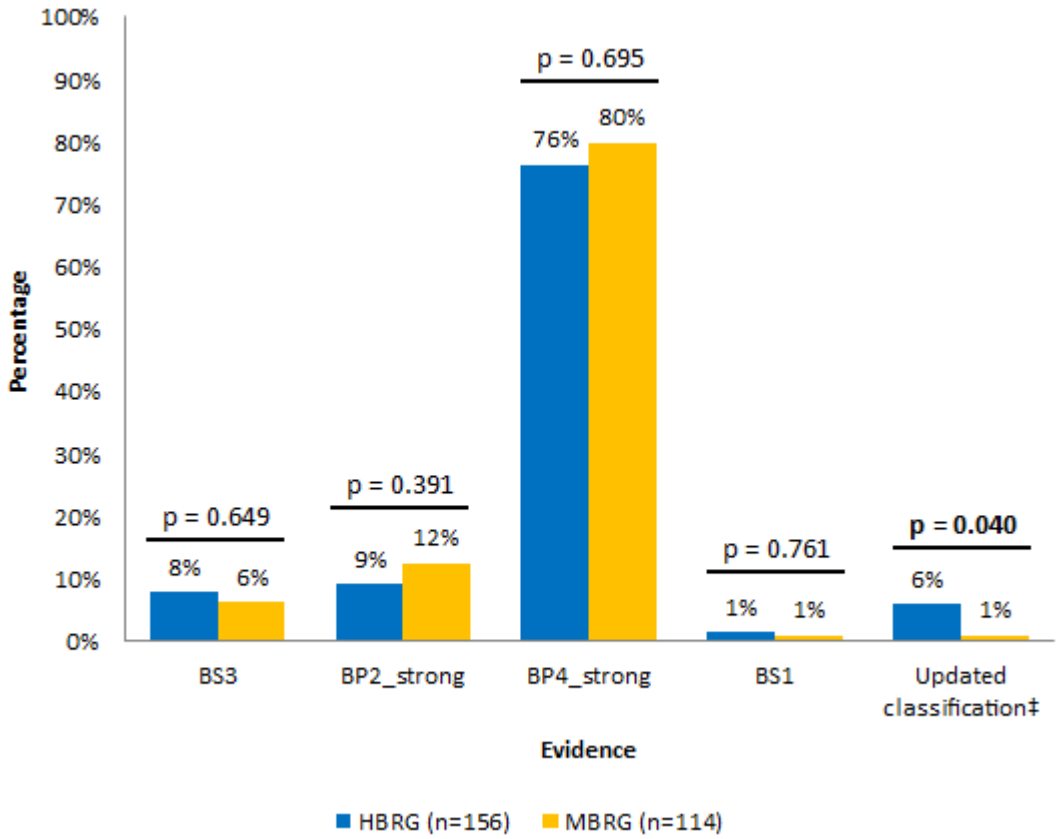


Figure 6. **Frequency of "strong to moderate evidence for benign" used for VUS reclassification in HBRGs and MBRGs.** N equals the number of variants. Percentages reflect frequency of criteria used for each category of genes. P-values in bold indicate statistical significance.

‡: Updated classification evidence and/or weighting
*See Table 2 for a full description of ACMG evidence criteria codes

A chi-square test was performed for evidence category "Supporting evidence for benign" to analyze differences between reclassified variants in HBRGs and MBRGs. Three criteria showed statistically significant differences in frequency between reclassified variants in HBRGs and MBRGs; "*BS1_supporting*", "*BP6*", and "*BS3*" ($p<0.001$ for both *BS1* and *BP6*; $p=0.021$ for *BS3*). "*BS1_supporting* Allele frequency is greater than expected for disorder" was cited more frequently for reclassified variants in MBRGs (56%) as compared to reclassified variants in HBRGs (10%). Alternatively, "*BP6* Reputable source recently

reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation" was cited solely for reclassified variants in HBRGs (37%). Finally, "*BS3* Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing" was cited more frequently for reclassified variants in HBRGs (10%) (p=0.021).

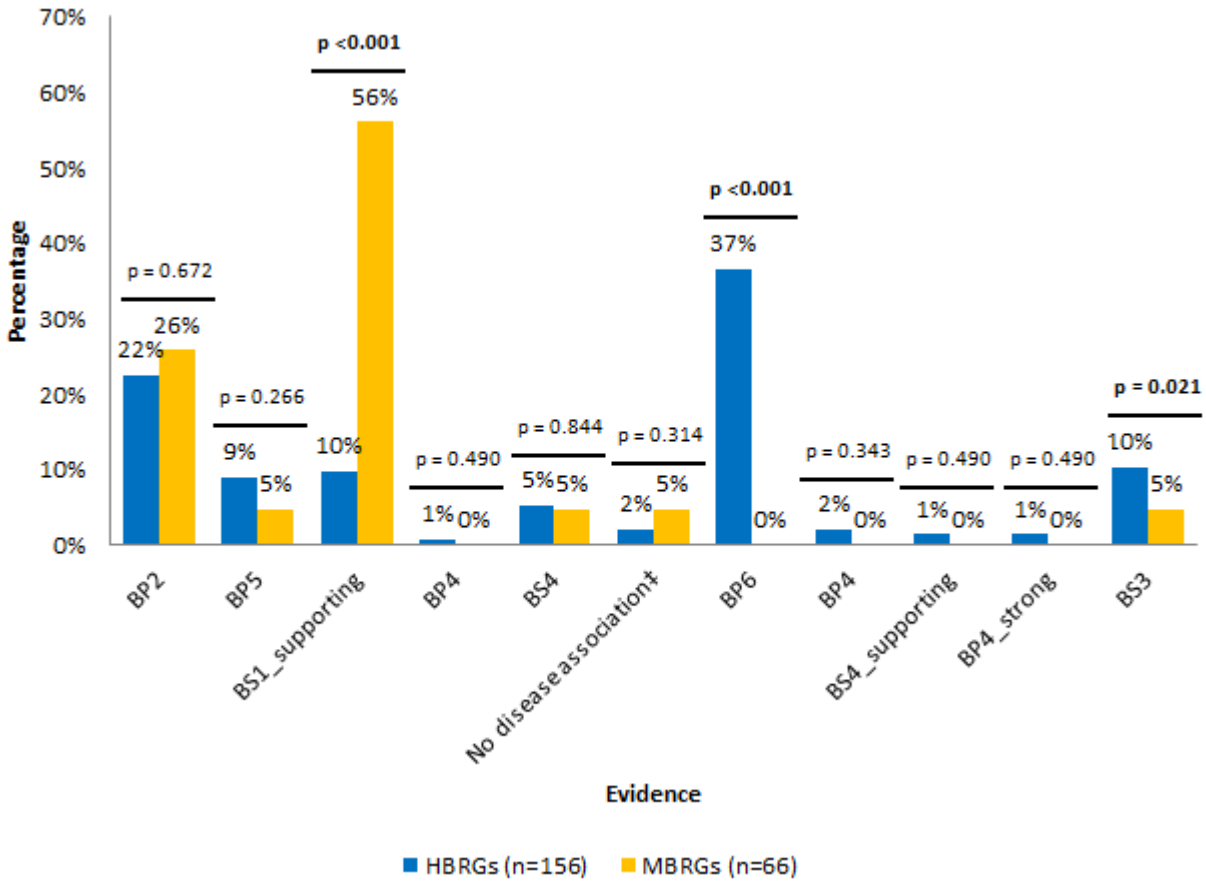


Figure 7. **Frequency of "supporting evidence for benign" used for VUS reclassification in HBRGs and MBRGs.** N equals the number of variants. Percentages reflect frequency of criteria used for each category of genes. P-values in bold indicate statistical significance.

‡: No disease association in a small case-control study
*See Table 2 for a full description of ACMG evidence criteria codes

3.3 Triggers that precede VUS reclassification

The frequencies of each trigger category, or events that triggered the reclassification process were calculated for reclassified variants in HBRGs and MBRGs. "Periodic re-review" and "New research/data available" were the triggers that were seen most often. Reclassified variants in MBRGs (58%) cited "Periodic re-review" significantly more than reclassified variants in HBRGs (45%) (p=0.034). Alternatively, "New research/data available" was cited significantly more often for reclassified variants in HBRGs (54%) versus MBRGs (38%) (p=0.005). "Variant identified in new patient sample" was cited significantly more often for reclassified variants in MBRGs (p=0.006).

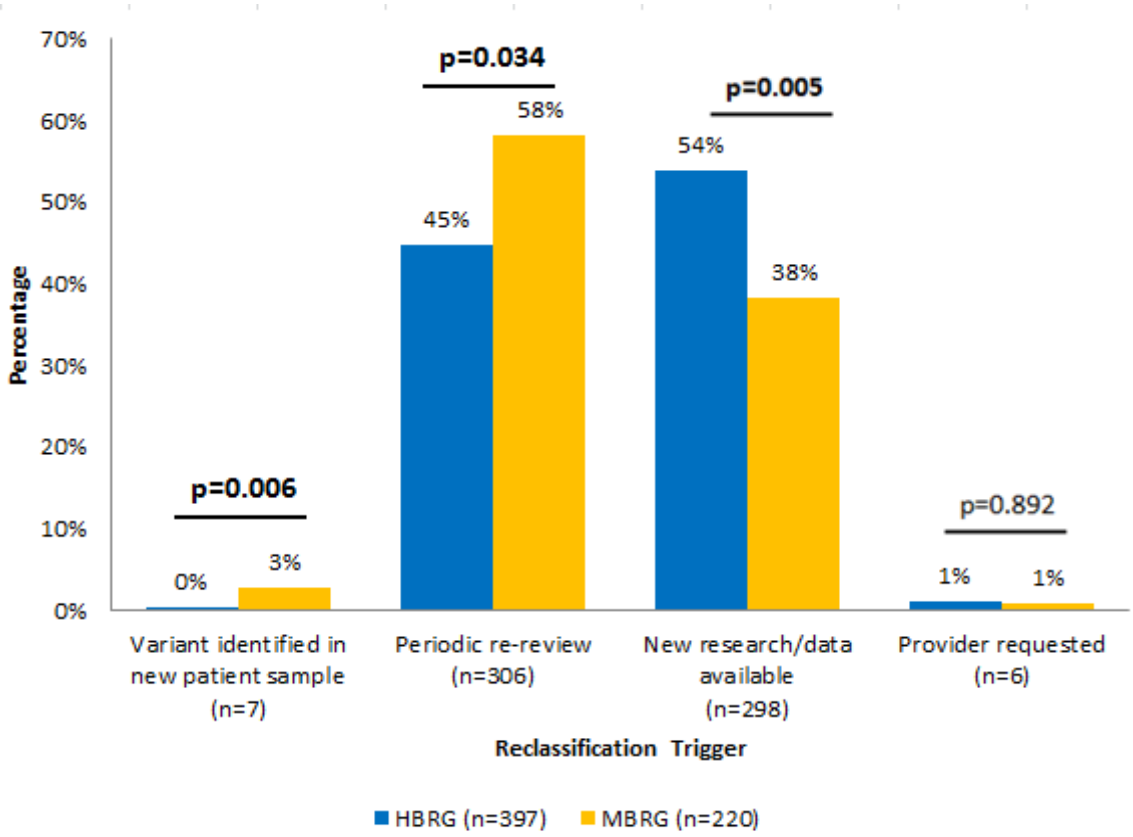


Figure 8. **Frequency of VUS reclassification triggers in HBRGs and MBRGs.** N equals the number of variants. Percentages reflect frequency of trigger used for each category of genes. P-values in bold indicate statistical significance.

3.4 Frequency of discrepant classification in public variant database

It was determined that a majority of the reclassified variants in this study (82%, 506/617) had a discrepant classification in ClinVar, as of February 2019, meaning that at least one classification from any other submitter was not equal to the classification given at the time of reclassification. The frequencies of discrepant classification between reclassified variants in HBRGs and MBRGs were compared, but there was no significant difference in the presence or absence of a discrepant classification (p=0.914 and 0.849, respectively).

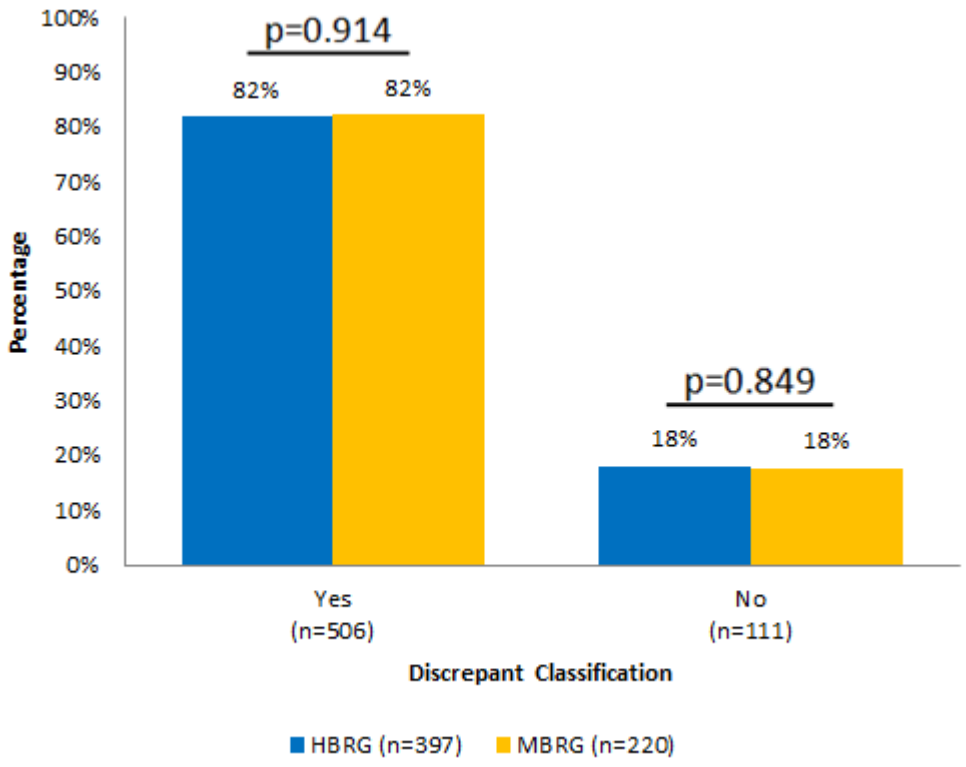


Figure 9. **Overall frequency of discrepant variant classification in HBRGs and MBRGs.** N equals the number of variants. Percentages reflect frequency of discrepant variant classification for each category of genes.

For reclassified variants with a discrepant classification, the level of discrepancy was calculated (Figure 10). A majority of the discrepant classifications seen were discrepancy level one (96%), meaning there was one level of classification difference between laboratories i.e., VUS vs. likely benign/likely pathogenic. There rest were discrepancy level two (4%), meaning there was two levels of classification difference between laboratories i.e., VUS vs. benign/pathogenic.

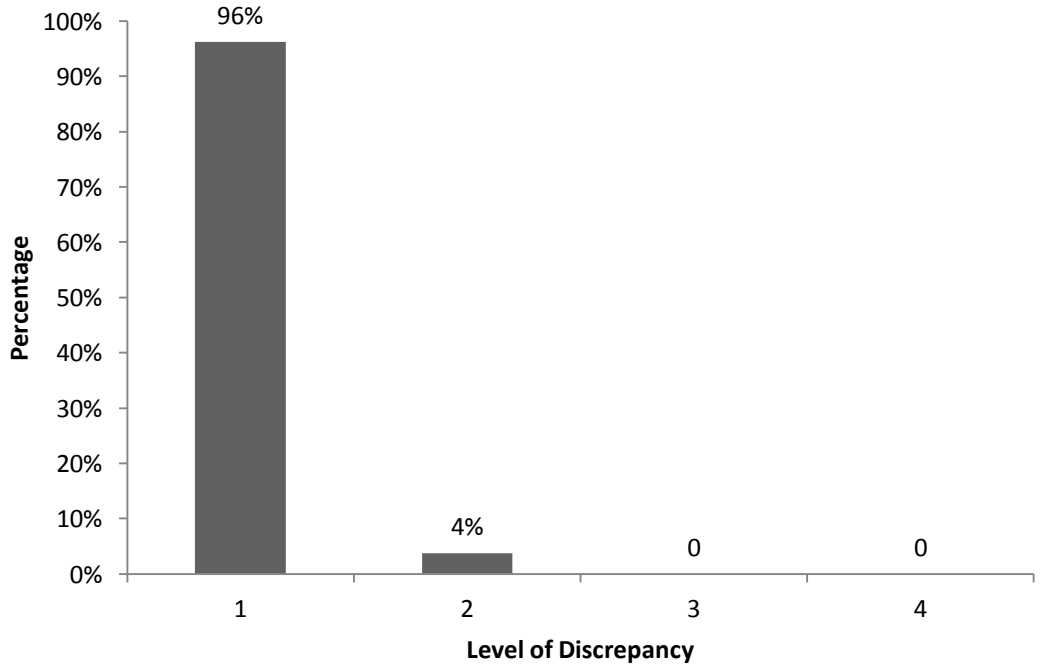


Figure 10. **Level of discrepancy for 506 (total number) variants with a discrepant variant classification as of February 2019.** Percentages reflect frequency of different levels of discrepant variant classification.

1: One level of discrepancy (ex. VUS vs. likely benign/likely pathogenic)
2: Two levels of discrepancy (ex. VUS vs. benign/pathogenic)
3: Three levels of discrepancy (ex. VLB vs. pathogenic)

The percentage of discrepant variant classifications (based on submissions in ClinVar as of February 2019) was also calculated for each year that variants were reclassified. The year of variant reclassification with the highest proportion of discrepant classifications observed in 2019 was 2014, with 94% (98/104), and the proportion of discrepant classifications decreased every year thereafter. The reclassification years that had the lowest proportion of discrepant classifications noted in 2019 were 2017 and 2018, with 71% in each year (150/211 and 5/7, respectively).

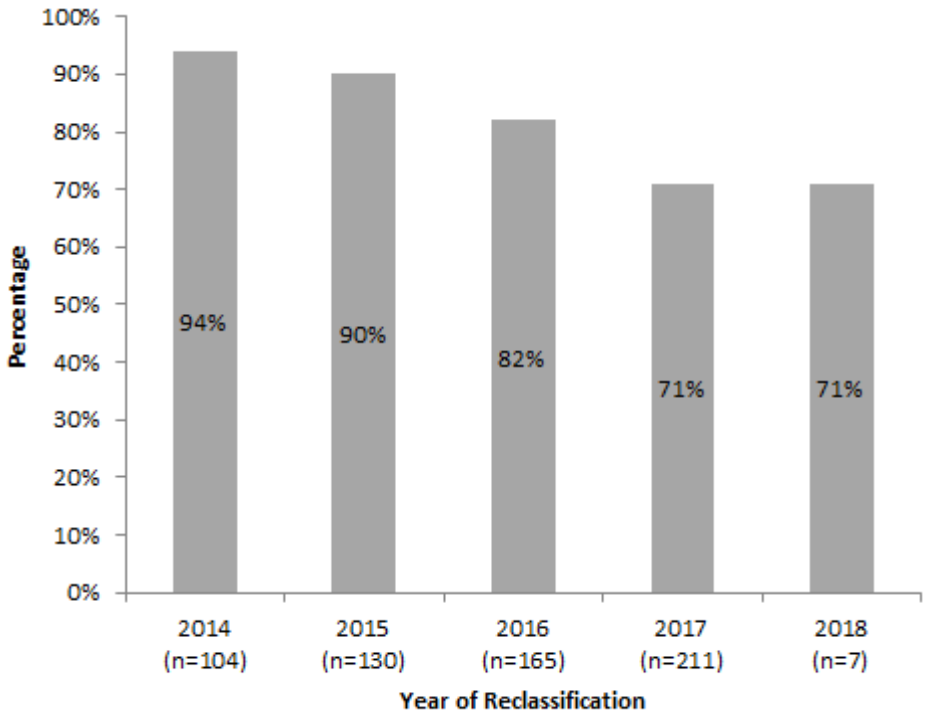


Figure 11. **Frequency of discrepant variant classification by year of reclassification.** Percentages reflect number of discrepant classifications (observed in February 2019) out of total variants reclassified in that year.

3.5 Average time to VUS reclassification

The mean time to reclassification differed for variants in HBRGs and MBRGs: 20.9 months vs. 25.9 months, respectively ($p<0.001$). The overall median time to reclassification was 20.6 months with the median time to reclassification for variants in HBRGs being 18.7 months and 23.5 months for variants in MBRGs.

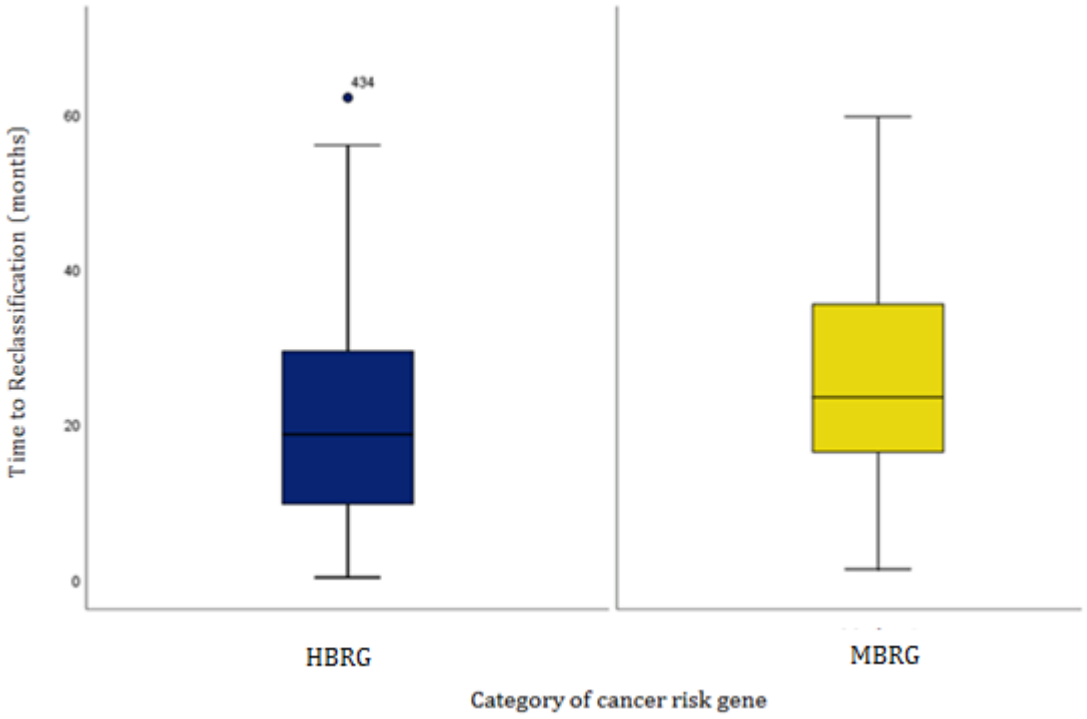


Figure 12. **Average time to VUS reclassification in HBRGs and MBRGs.** Blue color indicates HBRGs; yellow color represents MBRGs. Box-plot diagram showing the time to VUS reclassification in HBRGs and MBRGs. The black center line denotes the median value (50th percentile), while the blue and yellow boxes, respectively, contain the 25th to 75th percentiles. The black whiskers mark the 5th and 95th percentiles, and values beyond these upper and lower bounds are considered outliers, marked with black dots.

The average time to reclassification for variants in each of the 15 genes was also calculated and is available in Appendix G. Variants in *NF1* and *RAD51D* had the shortest average time to reclassification, while variants in *ATM* and *RAD51C* had the longest average time to reclassification.

IV. DISCUSSION

This study aimed to identify significant trends in VUS reclassification, in particular by comparing reclassifications of variants in HBRGs and MBRGs. The analysis included evaluation of reclassification direction (upgrade or downgrade), reclassification criteria, triggers that preceded VUS reclassification, and time to reclassification. Consistent with previously published studies, our data showed an overwhelming majority of downgrades in 617 VUS reclassifications across 15 genes related to breast cancer risk (Mersch *et al.* 2018, Macklin *et al.* 2017). However, this study did identify a significant trend in the odds of upgrade for reclassified variants in HBRGs over the study period. The most frequently used criteria for VUS reclassification in HBRGs were *BP6*, *BS3*, *BP4_strong*, *PS2/PM6*, and updated classification evidence and/or weighting (in support of pathogenicity). Alternatively, the most frequently used criteria for VUS reclassification in MBRGs were *BS1*, *BP4_strong*, and updated classification evidence and/or weighting (in support of benign classification). "Periodic re-review" and "variant identified in new patient sample" were the two triggers that preceded VUS reclassification for variants in MBRGs most often. "New research and/or data available" was the trigger that preceded VUS reclassification in HBRGs most often. Additionally, VUSs in HBRGs were reclassified five months sooner, on average, than variants in MBRGs.

4.1 VUS reclassification direction over study period

A notable observation from the analysis concerned the odds of VUS upgrade in HBRGs over the study period. The odds of upgrade were 1.68 times higher every year for reclassified variants in HBRGs in the study period. This increase in odds of upgrade is

likely due to a number of factors, one being an increase in our variant knowledge base. The increase in availability of data, such as *de novo* events and phenotype, over time seems to have led to many of the VUS upgrades in HBRGs observed in this study. This was supported by the significant frequency with which VUS reclassification in HBRGs was triggered by "new research/data available". However, it is important to note that the accumulation of new data can support both benign and pathogenic directions. Furthermore, guidelines, such as those published by ClinGen expert panels in 2018, will help further our understanding and application of criteria for variant classification in HBRGs in the future (Lee *et al.* 2018, Mester *et al*. 2018).

Another factor that influences reclassification is the rapid uptake of multigene panel testing in the cancer genetics setting. As the number of individuals being tested increases so does the knowledge base for both common and rare variants. For example, an individual's phenotype and family history are important to consider when weighting evidence (*PS2/PM6* and *PP4*). Since it is uncommon for variant classification to be based on one individual's phenotype or family history the more individuals that undergo testing the greater the availability of aggregate phenotypic and family history data will be available for individual variants. Aggregate data of this nature is more applicable to variants in HBRGs due to their higher phenotype penetrance i.e., a greater proportion of individuals carrying a pathogenic variant will have breast cancer or a history of breast cancer. Additionally, as the number of individuals undergoing testing increases so does our understanding of benign genetic variation in MBRGS and HBRGs in the population. To date, public databases that catalog human genetic variation, such as the Genome Aggregation Database (gnomAD),

have been overrepresented by genetic data from Caucasian individuals (Hall *et al.* 2009), but as more non-Caucasian individuals undergo genetic testing our understanding of genetic variation in those populations will grow. Furthermore, population-level genetic screening for conditions such as hereditary breast and ovarian cancer syndrome, Lynch syndrome, and familial hypercholesterolemia may allow for greater understanding of benign variation amongst unaffected individuals (Akbari *et al.* 2017, Gabai-Kapara *et al.* 2014).

VUS upgrades to likely pathogenic or pathogenic are of particular clinical importance because of the subsequent medical management changes that accompany these upgrades (Daly *et al.* 2017). In this study, 19% (119/617) of the total reclassified VUSs were upgraded to pathogenic or likely pathogenic. This is higher than two previous studies on variant reclassification (Mersch *et al.* 2018, Macklin *et al.* 2017). In those studies, 8.7% (178/2,048) and 3.3% (1/30) of reclassified VUSs were upgraded. These differences demonstrate that the rate of VUS upgrade is not only laboratory dependent but potentially time dependent. Differences in test volume and ascertainment of tested individuals amongst laboratories likely contribute to discrepancies in frequency and direction of VUS reclassification. For instance, laboratories with greater volume will aggregate more variant and phenotype data which will aid in the classification or reclassification of variants. Additionally, the timeframe of this study and previous studies was not exactly the same which makes it difficult to make direct comparisons. However, the differences in timeframe (with this study extending into 2018) could explain the higher incidence of VUS upgrades seen in this study as compared to previous studies. Availability of new research or data in a

specific year could have a significant impact on reclassification of previously reported variants. For example a large scale clinically validated functional study testing hundreds of variants could give strong data towards pathogenicity and therefore lead to reclassification of variants.

The observed differences in VUS reclassification direction across the 15 genes highlight potential exceptions to the concept that most VUS reclassifications are downgrades. For example, *PTEN* was shown to have a high proportion of upgrades, 53% (8/15). This is consistent with the knowledge that *PTEN* is a gene with a low rate of benign missense variants, and therefore when a missense variant is present it is more likely to be pathogenic (Mester *et al.* 2018). In addition, there are *PTEN*-specific phenotypic criteria and *de novo* rates which provide substantial weight for criteria in support of pathogenicity. Furthermore, because pathogenic variants in *PTEN* are associated with a heterogeneous clinical spectrum an expert panel (EP) was formed to weigh in on *PTEN* variant classification (Mester *et al.* 2018). Implementation of these refined criteria will likely lead to more accurate and timely *PTEN* variant classification in the future.

Overall, there are many factors that influence VUS reclassification direction, some of which include internal laboratory data, availability of gene-specific criteria, and numbers of tested individuals. To further understand VUS reclassifications we looked at the criteria that were frequently cited as evidence for reclassification.

4.2 Significant differences in criteria used for VUS reclassifications

"*PS2/PM6 De novo* (both maternity and paternity confirmed) in a patient with the disease and no family history or assumed *de novo*, but without confirmation of paternity and maternity" was cited exclusively for reclassified variants in HBRGs (13%). None of the reclassified VUSs in MBRGs used *PS2/PM6* as the criteria for upgrade. This difference can be explained by the inability to use *de novo* occurrences as criteria for classification for variants in MBRGs due to the lack of phenotypic specificity. As a result, parental studies are not typically offered as a part of the reclassification process for variants in MBRGs therefore that data is not readily available. On the contrary, parental studies for variants in HBRGs are routinely performed due to the specific phenotype associated with pathogenic variants in these genes. For example, pathogenic variants in *TP53* cause Li-Fraumeni syndrome which is associated with the development of the following tumors: soft tissue sarcoma, osteosarcoma, pre-menopausal breast cancer, brain tumors, adrenocortical carcinoma, and leukemias. The presence of these tumors in an individual is more informative for interpreting *de novo* data because of their relative scarcity in the general population, as compared to breast cancer. In March of 2018, SVI published recommendations for the interpretation of *de novo* criteria (*PMS2/PM6*) which included a point-based system to determine the strength of *de novo* evidence based upon three parameters: confirmed versus assumed *de novo* status, phenotypic consistency, and number of *de novo* observations (https://clinicalgenome.org/working-groups/sequence-variant-interpretation/). *De novo* criteria has since been applied to variant classification in MBRGs with the appropriate weighting i.e., *PS2_moderate*, which may lead to greater availability and application of this criteria in the future.

Overall, "*BP4_strong* Amino acid is reference allele in ≥3 primates OR ≥15 mammals" was cited 34% (212/617) of the time and showed no bias towards reclassified variants in either HBRGs or MBRGs. This criterion was introduced in the Ambry variant classification scheme in April of 2015 and accounts for a large proportion of VUS downgrades seen in the following years: 2015 (45 of the 107 VUS downgrades), 2016 (95 of the 139 VUS downgrades), and 2017 (67 of the 152 downgrades). This suggests that changes to a clinical laboratory's classification scheme can have a significant impact on VUS reclassification due to continued refinement of criteria and their weighting.

"*BS3* Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing" was cited significantly more often for reclassified variants in HBRGs, specifically *BRCA1*, *BRCA2* and *TP53,* suggesting a greater availability or validation of functional or splicing criteria for these genes. Parsing this data further we see that a significant proportion of *BS3* criteria were citing splicing variant impact. A splice site alteration could potentially affect normal pre-mRNA splicing and be pathogenic via disruption of consensus sequences, creation of *de novo* sequences, or alteration of splicing regulatory elements (Spurdle *et al.* 2008). Traditionally, clinical laboratories have relied on published research to provide evidence of splicing variant impact on gene expression or protein function. To date, there have been multiple studies to assess the effects of germline splicing alterations in breast cancer genes via both *in vitro* and *in vivo* studies (Farber-Katz *et al.* 2018, Farrugia *et al.* 2008, Houdayer *et al.* 2012, Pesaran *et al.* 2016, Tesoriero *et al.* 2005, Thomassen *et al.* 2012). More recently, some clinical laboratories have implemented

internal research labs to develop and perform assays to evaluate the functional impact of a VUS (Pesaran *et al.* 2016). This integrated approach likely contributed to the frequent use of this criterion for VUS reclassification in HBRGs. Furthermore, large-scale functional studies which used massively parallel sequencing to measure the effects of thousands of missense variants on *PTEN* protein intracellular abundance (Matreyek *et al.* 2018), will likely provide additional functional criteria for variant classification or reclassification.

In addition to advances in laboratory assays, advancements in computational tools have led to greater accuracy and efficiency of variant classification (Fortuno *et al.* 2018, Houdayer *et al.* 2012). In this study, computational data was cited in a number of evidence categories: "*PP1* Other data supporting pathogenic classification" and "*BP6/BP4_strong/BS4/BS4_supporting* Other data supporting benign classification". About 31% (37/119) of VUSs that were reclassified to VLP or pathogenic cited "*PM1/5* Mutational hotspot, computational data, including structural analysis and/or modeling" as the criteria for upgrade. In addition to assessing mutational hotspots, structural biologists assess the pathogenicity of variants by analyzing a number of parameters, including but not limited to impact on protein stability, impact of the variant on functional domains and protein-protein interactions. However, these computational tools cannot be applied to all variants and all genes and require expertise for appropriate interpretation.

A criterion that was cited exclusively for reclassified variants in HBRGs was "*BP6* Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation" ($p<0.001$). Although this was a line of

evidence in the 2015 ACMG-AMP guidelines, in December of 2018 the SVI working group appealed to ACMG to remove *BP6* from the classification framework (Biesecker *et al.* 2018). The rationale for removal was that alternative criteria should be used instead of *BP6* because it relies on assertions that are not directly linked to the evidence on which it was based. The SVI working group further explains that this criterion was appropriate in 2015 as it was intended to bridge the gap in knowledge amongst clinical laboratories however this bridge is no longer needed due to the implementation of resources of public variant databases, such as ClinVar. Based on these considerations, laboratories no longer use *BP6* as criteria for variant classification. If this study were to be repeated in the future we would likely see a significant decrease or altogether elimination of this criterion as evidence for reclassification.

Differences in criteria and weight of criteria used in variant classification exist amongst clinical laboratories and likely contribute to observed differences in VUS reclassification (Harrison *et al.* 2017, Harrison *et al.* 2018, Yang *et al.* 2017). One study analyzed all ClinVar variants classified by two or more submitters and found that 11.7% of variants had discrepant interpretations; these were further categorized by level of discrepancy and 3.5% of all variants were considered to have medically significant discrepancies i.e., pathogenic/VLP versus VUS/benign/VLB (Harrison *et al.* 2017). Another study found that the year of submission to ClinVar played a significant role in discrepancy; submissions prior to 2014 were more likely to have discrepant classification due to outdated criteria (Yang *et al.* 2017). These results are generally in agreement with the results of our study showing that the percentage of discrepant classifications decreased

every year, with the years 2017 and 2018 being the lowest at 71% each (Figure 11, 150/211 and 5/7 respectively). However, in this study the discrepancy frequencies are likely over inflated, as we did not delineate the number of discrepant or concordant submissions in Clinvar nor did we differentiate the source of the discrepant classification. For example, if there were five submissions in total and one out of the five had a discrepant classification this was counted as discrepant. Additionally, there are over 1200 submitters in the ClinVar database, of which a handful are considered reputable sources. These factors likely explain the high proportion of discrepant classifications seen in this study. A more recent study found that out of 49,242 variants, 77.8% had concordant classifications, 17.9% had VUS to VLB/benign conflicting classifications, 3.1% had pathogenic/VLP versus VUS differences and 1.2% had pathogenic/VLP versus benign/VLB difference. This resulted in 4.3% of variants having a medically significant difference in classification (Harrison *et al.* 2018). It is important to note that the scope and frequency of ClinVar submissions can vary significantly by clinical laboratory. In addition prioritizing resubmission of reclassified variants and providing an evaluation date can provide a better representation of a laboratory's current classification (Harrison *et al.* 2018).

4.3 Significant differences in triggers that precede VUS reclassification

"Periodic re-review" was cited significantly more often for reclassified variants in MBRGs as compared to HBRGs. The clinical laboratory that provided this dataset re-reviews variants every six months to assess for updates or availably of new evidence. Data from this study suggests that periodic re-review plays an important role in VUS reclassification as opposed to other potential triggers. However this could vary between

clinical laboratories and not all may have standardized periodic re-review guidelines (David *et al.* 2018). Alternatively, "new research/data available" was cited significantly more often for reclassified variants in HBRGs. This difference could be explained by the greater availability of new research and data for variants in HBRGs. It is possible that researchers are choosing to study HBRGs as opposed to MBRGs, due to the stronger association of pathogenic variants identified in an individual and presence of disease (genotype-phenotype correlation). Importantly, one reclassification trigger that was infrequently cited was "provider requested". The absence of this trigger is notable given that genetic testing laboratories are often inundated with communications from healthcare providers to inquire about the status of VUSs. While providers are well within their right to re-contact the laboratories, it is worth mentioning that we found no evidence that these requests played a significant role in the VUS reclassification process. However, this should not discourage patients and providers from re-contacting a laboratory about the status of a particular variant, especially in the context of pursing additional genetic studies or updates to family history or clinical information.

4.4 Differences in time to VUS reclassification

The median time to VUS reclassification was significantly shorter for variants in HBRGs than MBRGs. This difference may be explained by factors previously outlined above such as "new research/data available" due to new internal variant data and implementation of protein functional assays. Overall, the median time to reclassification was 20.6 months. This is lower than previously published studies, which showed medians of 39 and 30.6 months (Garcia *et al.* 2014, Mersch *et al.* 2018). The median time to VUS

reclassification in HBRGs in our data was 18.7 months and the difference in median time to VUS reclassification between HBRGs and MBRGs was 4.8 months. This difference could impact the timeframe of variant classification inquiry by health-care providers as well as the timeframe of patient follow-up. Reclassification from VUS to VLB/benign in a timely manner may help minimize the risk of inappropriate management and unnecessary anxiety. Traditionally, patients are told to return to clinic for updates anywhere from 1 to 3 years following the results of their genetic testing, should a VUS be identified (Murray *et al.* 2011). Given the VUS reclassification times shown in this study, healthcare providers should consider revising their recommendation based on the category of gene (HBRG or MBRG) the VUS is identified in.

4.5 Events following VUS reclassification

While laboratories make every effort to resolve VUS classification, it can be challenging for laboratories to provide efficient and effective communication of variant reclassification when it occurs. There is currently no clear legal duty on either the laboratory or healthcare provider to re-contact patients regarding variant reclassification. Although there are no clear legal guidelines on the duty to re-contact patients following variant reclassification, ACMG has published a position statement which includes legal, ethical and practical issues that should be considered (David *et al.* 2018). Ultimately, the issue of re-contact comes down to the principle of beneficence, defined as "the promotion of well-being in others i.e., to do good or prevent harm" (Uhlmann *et al.* 2011). This ethical principle applies to the duty to re-contact particularly in circumstances that may affect medical management. There are several practical issues which impede re-contacting

patients including: relocation of patients, providers or healthcare networks and difficulty navigating electronic health records. While recognizing that variant reclassification is inevitable, ACMG acknowledges that the responsibility of re-contact is shared among the ordering healthcare provider, the clinical testing laboratory, and the patient. However, it is important to note that clinical testing laboratories do not have direct contact with patients regarding their test results or reclassification of results. Therefore, it is important for the ordering healthcare provider to inform the patient that there is a possibility of variant reclassification, particularly in the setting of a VUS result, and that up-to-date contact information should be provided. Similarly, healthcare providers need to make every effort to provide clinical laboratories with the most effective way of communicating and recontacting the provider (fax, email, provider portal, etc.), considering that the responsibility of informing the ordering provider of variant reclassification rests solely with the clinical laboratory (David *et al.* 2018).

4.6 Limitations

The first limitation of this study is that it only included VUS reclassifications and did not ascertain VUSs whose classification did not change over the study period. Due to this, an overall VUS reclassification rate was not described for this data set, and an overall reclassification rate is useful to both patients and providers.

A second limitation of this study is that variant data was collected from a single commercial laboratory. In addition to collecting from a single commercial laboratory the data was collected by a single individual. However, the error rate, which was estimated as

1.45%, is low enough that it is unlikely to affect any of the major conclusions of this study. While many laboratories follow the ACMG-AMP variant classification criteria, (Richards *et al.* 2015) each clinical laboratory has their own specified policies and practices and therefore the results of this study are limited by those for this individual laboratory.

A third limitation is that several of the genes such as *PTEN*, *BARD1*, *CHEK2*, *RAD50*, *RAD51C*, and *RAD51D,* had less than 20 variants included in this study. An analysis including low variant counts will be underpowered with respect to detecting a difference.

A fourth limitation of this study involves the collection of discrepant classification in ClinVar. As previously stated, the presence of at least one discrepant classification from another laboratory was counted as discrepant, regardless of the number of other concordant classifications. In future analyses, discrepant classification would be more accurately represented by collecting the number and source of discrepant and concordant classifications in ClinVar. Additionally, quantifying the presence of medically significant discrepancies ex., VUS versus VLP or pathogenic, would help provide representation of differences that impact medical management.

A final limitation of this study involves the logistic regression analysis. The analysis assumes that the opportunity for variant reclassification is the same across the study period. However, as previously mentioned the clinical laboratory that provided this data set incorporated numerous updates to their classification scheme including “new

classification evidence and/or weighting of evidence" during the study period. These updates were not accounted for in the logistic regression model.

4.7 Future Directions

An important next step in continuing similar research would be to ascertain the overall VUS reclassification rate, direction, evidence used for reclassification, and reclassification triggers from several commercial laboratories. An analysis that includes variant information from multiple commercial laboratories would help substantiate the results seen in this study or highlight differences between laboratories. Additionally, collecting this data over a longer time period might help reveal trends that were not seen in this study. Finally, an analysis of evidence used across multiple laboratories might help elucidate gaps or misinformation in variant classification.

This study did not document the clinical phenotypes associated with the patients and families carrying each variant, which would have made for an interesting analysis. An analysis of this nature could include demographic, phenotypic and family history information for each patient and variant which would allow for further analysis including aggregate phenotypic data and likelihood of VUS upgrade. This study was able to tangentially analyze this by quantifying "*PP4* Patient's phenotype or family history is highly specific for a disease with a single genetic etiology" and "*PP1_strong/PP1_moderate* cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease". However, a likelihood of upgrade for each variant was not quantified in this study. Additionally, sharing one of the overall conclusions of this study,

that most reclassified VUSs were downgraded, with patients and healthcare providers may reduce their VUS-related anxiety. Surveying or interviewing patients or healthcare providers and their attitudes toward this information could glean useful insight into VUS-related anxiety. For instance, patients who are presented with the information that a majority of reclassified VUSs are downgraded may have a significant reduction in their VUS-related anxiety.

4.8 Conclusions

The results of this study support previously published data showing an overall trend toward VUS downgrades when combining HBRGs and MBRGs (Mersch *et al.* 2018, Macklin *et al.* 2017). However, when looking at variants that were reclassified in HBRGs the odds of upgrade increased in each subsequent year. The odds of upgrade were 1.68 higher every year for variants in HBRGS in the study period. The differences in criteria used for reclassification between reclassified variants in HBRGs and MBRGs highlight the complexity of variant classification. Additionally, the difference in triggers that precede VUS reclassification sheds new light on the process of reclassification initiation and what role health care providers and patients might play in this process, and the timing of that intervention.

The primary purpose of clinical laboratory testing in hereditary breast cancer predisposition is to support medical management of cancer risk. In the clinic, genetic testing is generally used to identify or confirm the cause of apparently hereditary disease. Given the complexity of genetic testing, the best outcomes result from health-care

providers and clinical laboratories working collaboratively. An important aspect of variant classification that was not addressed in this study is detailed clinical information such as patient and family history, physical exam findings, and previous laboratory testing. For instance, further clarification with the health-care provider and patient may uncover additional evidence to support a variant classification. It is important to note that variant analysis and interpretation is based on the available information at that time and can evolve over time as new data become available. A Bayesian framework of the ACMG-AMP guidelines is available which provides mathematical probabilities of pathogenicity for each classification category (Tavtigian *et al.* 2018). The results of the Tavtigian *et al*. study provide a quantitative framework for the ACMG-AMP guidelines, allowing for further refinement of the evidence categories and combination rules. The results of our study provide a better understanding of the VUS reclassification process and facilitate effective VUS reclassification counseling and education.

REFERENCES

ACMG Board of Directors. 2017. Laboratory and clinical genomic data sharing is crucial to improving genetic health care: a position statement of the American College of Medical Genetics and Genomics. Genet Med 19: 721–722.

Akbari MR, Gojska N, Narod SA. Coming of age in Canada: a study of population-based genetic testing for breast and ovarian cancer. *Curr Oncol.*2017 Oct;24(5):282–283. Doi: 10.3747/co.24.382

Amendola, L. M., Jarvik, G. P., Leo, M. C., Mclaughlin, H. M., Akkari, Y., Amaral, M. D., . . . Rehm, H. L. (2016). Performance of ACMG-AMP Variant-Interpretation Guidelines among Nine Laboratories in the Clinical Sequencing Exploratory Research Consortium. *The American Journal of Human Genetics, 98*(6), 1067-1076. Doi:10.1016/j.ajhg.2016.03.024

American Medical Association. 2013. Genome Analysis and Variant Identification Policy D-460.971

American Society of Clinical Oncology Policy Statement Update: Genetic Testing for Cancer Susceptibility. (2003). *Journal of Clinical Oncology, 21*(12), 2397-2406. Doi:10.1200/jco.2003.03.189

An Overview of the Human Genome Project. (n.d.). Retrieved from https://www.genome.gov/12011238/an-overview-of-the-human-genome-project/

Antoniou, A. C., PhD, Casadei, S., PhD, & Heikkinen, T., PhD. (2014). Breast-Cancer Risk in Families with Mutations in PALB2. *The New England Journal of Medicine, 371*, 497-506. Doi:10.1056/NEJMoa1400382

Bell, J., Bodmer, D., Sistermans, E., & Ramsden, S. C. (2007). Practice guidelines for the Interpretation and Reporting of Unclassified Variants (Uvs) in Clinical Molecular Genetics. *UK Clinical Molecular Genetics Society and and the Dutch Society of Clinical Genetic Laboratory Specialists*.

Biesecker, L. G., & Harrison, S. M. (2018). The ACMG/AMP reputable source criteria for the interpretation of sequence variants. *Genetics in Medicine, 20*(12), 1687-1688. Doi:10.1038/gim.2018.42

Bowcock, A. M. (1993). Molecular cloning of BRCA1: A gene for early onset familial breast and ovarian cancer. *Breast Cancer Research and Treatment, 28*(2), 121-135. Doi:10.1007/bf00666425

Brose MS, Rebbeck TR, Calzone KA, *et al.* Cancer risk estimates for BRCA1 mutation carriers identified in a risk evaluation program. *Journal of the National Cancer Institute* 2002; 94(18):1365–1372.

Chong HK, Wang T, Lu HM, *et al.* The validation and clinical implementation of BRCAplus: a comprehensive high-risk breast cancer diagnostic assay. (2014). *PloS One, 9*(9). Doi:10.1371/journal.pone.0110156

Couch FJ, *et al.* Inherited mutations in 17 breast cancer susceptibility genes among a large triple-negative breast cancer cohort unselected for family history of breast cancer. *J. Clin. Oncol.* 2015;33:304–311.

Couch, F. J., Shimelis, H., Hu, C., Hart, S. N., Polley, E. C., Na, J., . . . Dolinsky, J. S. (2017). Associations Between Cancer Predisposition Testing Panel Genes and Breast Cancer. *JAMA Oncology, 3*(9), 1190. Doi:10.1001/jamaoncol.2017.0424

Cragun D, *et al.* Panel-based testing for inherited colorectal cancer: a descriptive study of clinical testing performed by a US laboratory. *Clin. Genet.* 2014;86:510–520.

Culver, J., Brinkerhoff, C., Clague, J., Yang, K., Singh, K., Sand, S., & Weitzel, J. (2013). Variants of uncertain significance in BRCAtesting: Evaluation of surgical decisions, risk perception, and cancer distress. *Clinical Genetics, 84*(5), 464-472. Doi:10.1111/cge.12097

Daly M, Pilarski R, BerryM, *et al.* Genetic/familial high-risk assessment: breast and ovarian. NCCN Clinical Practice Guidelines in Oncology. (Version 2.2017). http://www.nccn.org/professionals/physician_gls/pdf/genetics_screening.pdf 2017.

David, K. L., Best, R. G., Brenman, L. M., Bush, L., Deignan, J. L., Flannery, D., . . . Pyeritz, R. E. (2018). Patient re-contact after revision of genomic test results: Points to consider—a statement of the American College of Medical Genetics and Genomics (ACMG). *Genetics in Medicine, 21*(4), 769-771. Doi:10.1038/s41436-018-0391-z

Domchek, S. M., Bradbury, A., Garber, J. E., Offit, K., & Robson, M. E. (2013). Multiplex Genetic Testing for Cancer Susceptibility: Out on the High Wire Without a Net? Journal of *Clinical Oncology, 31*(10), 1267-1270. Doi:10.1200/jco.2012.46.9403

Easton DF, *et al.* Gene-panel sequencing and the prediction of breast-cancer risk. *N. Engl. J. Med.* 2015;372:2243–2257.

Farber-Katz, S., Hsuan, V., Wu, S., Landrith, T., Vuong, H., Xu, D., . . . Karam, R. (2018). Quantitative Analysis of BRCA1 and BRCA2 Germline Splicing Variants Using a Novel RNA-Massively Parallel Sequencing Assay. *Frontiers in Oncology, 8*. Doi:10.3389/fonc.2018.00286

Farrugia DJ, Agarwal MK, Pankratz VS, Deffenbaugh AM, Pruss D, Frye C, *et al.* Functional assays for classification of BRCA2 variants of uncertain significance. *Cancer Res.* (2008) 68:3523–31. Doi: 10.1158/0008-5472.CAN-07-1587

Fortuno, C., James, P. A., Young, E. L., Feng, B., Olivier, M., Pesaran, T., . . . Spurdle, A. B. (2018). Improved, ACMG-compliant, in silico prediction of pathogenicity for missense

substitutions encoded by TP53 variants. *Human Mutation, 39*(8), 1061-1069. Doi:10.1002/humu.23553

Francioli, L. C., Polak, P. P., Koren, A., Menelaou, A., Chun, S., Renkens, I., . . . Sunyaev, S. R. (2015). Genome-wide patterns and properties of de novo mutations in humans. *Nature Genetics,47*(7), 822-826. Doi:10.1038/ng.3292

Garcia, C., Lyon, L., Littell, R. D., & Powell, C. B. (2014). Comparison of risk management strategies between women testing positive for a BRCA variant of unknown significance and women with known BRCA deleterious mutations. *Genetics in Medicine, 16*(12), 896-902. Doi:10.1038/gim.2014.48

Gabai-Kapara E, et al. Population-based screening for breast and ovarian cancer risk due to BRCA1 and BRCA2. *PNAS*. 2014 Sep 30;111(39):14205–10. Doi: 10.1073

Gold, E. R., & Carbone, J. (2008). Myriad Genetics: In the Eye of a Policy Storm. SSRN Electronic Journal. Doi:10.2139/ssrn.1260098

Hall, J., Lee, M., Newman, B., Morrow, J., Anderson, L., Huey, B., & King, M. (1990). Linkage of early-onset familial breast cancer to chromosome 17q21. *Science, 250*(4988), 1684-1689. Doi:10.1126/science.2270482

Hall, M. J., MD, Reid, J. E., & Burbidge, L. A., BS. (2009). BRCA1 and BRCA2 mutations in women of different ethnicities undergoing testing for hereditary breast-ovarian cancer. *American Cancer Society, 115*(12), 2222-2233. Doi:10.1002/cncr.24200

Han, M., Zheng, W., Cai, Q., Gao, Y., Zheng, Y., & Bolla, M. K. (2017). Evaluating genetic variants associated with breast cancer risk in high and moderate-penetrance genes in Asians. *Carcinogenesis, 38*(5), 511-518. Retrieved from https://doi.org/10.1093/carcin/bgx010.

Harrison, S. M., Dolinksy, J. S., Chen, W., Collins, C. D., Das, S., Deignan, J. L., . . . Rehm, H. L. (2018). Scaling resolution of variant classification differences in ClinVar between 41 clinical laboratories through an outlier approach. *Human Mutation, 39*(11), 1641-1649. Doi:10.1002/humu.23643

Harrison, S. M., Dolinsky, J. S., Johnson, A. E., Pesaran, T., Azzariti, D. R., Bale, S., . . . Rehm, H. L. (2017). Clinical laboratories collaborate to resolve differences in variant interpretations submitted to ClinVar. *Genetics in Medicine, 19*(10), 1096-1104. Doi:10.1038/gim.2017.14

Hollestelle, A., Wasielewski, M., Martens, J. W., & Schutte, M. (2010). Discovering moderate-risk breast cancer susceptibility genes. *Current Opinion in Genetics & Development, 20*(3), 268-276. Doi:10.1016/j.gde.2010.02.009

Hooker, G. W., Clemens, K. R., Quillin, J., Postula, K. J., Summerour, P., Nagy, R., & Buchanan, A. H. (2017). Cancer Genetic Counseling and Testing in an Era of Rapid Change. *Journal of Genetic Counseling, 26*(6), 1244-1253. Doi:10.1007/s10897-017-0099-2

Houdayer, C., Caux-Moncoutier, V., Krieger, S., Barrois, M., Bonnet, F., Bourdon, V., . . . Stoppa-Lyonnet, D. (2012). Guidelines for splicing analysis in molecular diagnosis derived from a set of 327 combined in silico/in vitro studies on BRCA1 and BRCA2 variants. *Human Mutation, 33*(8), 1228-1238. Doi:10.1002/humu.22101

John EM, Miron A, Gong G, Phipps AI, Felberg A, Li FP, West DW,Whittemore AS. 2007. Prevalence of pathogenic BRCA1 mutation carriers in 5 US racial/ethnic groups. *JAMA* 298:2869–2876

Kapoor, N. S., Curcio, L. D., Blakemore, C. A., Bremner, A. K., Mcfarland, R. E., West, J. G., & Banks, K. C. (2015). Multigene Panel Testing Detects Equal Rates of Pathogenic BRCA1/2 Mutations and has a Higher Diagnostic Yield Compared to Limited BRCA1/2 Analysis Alone in Patients at Risk for Hereditary Breast Cancer. *Annals of Surgical Oncology, 22*(10), 3282-3288. Doi:10.1245/s10434-015-4754-2

Kean-Cowdin R, Spencer FH, Xia LY, Pearce CL, Thomas DC, Stram DO, Henderson BE. 2005. BRCA1 variants in a family study of African-American and Latina women. *Hum Genet* 116:497–506.

Keating NL, Stoeckert KA, Regan MM, DiGianni L, Garber JE. (2008) Physicians' experiences with BRCA1/2 testing in community settings. J Clin Oncol 26:5789–5796

Kong, A. *et al.* Rate of de novo mutations and the importance of father's age to disease risk. Nature 488, 471–475 (2012).

Kuchenbaecker KB, Hopper JL, Barnes DR, *et al.* Risks of breast, ovarian, and contralateral breast cancer for BRCA1 and BRCA2 mutation carriers. *JAMA* 2017; 317(23):2402-2416.

Kurian AW, *et al.* Clinical evaluation of a multiple-gene sequencing panel for hereditary cancer risk assessment. *J. Clin. Oncol.* 2014;32:2001–2009.

Kurian, A. W., & Ford, J. M. (2015). Multigene Panel Testing in Oncology Practice. *JAMA Oncology, 1*(3), 277. Doi:10.1001/jamaoncol.2015.28

Kurian, A. W., Hughes, E., Handorf, E. A., Gutin, A., Allen, B., Hartman, A., & Hall, M. J. (2017). Breast and Ovarian Cancer Penetrance Estimates Derived From Germline Multiple-Gene Sequencing Results in Women. *JCO Precision Oncology,* (1), 1-12. Doi:10.1200/po.16.00066

Kurian AW, Li Y, Hamilton AS *et al* (2017) Gaps in incorporating germline genetic testing into treatment decision-making for early-stage breast cancer. J Clin Oncol 35:2232–2239

LaDuca H, *et al.* Utilization of multigene panels in hereditary cancer predisposition testing: analysis of more than 2,000 patients. *Genet. Med.* 2014;16:830–837.

Levy-Lahad E, Friedman E. Cancer risks among BRCA1 and BRCA2 mutation carriers. *British Journal of Cancer* 2007; 96(1):11–15.

Li, M. M., Datto, M., Duncavage, E. J., Kulkarni, S., Lindeman, N. I., Roy, S., . . . Nikiforova, M. N. (2017). Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer. *The Journal of Molecular Diagnostics, 19*(1), 4-23. Doi:10.1016/j.jmoldx.2016.10.002

Lincoln SE, *et al.* A systematic comparison of traditional and multigene panel testing for hereditary breast and ovarian cancer genes in more than 1000 patients. *J. Mol. Diagn.* 2015;17:533–544.

Macklin, S., Durand, N., Atwal, P., & Hines, S. (2017). Observed frequency and challenges of variant reclassification in a hereditary cancer clinic. *Genetics in Medicine, 20*(3), 346-350. Doi:10.1038/gim.2017.207

Macklin, S. K., Jackson, J. L., Atwal, P. S., & Hines, S. L. (2018). Physician interpretation of variants of uncertain significance. *Familial Cancer, 18*(1), 121-126. Doi:10.1007/s10689-018-0086-2

Madanikia, S. A., Bergner, A., Ye, X., & Blakeley, J. O. (2012). Increased risk of breast cancer in women with NF1. *American Journal of Medical Genetics Part A, 158A*(12), 3056-3060. doi:10.1002/ajmg.a.35550

Makhnoon, S., Shirts, B. H., & Bowen, D. J. (2019). Patients perspectives of variants of uncertain significance and strategies for uncertainty management. *Journal of Genetic Counseling, 28*(2), 313-325. Doi:10.1002/jgc4.1075

Matreyek, K. A., Starita, L. M., Stephany, J. J., Martin, B., Chiasson, M. A., Gray, V. E., . . . Fowler, D. M. (2018). Multiplex Assessment of Protein Variant Abundance by Massively Parallel Sequencing. Doi:10.1101/211011

Maxwell KN, *et al.* Prevalence of mutations in a panel of breast cancer susceptibility genes in BRCA1/2-negative patients with early-onset breast cancer. *Genet. Med.* 2015;17:630–638.

Mersch, J., Brown, N., Pirzadeh-Miller, S., Mundt, E., Cox, H. C., Brown, K., . . . Ross, T. (2018). Prevalence of Variant Reclassification Following Hereditary Cancer Genetic Testing. *Jama,320*(12), 1266. Doi:10.1001/jama.2018.13152

Mester, J. L., Ghosh, R., Pesaran, T., Huether, R., Karam, R., Hruska, K. S., . . . Eng, C. (2018). Gene-specific criteria for PTEN variant curation: Recommendations from the ClinGen PTEN Expert Panel. *Human Mutation, 39*(11), 1581-1592. Doi:10.1002/humu.23636

Michaelson, J.J. *et al.* (2012). Whole-genome sequencing in autism identifies hot spots for de novo germline mutation. *Cell* 151, 1431–1442 .

Minion LE, *et al.* Hereditary predisposition to ovarian cancer, looking beyond BRCA1/BRCA2. *Gynecol. Oncol.* 2015;137:86–92.

Møller, P., Dominguez-Valentin, M., Rødland, E., & Hovig, E. (2019). Causes for Frequent Pathogenic BRCA1 Variants Include Low Penetrance in Fertile Ages, Recurrent De-Novo Mutations and Genetic Drift. *Cancers, 11*(2), 132. Doi:10.3390/cancers11020132

Murray, M. L., Cerrato, F., Bennett, R. L., & Jarvik, G. P. (2011). Follow-up of carriers of BRCA1 and BRCA2 variants of unknown significance: Variant reclassification and surgical decisions. *Genetics in Medicine, 13*(12), 998-1005. Doi:10.1097/gim.0b013e318226fc15

Nagy, R., Ganapathi, S., Comeras, I., Peterson, C., Orloff, M., Porter, K., … Kloos, R. T. (2011). Frequency of germline PTEN mutations in differentiated thyroid cancer. Thyroid: Official Journal of the American Thyroid Association, 21(5), 505–510. https://doi.org/10.1089/thy.2010.0365

Najafzadeh M, Lynd LD, Davis JC *et al*. (2012) Barriers to integrating personalized medicine into clinical practice: a best-worst scaling choice experiment. Genet Med 14:520–526

National Institutes of Health. 2014. Genomic Data Sharing 79 FR 51345.

National Society of Genetic Counselors. 2015. Clinical Data Sharing.

Oneill, S. C., Rini, C., Goldsmith, R. E., Valdimarsdottir, H., Cohen, L. H., & Schwartz, M. D. (2009). Distress among women receiving uninformativeBRCA1/2results: 12-month outcomes. *Psycho-Oncology, 18*(10), 1088-1096. Doi:10.1002/pon.1467

Pesaran, T., Karam, R., Huether, R., Li, S., Farber-Katz, S., Chamberlin, A., . . . Elliott, A. (2016). Beyond DNA: An Integrated and Functional Approach for Classifying Germline Variants in Breast Cancer Genes. *International Journal of Breast Cancer, 2016*, 1-10. Doi:10.1155/2016/2469523

Plon, S. E., Eccles, D. M., Easton, D., Foulkes, W. D., Genuardi, M., Greenblatt, M. S., . . . Tavtigian, S. V. (2008). Sequence variant classification and reporting: Recommendations for improving the interpretation of cancer susceptibility genetic test results. *Human Mutation, 29*(11), 1282-1291. Doi:10.1002/humu.20880

Rahman, N., Seal, S., Thompson, D., Kelly, P., Renwick, A., Elliott, A., . . . Stratton, M. R. (2006). PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nature Genetics, 39*(2), 165-167. Doi:10.1038/ng1959

Richter, S., Haroun, I., Graham, T. C., Eisen, A., Kiss, A., & Warner, E. (2013). Variants of unknown significance in BRCA testing: Impact on risk perception, worry, prevention and counseling. *Annals of Oncology, 24*(Suppl 8), Viii69-Viii74. Doi:10.1093/annonc/mdt312

Richards, S., PhD. (2015). Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in Medicine, 17*, 405-423.

Ring, K. L., Bruegl, A. S., Allen, B. A., Elkin, E. P., Singh, N., Hartman, A.-R., … Broaddus, R. R. (2016). Germline multi-gene hereditary cancer panel testing in an unselected endometrial cancer cohort. Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc., 29(11), 1381–1389. https://doi.org/10.1038/modpathol.2016. 135

Seminog, O. O., & Goldacre, M. J. (2015). Age-specific risk of breast cancer in women with neurofibromatosis type 1. *British Journal of Cancer, 112*(9), 1546-1548. doi:10.1038/bjc.2015.78

Slavin, T. P., Tongeren, L. R., Behrendt, C. E., Solomon, I., Rybak, C., Nehoray, B., . . . Weitzel, J. N. (2018). Prospective Study of Cancer Genetic Variants: Variation in Rate of Reclassification by Ancestry. JNCI: *Journal of the National Cancer Institute, 110*(10), 1059-1066. Doi:10.1093/jnci/djy027

Slavin, T. P., Niell-Swiller, M., Solomon, I., Nehoray, B., Rybak, C., Blazer, K. R., & Weitzel, J. N. (2015). Clinical Application of Multigene Panels: Challenges of Next-Generation Counseling and Cancer Risk Management. *Frontiers in Oncology, 5*. Doi:10.3389/fonc.2015.00208

Southey MC, Winship I, Nguyen-Dumont T. PALB2: research reaching to clinical outcomes for women with breast cancer. *Hered Cancer Clin Pract*. 2016;14:9. Published 2016 Apr 19. Doi:10.1186/s13053-016-0049-2

Spurdle, A. B., Couch, F. J., Hogervorst, F. B., Radice, P., & Sinilnikova, O. M. (2008). Prediction and assessment of splicing alterations: Implications for clinical testing. *Human Mutation, 29*(11), 1304-1313. Doi:10.1002/humu.20901

Starita, L. M., Ahituv, N., Dunham, M. J., Kitzman, J. O., Roth, F. P., Seelig, G., . . . Fowler, D. M. (2017). Variant Interpretation: Functional Assays to the Rescue. *The American Journal of Human Genetics, 101*(3), 315-325. Doi:10.1016/j.ajhg.2017.07.014

Suarez-Kelly, L. P., Yu, L., Kline, D., Schneider, E. B., Agnese, D. M., & Carson, W. E. (2019). Increased breast cancer risk in women with neurofibromatosis type 1: A meta-analysis and systematic review of the literature. *Hereditary Cancer in Clinical Practice, 17*(1). Doi:10.1186/s13053-019-0110-z

Tavtigian, S. V., Greenblatt, M. S., Harrison, S. M., Nussbaum, R. L., Prabhu, S. A., Boucher, K. M., & Biesecker, L. G. (2018). Modeling the ACMG/AMP variant classification guidelines as a Bayesian classification framework. *Genetics in Medicine, 20*(9), 1054-1060. Doi:10.1038/gim.2017.210

Tesoriero AA, Wong EM, Jenkins MA, Hopper JL, Brown MA, Chenevix-Trench G, *et al.* Molecular characterization and cancer risk associated with BRCA1 and BRCA2 splice site variants identified in multiple-case breast cancer families. *Hum Mutat.* (2005) 26:495. Doi: 10.1002/humu.9379

Thomassen M, Blanco A, Montagna M, Hansen TV, Pedersen IS, Gutierrez-Enriquez S, *et al.* Characterization of BRCA1 and BRCA2 splicing variants: a collaborative report by ENIGMA consortium members. *Breast Cancer Res Treat.* (2012) 132:1009–23. Doi: 10.1007/s10549-011-1674-0

Tung N, *et al.* Frequency of mutations in individuals with breast cancer referred for BRCA1 and BRCA2 testing using next-generation sequencing with a 25-gene panel. *Cancer.* 2015;121:25–33.

Tung, N., Lin, N. U., Kidd, J., Allen, B. A., Singh, N., Wenstrup, R. J., … Garber, J. E. (2016). Frequency of germline mutations in 25 cancer susceptibility genes in a sequential series of patients with breast cancer. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology, 34(13), 1460–1468. https://doi.org/10.1200/JCO.2015.65.0747

Tung, N., Domchek, S. M., Stadler, Z., Nathanson, K. L., Couch, F., Garber, J. E., Robson, M. E. (2016). Counseling framework for moderate-penetrance cancer-susceptibility mutations. *Nature Reviews Clinical Oncology, 13*(9), 581-588. Doi:10.1038/nrclinonc.2016.90

Uhlmann, W. R., Schuette, J. L., & Yashar, B. (2011). *A Guide to Genetic Counseling*. Somerset: Wiley.

Venkitaraman, A. R. (2002). Cancer Susceptibility and the Functions of BRCA1 and BRCA2. *Cell,108*(2), 171-182. Doi:10.1016/s0092-8674(02)00615-3

Wall, D. (2008). Faculty of 1000 evaluation for ACMG recommendations for standards for interpretation and reporting of sequence variations: Revisions 2007. *F1000 – Post-publication Peer Review of the Biomedical Literature*. Doi:10.3410/f.717297951.792752804

Walsh, T., Lee, M. K., Casadei, S., Thornton, A. M., Stray, S. M., Pennil, C., . . . King, M. (2010). Detection of inherited mutations for breast and ovarian cancer using genomic capture and massively parallel sequencing. *PNAS, 107*(28), 12629-12633. Doi:1007983107

Walsh T, *et al.* Mutations in 12 genes for inherited ovarian, fallopian tube, and peritoneal carcinoma identified by massively parallel sequencing. *Proc. Natl Acad. Sci. USA.* 2011;108:18032–18037.

Welsh JL, Hoskin TL, Day CN et al (2017) Clinical decision making in patients with variant of uncertain significance in BRCA1or BRCA2 genes. Ann Surg Oncol. https://doi.org/10.1245/s10434-017-5959-3

Whole-genome sequence variation, population structure and demographic history of the Dutch population. (2014). *Nature Genetics, 46*(8), 818-825. Doi:10.1038/ng.3021

Yang, C., Ceyhan-Birsoy, O., Mandelker, D., Jairam, S., Catchings, A., O'Reilly, E. M., . . . Zhang, L. (2018). A synonymous germline variant PALB2 c.18GT (p.Gly6=) disrupts normal splicing in a family with pancreatic and breast cancers. *Breast Cancer Research and Treatment, 173*(1), 79-86. Doi:10.1007/s10549-018-4980-y

Yang, S., Lincoln, S. E., Kobayashi, Y., Nykamp, K., Nussbaum, R. L., & Topper, S. (2017). Sources of discordance among germ-line variant classifications in ClinVar. *Genetics in Medicine, 19*(10), 1118-1126. Doi:10.1038/gim.2017.60

Yurgelun MB, *et al.* Identification of a variety of mutations in cancer predisposition genes in patients with suspected Lynch syndrome. *Gastroenterology.* 2015;149:604–613. E20.

Zhang, L., Fleischut, M., Kohut, K., Spencer, S., Wong, K., Stadler, Z., . . . Robson, M. (2011). Assessment of the prevalence of de novo mutations in the BRCA1 and BRCA2 genes. *Clinical Genetics, 80*(1), 97-98. Doi:10.1111/j.1399-0004.2011.01691.x

APPENDIX A

Evidence Category	Criteria
Very strong for pathogenicity	*PVS1* null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where LOF is a known mechanism of disease Caveats: • Beware of genes where LOF is not a known disease mechanism (e.g., GFAP, MYH7) • Use caution interpreting LOF variants at the extreme 3′ end of a gene • Use caution with splice variants that are predicted to lead to exon skipping but leave the remainder of the protein intact • Use caution in the presence of multiple transcripts
Strong for pathogenicity	*PS1* Same amino acid change as a previously established pathogenic variant regardless of nucleotide change • Example: Val→Leu caused by either G>C or G>T in the same codon • Caveat: Beware of changes that impact splicing rather than at the amino acid/protein level *PS2* De novo (both maternity and paternity confirmed) in a patient with the disease and no family history • Note: Confirmation of paternity only is insufficient. Egg donation, surrogate motherhood, errors in embryo transfer, and so on, can contribute to nonmaternity. *PS3* Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product • Note: Functional studies that have been validated and shown to be reproducible and robust in a clinical diagnostic laboratory setting are considered the most well established. *PS4* The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls • Note 1: Relative risk or OR, as obtained from case–control studies, is >5.0, and the confidence interval around the estimate of relative risk or OR does not include 1.0. See the article for detailed guidance. • Note 2: In instances of very rare variants where case–control studies may not reach statistical significance, the prior observation of the variant in multiple unrelated patients with the same phenotype, and its absence in controls, may be used as moderate level of evidence.
Moderate for pathogenicity	*PM1* Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation *PM2* Absent from controls (or at extremely low frequency if recessive) (Table 6) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium • Caveat: Population data for insertions/deletions may be poorly called by next-generation sequencing. *PM3* For recessive disorders, detected in trans with a pathogenic variant • Note: This requires testing of parents (or offspring) to determine phase. *PM4* Protein length changes as a result of in-frame deletions/insertions in a nonrepeat region or stop-loss variants *PM5* Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before • Example: Arg156His is pathogenic; now you observe Arg156Cys • Caveat: Beware of changes that impact splicing rather than at the amino acid/protein level. *PM6* Assumed de novo, but without confirmation of paternity and maternity
Supporting for pathogenicity	*PP1* Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease • Note: May be used as stronger evidence with increasing segregation data *PP2* Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease *PP3* Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.) • Caveat: Because many in silico algorithms use the same or very similar input for their predictions, each algorithm should not be counted as an independent criterion. PP3 can be used only once in any evaluation of a variant. *PP4* Patient's phenotype or family history is highly specific for a disease with a single genetic etiology *PP5* Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation

Stand-alone for benign	*BA1* Allele frequency is >5% in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium
Strong for benign	*BS1* Allele frequency is greater than expected for disorder (see Table 6) *BS2* Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder, with full penetrance expected at an early age *BS3* Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing *BS4* Lack of segregation in affected members of a family • Caveat: The presence of phenocopies for common phenotypes (i.e., cancer, epilepsy) can mimic lack of segregation among affected individuals. Also, families may have more than one pathogenic variant contributing to an autosomal dominant disorder, further confounding an apparent lack of segregation.
Supporting for benign	*BP1* Missense variant in a gene for which primarily truncating variants are known to cause disease *BP2* Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern *BP3* In-frame deletions/insertions in a repetitive region without a known function *BP4* Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.) • Caveat: Because many in silico algorithms use the same or very similar input for their predictions, each algorithm cannot be counted as an independent criterion. BP4 can be used only once in any evaluation of a variant. *BP5* Variant found in a case with an alternate molecular basis for disease *BP6* Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation *BP7* A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved

*Adapted from Richards *et al.* 2015

APPENDIX B

Scheme For Autosomal Dominant And X-Linked Mendelian Diseases (V2-20-17)

CLASS	AMBRY CLASSIFICATION	CATEGORY	CRITERIA	EXCEPTIONS (NEW BASELINE CLASS)
5	Pathogenic Mutation	A 1 needed	Alterations resulting in premature truncation (e.g.reading frame shift, nonsense)	Truncation in close proximity to 3' terminus (3/4 gene specific)
				LOF has not been established as mechanism of pathogenicity (e.g. MYH7) (3)
			Other ACMG-defined mutation (i.e. initiation codon or gross deletion)	In-frame gross deletion of a single exon not in a known protein functional domain (3), Initiation codon that is not well conserved or possible alternate start (3/4), LOF has not been established as a mechanism of pathogenicity (3)
		B 4 needed	Confirmed de novo alteration	Weight is gene and disease specific (2B, 1B, 1C)
			Functionally validated splicing mutation	In-frame skipping of a single exon not in a known protein functional domain. LOF has not been established as a mechanism of pathogenicity. Biologically relevant, naturally occurring, in-frame isoforms with data supporting normal function (3)
			Significant disease association in appropriately sized case-control study(ies)	
			Detected in an individual satisfying established diagnostic critera for classic disease without a clear mutation	
			Last nucleotide of exon	Last nucleotide poorly conserved or non-G, in silico not consistent with U2-dependent intron
			Good segregation with disease	1B=(LOD 1.5-3 = 5-9 meioses); 2 or 3B=(LOD >3 = >10 meioses) gene specific
			Deficient protein function in appropriate functional assay(s)	
			Well-characterized mutation at same position	
			Other strong data supporting pathogenic classification	
4	Variant, Likely Pathogenic	3B	Alterations at the canonical donor/acceptor sites (± 1, 2) without other strong (B-level) evidence supporting pathogenicity	LOF has not been established as a mechanism of pathogenicity (3)
		C 4 needed	Rarity in general population databases (dbSNP, ESP, 1000 Genomes)	Dependent on disease penetrance and inheritance pattern
			In silico models in agreement (deleterious) and/or completely conserved position in appropriate species	*In silico* splicing predictions not used as independent line of evidence for last nucleotide of exon
			Moderate segregation with disease (at least 3 informative meioses) for rare diseases	
			Other data supporting pathogenic classification	
		3 of B		
		2 of B and at least 1 of C		
		1 of B and at least 3 of C		
3	VUS	Insufficient or conflicting evidence		
		Gross duplications without strong evidence for pathogenic or benign		
2	Variant, Likely Benign	D 1 needed	Intact protein function observed in appropriate functional assay(s)	
			Intronic alteration with no splicing impact by RT-PCR analysis or other splicing assay	
			Seen *in trans* with a mutation or in homozygous state in individuals without severe disease for that gene	Genes without a defined, severe biallelic phenotype (3)
			Other strong data supporting benign classification	
		E 2 needed	Co-occurence with mutations in the same gene (phase unknown)	Genes without a defined, severe biallelic phenotype (3) When always linked to a the same mutation (can't rule out synergetic effect)
			Co-occurence with mutations in other highly penetrant genes that clearly explain a proband's phenotype	
			Subpopulation frequency in support of benign classification	
			In silico models in agreement (benign)	
			Does not segregate with disease in a family study (genes with incomplete penetrance)	
			No disease association in a small case-control study	
			Other data supporting benign classification	
1	Benign	F 1 needed	General population or subpopulation frequency is too high to be a pathogenic mutation based on disease/syndrome prevalence and penetrance	
			Does not segregate with disease in a family study (genes with complete penetrance)	
			Internal frequency is too high to be a pathogenic mutation based on disease/syndrome prevalence and penetrance	
			No disease association in appropriately sized case-control study(ies)	
		1 of D and at least 2 of E		
		2 or more of D		
		>3 of E w/o conflicting data		
		>4 of E w/conflicting data		

The variant classification scheme is not intended for the interpretation of alterations considered epigenetic factors including genetic modifiers, multifactorial disease, or low-risk disease association alleles and may be limited in the interpretation of alterations confounded by incomplete penetrance, variable expressivity, phenocopies, triallelic or oligogenic inheritance, or skewed X-inactivation.

s0339.48b4_v1 | 7.19.17

This scheme is available at

https://www.ambrygen.com/file/material/view/272/Variant_ClassificationScheme_0617_Final.pdf

APPENDIX C

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD
PAGE 1 OF 2
December 17, 2017

KIRSTEN ANNE KELLY
PEDIATRICS

HS# 2018-4835: *Investigating Variants of Uncertain Significance Reclassification Drivers in Breast Cancer Genes* [Application for IRB Review (APP) # 13125]
Dear Kirsten Kelly,
A sub-committee of Institutional Review Board (IRB) reviewed the above-referenced item at its meeting on December 13, 2018 and concluded that the project, as described, may not qualify as human subjects research, however, further clarification is required from the team.
The following guidelines help determine whether the study constitutes human subjects research:
Research - a ***systematic investigation***, including research development, testing and evaluation, *designed to develop or contribute to* ***generalizable knowledge***.
A ***systematic*** approach involves a predetermined system, method or a plan for studying a specific topic, answering a specific question, testing a specific hypothesis, or developing theory. A systematic approach includes the collection of information and/or biospecimens, and analysis either quantitative or qualitative.
Activities **designed to develop or contribute to generalizable knowledge** are those activities designed to draw general conclusions, inform policy, or generalize outcomes beyond the specific group, entity, or institution (i.e., to elaborate, to be an important factor in identifying or expanding truths, facts, information that are universally applicable).
Human subject - a *living individual* about whom an investigator (whether faculty, student, or staff) conducting research obtains: **(1)** data through ***intervention*** *or* ***interaction*** with the individual; or **(2)** ***identifiable private information***.
Intervention includes both physical procedures by which information is gathered (for example, venipuncture) and manipulations of the subject or the subject's environment that are performed for research purposes.
Interaction includes communication or interpersonal contact between investigator and subject.
Please note that for the proposed activities to be considered Human Subjects Research, it needs to meet the criteria for both ***Research*** and ***Human Subjects***. In this case, the project does not meet the criteria for **Human Subjects** as the information abstracted *appears* to be de-identifiable.
The Committee required the following clarifications:
·Although UCI members are part-time employees at Ambry Genetics, it may be that the study could be considered Non-Human Subjects Research. Please **confirm** that UCI part-time employees at Ambry Genetics would not seek to re-identify subjects when abstracting data.

As such, the project would not meet the definition of human subjects/research and UCI IRB approval is not required to pursue your activities. Instead, you will receive an official Confirmation of Non-Human Subjects Research stating that UC Irvine does not require that you receive IRB Approval.
Please **indicate** whether you agree with this assessment.

- Otherwise, if UCI IRB Approval is crucial, please **address** the following:

    - After reading the procedures for this project, it appears that all research procedures will be conducted at Ambry Genetics. As such, please **clarify** UCI's intellectual contribution to the project.

    - Please further **clarify** how this study is **human subjects research.**

    - In addition, please **clarify** the HIPAA procedures at Ambry Genetics. As the data collected will be done at a non-UCI site, UCI may be unable to offer a Total Waiver of HIPAA Authorization.

Note: After a response is received, the responses will be re-assessed and the study team will receive additional comments and revisions needed to the submitted documents.
If you have any questions regarding the IRB's review, please respond to this e-mail, or call the Office of Research at 949-824-5047.

RE: Activities that Do Not Constitute Human Subjects Research
The University of California, Irvine (UCI) Human Research Protections Program complies with all review requirements defined in 45 CFR Part 46, Protection of Human Subjects. 45 CFR 46.102(e) defines research as "a systematic investigation, including research development, testing and evaluation, designed to develop or contribute to generalizable knowledge; and 45 CFR 46.102(f) defines a human subject as "a living individual about whom an investigator conducting research obtains 1) data through intervention or interaction with the individual; or 2) identifiable private information." Private information must be individually identifiable (i.e., the identity of the subject is or may readily be ascertained by the investigator or associated with the information) to meet the definition of human subject.

The UCI Human Research Protections (HRP) staff reviewed the information you submitted pertaining to your project "Investigating Variants of Uncertain Significance Reclassification Drivers in Breast Cancer Genes" and concluded that the project, as described, does not qualify as human subjects research because the activities do not involve human subjects. The team will not seek to re-identify subjects when abstracting data from Ambry Genetics. Therefore, the activities are not subject to UCI IRB review and approval. If your project changes in ways that may affect this determination, please contact the HRP staff for additional guidance.

Beverley Alberola, CIP
IRB Alternate Member

APPENDIX D

Criteria Cited
Other moderate data supporting pathogenic classification
PM1/5 Mutational hotspot, computational data, including structural analysis and/or modeling
Updated classification evidence and/or weighting
Other data supporting pathogenic classification
PM1_supporting Computational data, including structural analysis and/or modeling
Other strong data supporting benign classification
BP4_strong Amino acid is reference allele in ≥3 primates OR ≥15 mammals
BS1 Internal allele frequency greater than expected based on disease prevalence
Updated classification evidence and/or weighting
Other data supporting benign classification
BP6 Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation
BP4_strong Amino acid is reference allele in ≥3 primates OR ≥15 mammals
BP4 Computational data, including structural analysis and/or modeling
BS4_supporting Cosegregation and family history data*

*International Agency for Research on Cancer (IARC) and Open Variation Database (LOVD) (https://www.lovd.nl/)

APPENDIX E

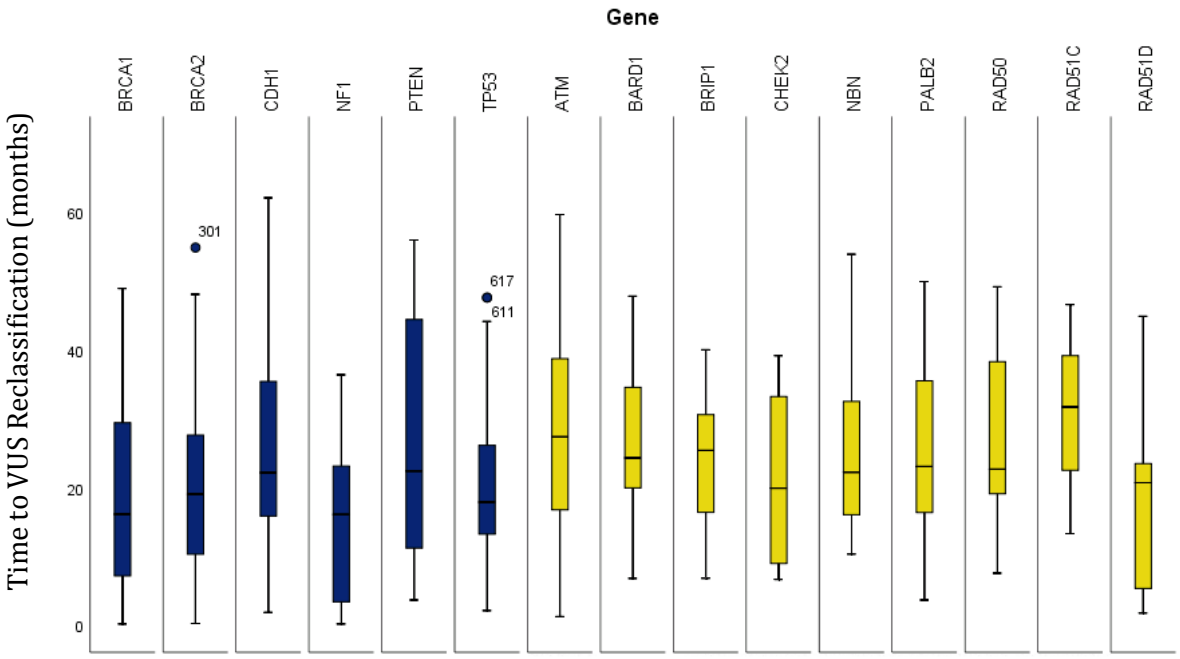
Categories of Triggers that Precede Reclassification	Variant identified in new patient sample
	Periodic re-review
	New research/data available
	Provider requested

APPENDIX F

RStudio logistic regression model code

```
#read in data and remove 2018
ds = read.csv("C:/Users/lishiz/Desktop/Book1.csv")
ds = ds[which(ds$Year != 2018), ]

#Create a new outcome named as outcome2. Upgrade is coded as 1 in outcome2.
ds$outcome2 = (ds$Outcome != "Upgrade")

#fit a logistic regression in each high and moderate penetrance group.

#high penetrance group
ds_high = ds[which(ds$Group == "High "),]
model1 = glm(outcome2 ~ Year, data = ds_high, family = "binomial")

summary(model1)
table(ds_high$Outcome, ds_high$Year)
#results
or = exp(summary(model1)$coefficients[2, 1])
lower = exp(summary(model1)$coefficients[2, 1]-1.96*summary(model1)$coefficients[2, 2])
higher = exp(summary(model1)$coefficients[2, 1]+1.96*summary(model1)$coefficients[2, 2])
print(c(or, lower, higher))   ##odds ratio and 95% confidence interval

#moderate penetrance group
ds_moderate = ds[which(ds$Group == "Moderate "),]
model2 = glm(outcome2 ~ Year, data = ds_moderate, family = "binomial")

summary(model2)
table(ds_moderate$Outcome, ds_moderate$Year)
#results
or = exp(summary(model2)$coefficients[2, 1])
lower = exp(summary(model2)$coefficients[2, 1]-1.96*summary(model2)$coefficients[2, 2])
higher = exp(summary(model2)$coefficients[2, 1]+1.96*summary(model2)$coefficients[2, 2])
print(c(or, lower, higher))   ##odds ratio and 95% confidence interval
```

APPENDIX G


Gene
BRCA1
BRCA2
CDH1
NF1
PTEN
TP53
ATM
BARD1
BRIP1
CHEK2
NBN
PALB2
RAD50
RAD51C
RAD51D
Time to VUS Reclassification (months)
0
20
40
60
301
617
611