UNIVERSIDADE FEDERAL DE MINAS GERAIS

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JOÃO PAULO GONZAGA DE FARIA

GERMLINE PANEL AS STANDARD METHOD FOR INVESTIGATING HEREDITARY BREAST AND OVARIAN CANCER SYNDROME IN THE BRAZILIAN SUPPLEMENTARY HEALTH SYSTEM

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João Paulo Gonzaga de Faria

GERMLINE PANEL AS STANDARD METHOD FOR INVESTIGATING HEREDITARY BREAST AND OVARIAN CANCER SYNDROME IN THE BRAZILIAN SUPPLEMENTARY HEALTH SYSTEM

Dissertação apresentada ao programa de Pós-Graduação em Medicina Molecular da Faculdade Medicina da Universidade de Minas Gerais, como parte dos requisitos para obtenção do título de Mestre em Medicina Molecular.

Orientador: Prof. Luiz Armando De Marco.
Coorientadora: Dra. Juliana Godoy Assumpção.

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FOLHA DE APROVAÇÃO

USO DE PAINEL GERMINATIVO COMO MÉTODO PADRÃO DE INVESTIGAÇÃO DE SÍNDROME DE CÂNCER DE MAMA E OVÁRIO HEREDITÁRIO NO SISTEMA DE SAÚDE SUPLEMENTAR BRASILEIRO

JOÃO PAULO GONZAGA DE FARIA

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DEDICATÓRIA

Dedicado aos pacientes.

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“The solution often turns out more beautiful than the puzzle.”

(Richard Dawkins)

RESUMO

Introdução: Cerca de 10% dos casos de câncer de mama são decorrentes de fatores hereditários, segundo dados dos Instituto Nacional do Câncer José de Alencar. No contexto brasileiro, a incidência de câncer de mama é de cerca de 59 mil novos casos por ano. Cerca de 50 milhões de brasileiros possui plano de saúde. Nesta população da saúde suplementar, a investigação molecular de câncer hereditário para casos suspeitos de Síndrome de Câncer de Mama e Ovário Hereditários são ditados pela Agência Nacional de Saúde Suplementar através das Diretrizes de Utilização publicadas no Anexo II do rol de procedimentos obrigatórios (Anexo 1). As diretrizes mais recentes preveem a investigação escalonada através de sequenciamento de nova geração e multiplex ligation-dependent probe amplification dos genes *BRCA1* e *BRCA2* e, em caso de resultado inconclusivo, a realização de painel germinativo através de sequenciamento de nova geração dos genes *ATM, BRCA1, BRCA2, BRIP1, CDH1, CHEK2, MLH1, MSH2, MSH6, PALB2, PMS2, PTEN, RAD51C, RAD51D, STK11, TP53* e, em caso de novo resultado inconclusivo e o painel em questão não estiver validado para a análise de contagem do número de cópias, a realização de multiplex ligation-dependent dos mesmos genes. Evidências recentes sugerem que essa investigação escalonada atualmente prevista na Agência Nacional de Saúde pode ser otimizada através do uso apenas do painel germinativo com contagem de número de cópias. Este trabalho avaliou 701 pacientes com critérios de investigação molecular de Síndrome de Câncer de Mama e Ovários Hereditários segundo o rol de procedimentos da Agência Nacional de Saúde Suplementar. Todos foram consultados por médico geneticista e submetidos à testagem através de painel germinativo através de sequenciamento de nova geração com contagem do número de cópias.

Métodos: Entre 2021 e 2022 foram avaliados 701 pacientes com critérios de Síndrome de Câncer de Mama e Ovários Hereditários segundo os critérios da Agência Nacional de Saúde Suplementar por médico geneticista em Belo Horizonte, Minas Gerais, sendo 683 mulheres e 18 homens. Todos foram submetidos em laboratórios privados à testagem por painel germinativo com sequenciamento nova geração e contagem do número de cópias utilizando a plataforma NovaSeq Illumina. Uma parte, 348 pacientes, foi analisada através de painel de 40 genes, enquanto outra, 353 pacientes, foi analisada através de painel de 141 genes.

Resultados: Foram identificadas variantes patogênicas e provavelmente patogênicas em 19,54% dos pacientes. Na amostragem analisada através de 40 genes, a taxa de detecção foi de 16,4% para uma variante e 0,29% para duas variantes diferentes, enquanto na amostragem analisada através de 141 genes a taxa de detecção foi de 22,7% para uma variante e 2,27% para duas variantes. Nove por cento dos pacientes apresentaram variantes em genes de herança autossômico dominante, 11% em genes recessivos e 2,2% em genes com ambos os mecanismos de herança. A incidência de variantes de significado clínico incerto foi de 47,12% nos painéis de 40 genes e 82,72% dos painéis de 141 genes. Os resultados foram submetidos à testes estatísticos. O teste Mann Whitney foi utilizado entre as amostragens do sexo feminino e masculino, sem diferença significativa entre os resultados. As incidências de variantes patogênicas e provavelmente patogênicas entre os painéis de 40 e 141 genes foram submetidas ao teste qui quadrado, sugerindo que testes com maiores quantidades de genes tem maior probabilidade de encontrar variantes patogênicas e provavelmente patogênicas.

Conclusões: Os resultados sugerem que a substituição da investigação escalonada prevista na Agência Nacional de Saúde Suplementar pelo uso de painel germinativo com sequenciamento de nova geração e contagem do número de cópias como método inicial para pacientes com critérios de Síndrome de Câncer de Ovário Hereditários pode detectar até 22,7% de incidência de variantes patogênicas e provavelmente patogênicas, representando redução do tempo e possivelmente o custo de investigação.

Palavras chaves: Hereditary Breast and Ovarian Cancer Syndrome; breast cancer; ovarian cancer; prostate cancer; gene BRCA1; gene BRCA2.

ABSTRACT

Introduction: Approximately 10% of breast cancer cases are due to hereditary factors, according to data from the José de Alencar National Cancer Institute. In the Brazilian context, the incidence of breast cancer is approximately 59,000 new cases per year. Around 50 million Brazilians have private health insurance. In this population with supplementary health coverage, the molecular investigation of hereditary cancer for suspected cases of Hereditary Breast and Ovarian Cancer Syndrome is governed by the National Supplementary Health Agency through the Utilization Guidelines published in Annex II of the mandatory procedures list. The latest guidelines provide for a stepped investigation using next-generation sequencing and multiplex ligation-dependent probe amplification of the *BRCA1* and *BRCA2* genes, and in case of inconclusive results, performing germline panel testing through next-generation sequencing of the *ATM, BRCA1, BRCA2, BRIP1, CDH1, CHEK2, MLH1, MSH2, MSH6, PALB2, PMS2, PTEN, RAD51C, RAD51D, STK11, TP53* genes, and in case of a new inconclusive result and if the panel in question is not validated for copy number analysis, performing multiplex ligation-dependent probe amplification of the same genes. Recent evidence suggests that this stepped investigation currently provided for by the National Supplementary Health Agency can be optimized by using only the germline panel with copy number analysis. This study evaluated 701 patients with criteria for molecular investigation of Hereditary Breast and Ovarian Cancer Syndrome according to the procedures list of the National Supplementary Health Agency. All patients were consulted by a geneticist and underwent testing using a germline panel through next-generation sequencing with copy number analysis.

Methods: Between 2021 and 2022, 701 patients with criteria for Hereditary Breast and Ovarian Cancer Syndrome according to the criteria of the National Supplementary Health Agency were evaluated by a geneticist in Belo Horizonte, Minas Gerais, including 683 women and 18 men. All patients underwent testing at private laboratories using a germline panel with next-generation sequencing and copy number analysis using the NovaSeq Illumina platform. A subset of 348 patients was analyzed using a 40-gene panel, while another subset of 353 patients was analyzed using a 141-gene panel.

Results: Pathogenic and likely pathogenic variants were identified in 19.54% of the patients. In the subset analyzed using 40 genes, the detection rate was 16.4% for one variant and 0.29% for two different variants, while in the subset analyzed using 141 genes, the detection rate was 22.7% for one variant and 2.27% for two variants. Nine percent of the patients had variants in autosomal dominant inheritance genes, 11% in recessive genes, and 2.2% in genes with both inheritance mechanisms. The incidence of variants with uncertain clinical significance was 47.12% in the 40-gene panels and 82.72% in the 141-gene panels. The results were subjected to statistical tests. The Mann-Whitney test was used between the female and male sample groups, with no significant difference between the results. The incidences of pathogenic and likely pathogenic variants between the 40-gene and 141-gene panels were subjected to the chi-square test, suggesting that tests with a larger number of genes are more likely to detect pathogenic and likely pathogenic variants.

Conclusions: The results suggest that replacing the stepped investigation currently provided for by the National Supplementary Health Agency with the use of a germline panel with next-generation sequencing and copy number analysis as the initial method for patients with Hereditary Ovarian Cancer Syndrome criteria can detect up to a 22.7%

incidence of pathogenic and likely pathogenic variants, representing a reduction in time and possibly the cost of investigation.

Key words: Hereditary Breast and Ovarian Cancer Syndrome; breast cancer; ovarian cancer; prostate cancer; gene BRCA1; gene BRCA2.

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INTRODUCTION

INTRODUCTION

Hereditary Breast and Ovarian Cancer syndrome is a genetic condition that makes it more likely that a person will get breast, ovarian, and other cancers (Centers for Disease Control and Prevention, 2016). It is estimated that about 10% of breast cancer cases may have some genetic predisposition (Instituto Nacional de Câncer José Alencar Gomes da Silva & Ministério da Saúde, 2019), 20% of ovarian cancer cases (Toss et al., 2015), 5 - 10% of pancreatic cancer cases (Amundadottir, 2016) and 5 - 10% of prostate cancer cases (Doan et al., 2021). Individuals who fulfill the criteria for Hereditary Breast and Ovarian Cancer Syndrome (Centers for Disease Control and Prevention, 2016) with a confirmed molecular diagnosis may have a risk of up to 70% of developing cancer throughout their lives (Dwyer & Mary, 2023).

Breast cancer is a major public health issue in Brazil, with an estimated incidence of 59,700 new cases per year, corresponding to 56.33 per 100,000 women nationally and 80.33 per 100,000 women in the capitals (Instituto Nacional de Câncer José Alencar Gomes da Silva & Ministério da Saúde, 2019). The Brazilian health system is composed of both public and private services, and it is administered by the Ministry of Health, state, and municipal governments, as well as private hospitals and clinics.

The Sistema Único de Saúde (SUS) is the main public health system in the country and provides free and universal care to all Brazilian citizens, regardless of their financial condition. It is financed by taxes and aims to ensure universal access to health care for all Brazilians, promoting disease prevention and treatment. Private health services in Brazil are provided through private health insurance plans, which are signed by individuals or legal entities to ensure medical, dental and hospital assistance. These plans are set by the

National Health Agency, which determines rules for their operation and monitors the quality of the services provided. The List of Procedures and Events in Health of the National Health Agency (Agência Nacional de Saúde Suplementar, 2021) is the list of procedures and treatments that private health insurance plans are required to offer to their beneficiaries. The objective of the List is to guarantee the minimum coverage necessary to meet the health needs of the beneficiaries. Molecular tests for the assessment of the risk of hereditary cancer are available to users of Brazilian private health insurance plans, while users of the Sistema Único de Saúde do not have access to these tests.

Approximately 50,409,611 Brazilians are clients of health insurance plans. Of these, 26,660,966 are women, and 20,860.118 are women above 18 years old (Agência Nacional de Saúde Suplementar & Ministério da Saúde, 2023). According to available data, 12% of this group may develop breast cancer during their lifetime (Centers for Disease Control and Prevention, 2016), adding up to 3,199,316 individuals. Among these, about 455,911 would have a variant associated with increased cancer risk and, according to high penetrance, about 60%, or 249.547, would develop cancer over their lifetime.

The Brazilian healthcare system provides investigation of patients who meet the criteria for Hereditary Breast and Ovarian Cancer Syndrome according to the following pipeline: first, Sequencing of the *BRCA1* and *BRCA2* genes, second, Multiplex Ligation-dependent Probe Amplification of the *BRCA1* and *BRCA2* genes, and if the results remain inconclusive, it is mandatory to perform germline panel testing covering the *ATM, BRCA1, BRCA2, BRIP1, CDH1, CHEK2, MLH1, MSH2, MSH6, PALB2, PMS2, PTEN, RAD51C, RAD51D, STK11,* and *TP53* genes. The procedure list does not require an analysis of the Copy Number Variation with next-generation sequencing (Agência Nacional de Saúde Suplementar, 2021). With the advance of next generation sequencing methodology and multi

gene panels price gradually decreasing over time, the affordability of next generation sequencing has greatly improved. Therefore, it is worthy assessing the cost and time-effectiveness of testing Hereditary Breast and Ovarian Cancer Syndrome straight by next generation sequencing multi gene panels.

The present study analyzed 701 patients who were evaluated by a geneticist between 2021 and 2022 and fulfilled Hereditary Breast and Ovarian Cancer Syndrome criteria. All patients underwent molecular analysis using a single step strategy based on next generation sequencing germline panel. Analyses were carried out by two private laboratories, and the patients were randomly distributed between them. Both laboratories performed germline panel testing through next-generation sequencing with copy number counting. The panel of one laboratory covered 40 genes, while the other laboratory's panel covered 141 genes.

OBJECTIVES

OBJECTIVES

The present study evaluates the use of germline panels as a first-tier technique for investigating germline mutations in Hereditary Breast and Ovarian Cancer Syndrome in the Brazilian healthcare system.

MATERIALS AND METHODS

MATERIALS AND METHODS

This project was approved by the IRB (Institutional Review Board) - Ethics Committee of the Faculty of Medicine of the Federal University of Minas Gerais (CAAE 47224115.7.0000.5149).

Between 2021 and 2022, the total sample included 706 individuals who were referred to a geneticist and met the criteria for Hereditary Breast and Ovary Cancer Syndrome according to the guidelines of the Brazilian National Agency for Supplementary Health (Appendix 1) were submitted to a single test through germ panel. These criteria include female patients diagnosed with breast cancer under the age of 35 years old; female patients diagnosed with breast cancer under the age of 50 years old with at least one relative also diagnosed with breast cancer; female patients diagnosed with breast cancer bilaterally under the age of 50 years; female patients diagnosed with breast cancer under the age of 60 years with immunohistochemistry data showing a triple negative pattern (negative progesterone and estrogen receptors and negative HER2); female patients diagnosed with breast cancer at any age with > 2 relatives diagnosed with breast cancer; female patients diagnosed with breast cancer at any age and at least one relative diagnosed with ovarian cancer; female patients diagnosed with breast cancer and at least two relatives diagnosed with pancreatic and/or prostate cancer (Gleason score > 7); male patients diagnosed with breast cancer at any age; patients diagnosed with pancreatic and/or prostate cancer (Gleason > 7) with at least two relatives diagnosed with breast, ovarian, pancreas and/or prostate cancer (Gleason > 7); patients with identified familial mutation. Only one member per family was investigated. Patients were randomized between two private laboratories. In one laboratory panels with 40 genes

were performed, while in the other 141 genes were performed. The identity of each patient was not associated with the analyzed data.

Commercial germline panels were performed using the NovaSeq Illumina platform Next generation sequencing panels with copy number variation. DNA from 348 (49.6%) patients were subject to a germline panel comprising 40 genes and DNA from 353 (50.4%) patients were subject to a germline panel comprising 141 genes. The DNA was extracted from peripheral blood sample, captured by custom probes, and enriched for the regions of interest. After next generation sequencing of the target sequences, using the Illumina platform (Illumina, Inc, San Diego, USA), alignment and detection of variants based on the GRCh37 version of the Human Genome were performed. The variants were analyzed considering the patient's clinical condition and the American College of Medical Genetics (ACMG) variant classification protocol.

According to Brazilian data (Instituto Nacional de Câncer José Alencar Gomes da Silva & Ministério da Saúde, 2019), the percentage of breast cancer patients with pathogenic variants is 10%. A sample size of at least 341 individuals would attain 80% statistical power to detect an absolute difference of at least 5% or 50% relative difference in relation to the percentage of gene prevalence according the guidelines of the Brazilian National Agency for Supplementary Health. Therefore, to obtain these results, the bilateral binomial test was used at a significance level of 0.05. The software used was G*Power Version 3.1.9.6 (Faul F, 2007).

RESULTS

RESULTS

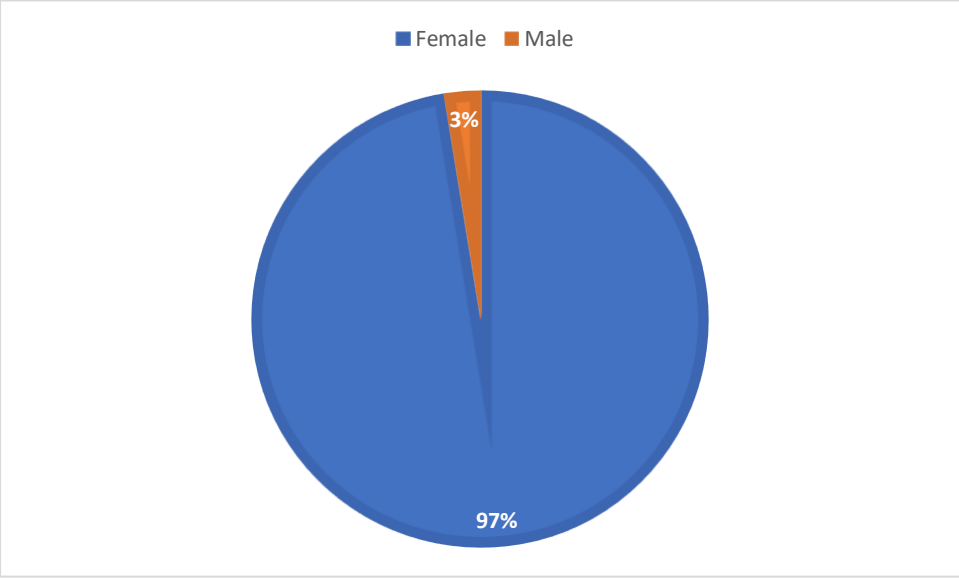
Study sample

Seven hundred and six individuals were examined during the medical genetic consultation. Three were not eligible because of exclusion criteria (not informing sex). Two were found to be siblings and were also excluded. From the analyzed samples, 683 individuals were females and 18 were males (Table 1, Figure 1).

Table 1 - Proportion of female and male patients

Variable	Sex	N	Total	Percentage (%)
	F	683	701	97,4
	M	18	701	2,6

Figure 1 - Proportion of female and male patients

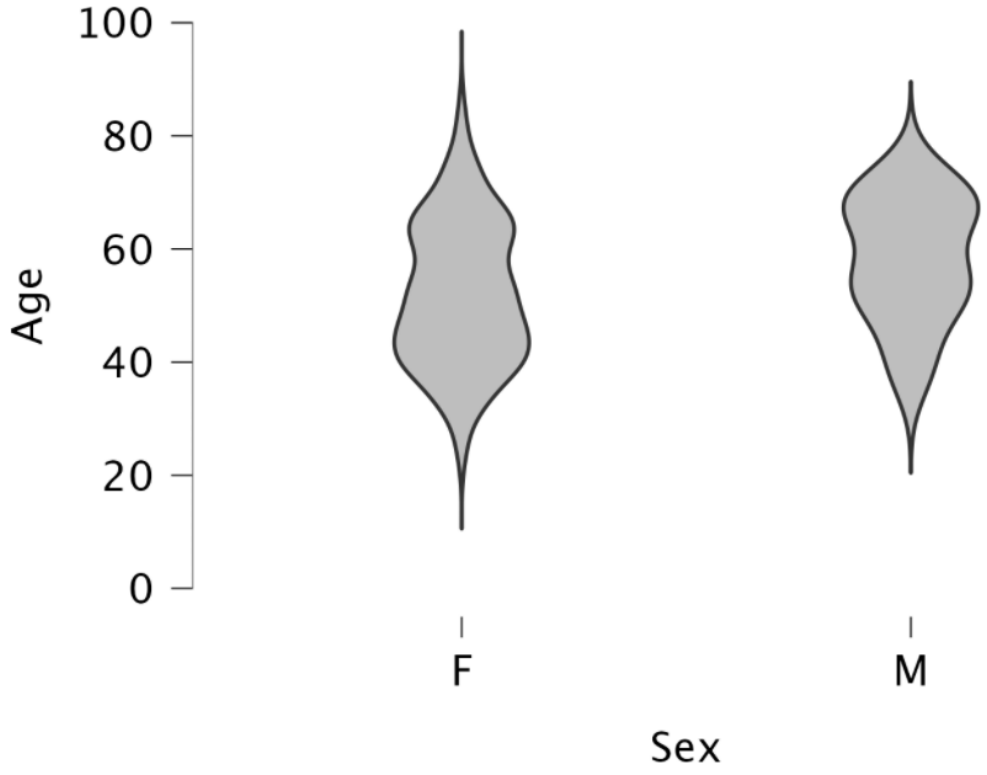


The average age of females was 51.97 years and males were 58.12 years (Table 2, Figure 2).

Table 2 - Proportion of female and male patients in relation to age.

	Age (Years)	
	F	**M**
N	683	18
Mean	51	58
Minimum	20	37
Maximum	89	73

Figure 2 - Proportion of female and male patients in relation to age.

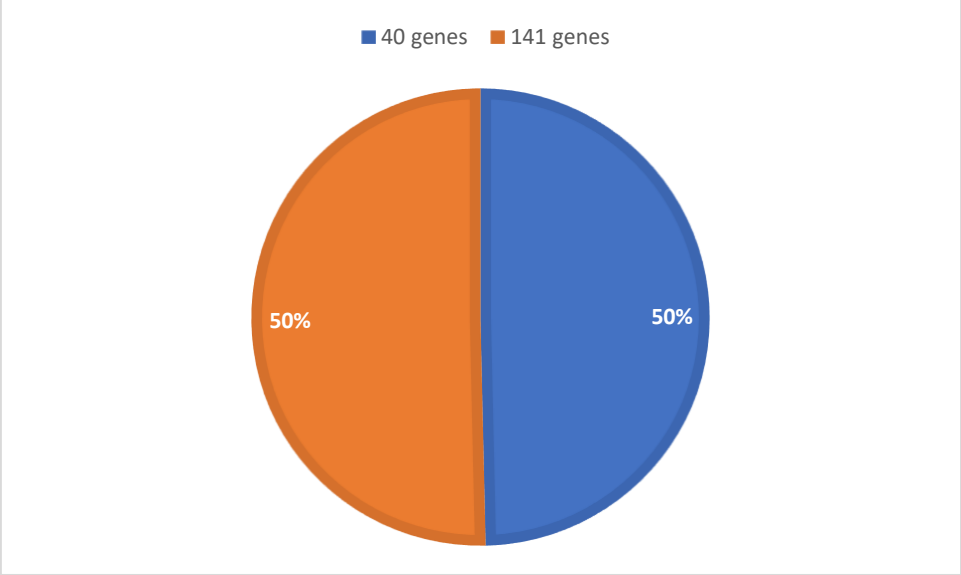


Three hundred forty-nine (49,5%) patients were analyzed using a panel of 40 genes and 355 (50,4%) patients were analyzed using a panel of 141 genes. (Table 3, Figure 3).

Table 3 - Proportion of genes analyzed per panel for each group (N) of patients.

Number of analyzed genes	N	Total	Percentage
40	348	701	49,6
141	353	701	50,4

Figure 3 - Proportion of genes analyzed per panel for each group of patients.



During medical examination, all patients referred a family history of personal cancer according to the Brazilian National Agency for Supplementary Health for Hereditary Breast and Ovarian Cancer Syndrome.

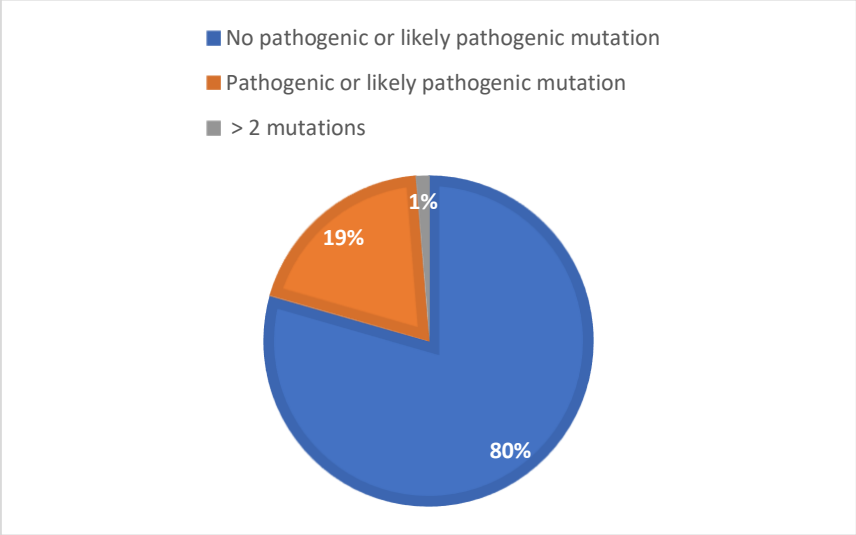
Genetic findings

Pathogenic and likely pathogenic variants were identified in 137 (19.6%). Nine (1.3%) had two pathogenic and/or probably pathogenic variants (Table 4, Figure 4).

Table 4 - Proportion of results without pathogenic variants and with pathogenic and/or probably pathogenic variants.

	N	Total	Percentage (%)
No pathogenic or likely pathogenic variant	564	701	80,5
Pathogenic or likely pathogenic variant	137	701	19,5
> 2 variants	9	701	1,3

Figure 4 - Proportion of results without pathogenic variants and with pathogenic and/or probably pathogenic variants.

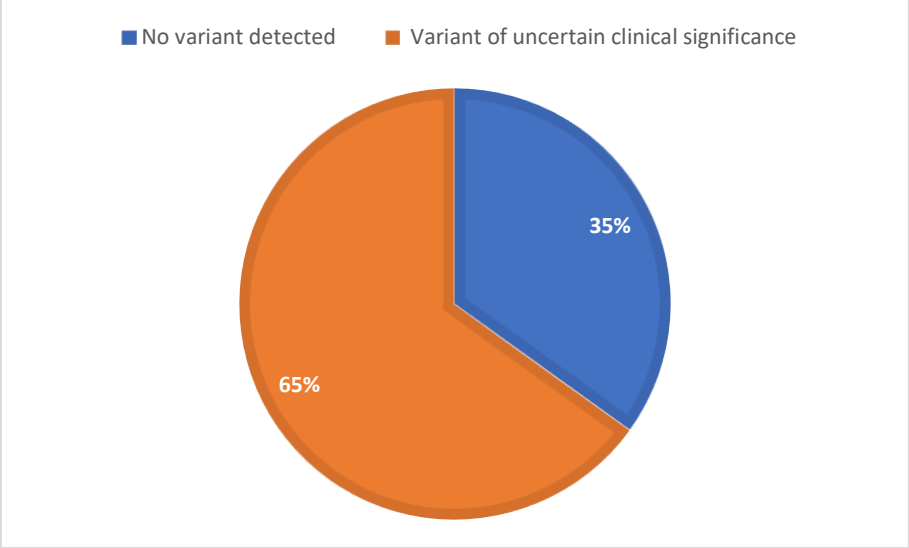


Variants of uncertain clinical significance were detected in 456 (65.2%) of the patients (Table 5 and Figure 5).

Table 5 - Proportion of results with variants of uncertain clinical significance.

	N	Total	Percentage (%)
No variant detected	245	701	34,8
Variant of uncertain clinical significance	456	701	65,2

Figure 5 - Proportion of results with variants of uncertain clinical significance.

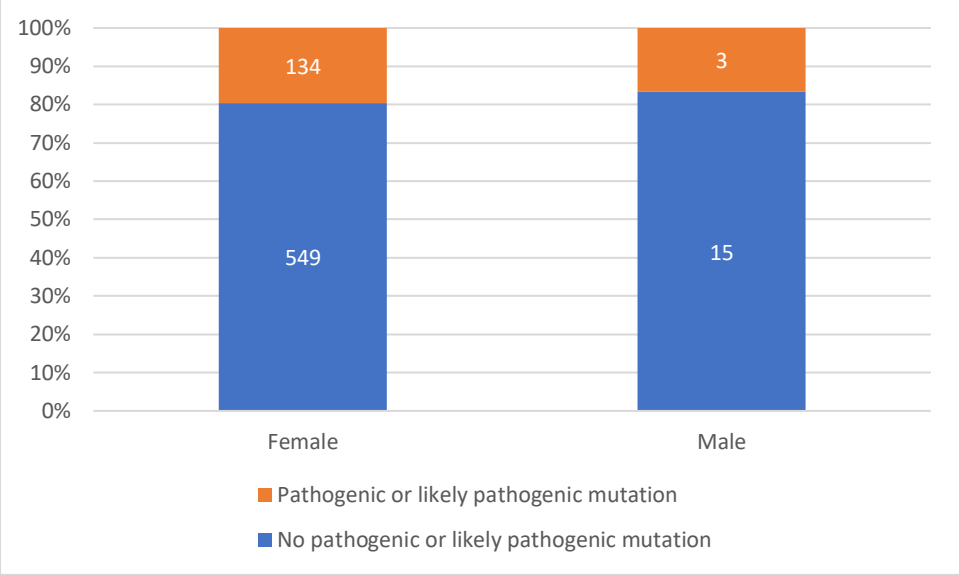


One hundred and thirty four (19.6%) of the women had pathogenic and/or probably pathogenic variants, while the result in the male sample was three (16.7%) (Table 6, Figure 6). Using the Mann-Whitney test, it was found that there is not enough statistical evidence to reject the null hypothesis of equality of medians between the groups (p-value = 0.756). This indicates that no significant difference was found in the distributions of positive genetic test results between women and men.

Table 6 - Proportion of results with pathogenic and/or probably pathogenic variants according to sex.

Sex	Pathogenic or likely pathogenic variant	N	Percentage (%)
Female	No pathogenic or likely pathogenic variant	549	80,3
	Pathogenic or likely pathogenic variant	134	19,7
	Total	683	100,00
Male	No pathogenic or likely pathogenic variant	15	83,3
	Pathogenic or likely pathogenic variant	3	16,7
	Total	18	100,00

Figure 6 - Proportion of results with pathogenic and/or probably pathogenic variants according to sex.

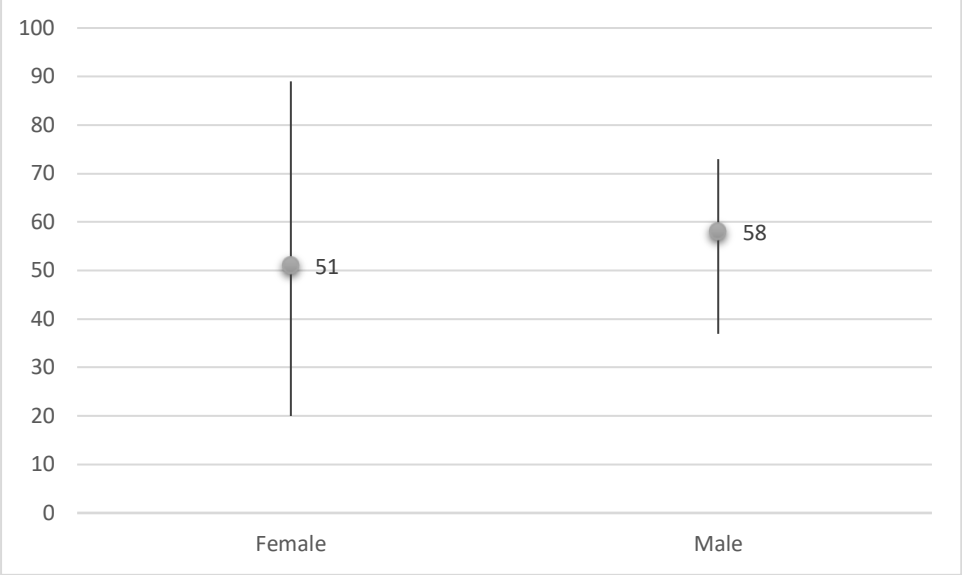


The average age in the female sample with pathogenic or probably pathogenic variants detected was 51.98 years and in the male sample it was 58.16 years. The youngest age to present a deleterious variant in the female sample was 20 years and the maximum age was 89 years, while in the male sample the minimum age was 37 years, and the maximum age was 73 years (Table 7, Figure 7).

Table 7 - Proportion of results with pathogenic and/or probably pathogenic variants according to sex and maximum age, minimum age and mean age with results showing pathogenic and/or probably pathogenic variants.

	Age	
	Female	**Male**
Medium age (years)	51	58
Minimum age (years)	20	37
Maximum age (years)	89	73

Figure 7 - Proportion of results with pathogenic and/or probably pathogenic variants according to sex and maximum age, minimum age and mean age with results showing pathogenic and/or probably pathogenic variants.



Genetic findings according to the number of genes analyzed between 40 vs 141 genes

The incidence of identification of pathogenic and probably pathogenic variants was 57 (16.4%) in the sample analyzed through a panel containing 40 genes and 80 (22,66%) in the sample analyzed through a panel containing 141 genes**.** A chi-square test was conducted to compare the positive results in a sample group that underwent a small-scale test consisting of 40 genes versus a sample group that underwent a larger-scale test consisting of 141 genes. The aim was to assess if there was a significant difference in the detection of positive results between the two testing approaches. The results of the chi-square test ($p = 0.036$) indicate that there is a statistically significant difference in the detection of pathogenic variants between the two tests, with the larger test identifying a higher number of pathogenic variants. The p-value of 0.036 suggests that the likelihood of observing such a difference by chance alone is relatively low. These findings provide evidence to support the hypothesis that the larger test has a greater ability to detect pathogenic variants compared to the smaller test. Table 8, Figure 8).

Table 8 - Proportion of pathogenic and probably pathogenic variants according to gene panel.

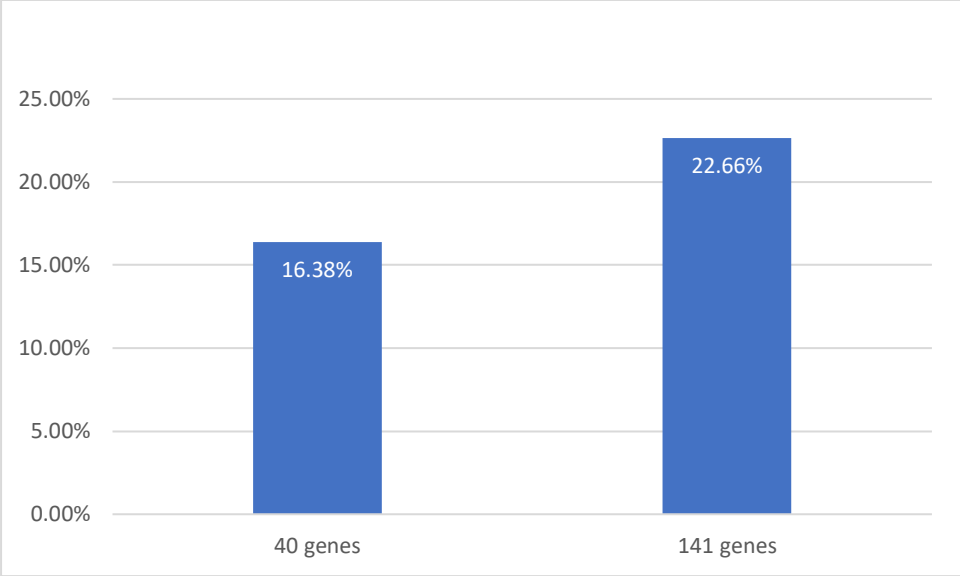
	Pathogenic or likely pathogenic mutation		
Number of genes	**0**	**1**	**Total**
141 genes panel	273	80	353
40 genes panel	291	57	348
Total	564	137	701

Chi-Squared Tests

	Value	**df**	**p**	**VS-MPR***
X^2	4.400	1	0.036	3.078
N	701			

* Vovk-Sellke Maximum *p* -Ratio: Based the *p* -value, the maximum possible odds in favor of H_1 over H_0 equals $1/(-e\ p \log(p))$ for $p \leq .37$ (Sellke, Bayarri, & Berger, 2001).

Figure 8 - Proportion of pathogenic or probably pathogenic variant according to gene panel.



Two individuals presented pathogenic and/or likely pathogenic copy number variations. One was a 53-years old female with *PALB2* microdeletion affecting exons 1-10. Another was a 61-years female with a microdeletion described as chr17:56.780.556-56.780.690.

The incidence of two simultaneous pathogenic or probably pathogenic variants was one (0,28%) in the sample analyzed through a panel containing 40 genes and 8 (2.26%) in the sample analyzed through a panel containing 141 genes (Table 9, Figure 9). A chi-square test was conducted to compare the positive results in a sample group that underwent a small-scale test consisting of 40 genes versus a sample group that underwent a larger-scale test consisting of 141 genes. The aim was to assess if there was a significant difference in the detection of positive results between the two testing approaches. The results of the chi-square test ($p = 0.036$) indicate that there is a statistically significant difference in the detection of pathogenic variants between the two tests, with the larger test identifying a higher number of pathogenic variants. Furthermore, the VS-MPR value of 3.708 further reinforces the statistical significance of the observed difference, indicating that the likelihood of obtaining such a large difference by chance alone is very low.

Table 9 - Proportion of two pathogenic and probably pathogenic variants according to gene panel.

Contingency Tables

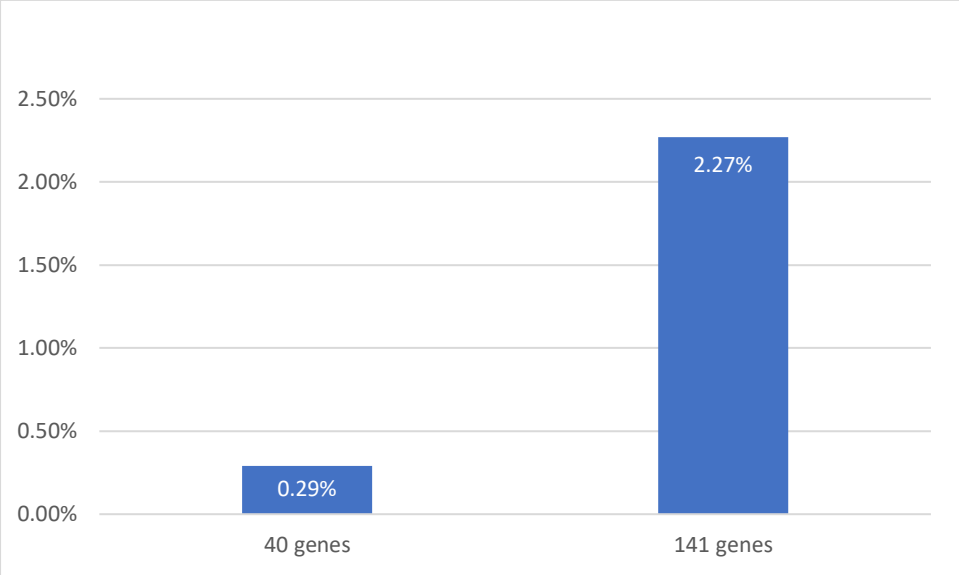
	> 2 mutations		
Number of genes	**0**	**2**	**Total**
141 genes panel	345	8	353
40 genes panel	347	1	348
Total	692	9	701

Chi-Squared Tests

	Value	**df**	**p**	**VS-MPR***
X^2	5.415	1	0.020	4.708
N	701			

* Vovk-Sellke Maximum *p* -Ratio: Based the *p* -value, the maximum possible odds in favor of H_1 over H_0 equals $1/(-e\ p \log(p))$ for $p \leq .37$ (Sellke, Bayarri, & Berger, 2001).

Figure 9 - Proportion of two pathogenic or probably pathogenic variants according to gene panel.



The proportion of variants of uncertain clinical significance was 164 (47.12%) in the sample analyzed through a panel containing 40 genes and 292 (82.72%) in the sample analyzed through a panel containing 141 genes (Figure 10). In the sample analyzed through a panel of 40 genes that presented variants of uncertain clinical significance, 115 (33.04%) presented only one variant, 40 (11.49%) presented two variants, seven (2.01%) presented three variants and two (0.57%) had four variants. In the group analyzed through a panel of 141 genes, 100 (28.32%) presented one single variant, 90 (25.49%) presented two variants, 61 (17.28%) presented three variants, 30 (8.49%) presented four variants, eight (2.26%) presented five variants, one (0.28%) presented six variants and two (0.56%) presented seven variants of uncertain clinical significance. The chi-square test comparing these results yielded a p-value of < 0.001 and a VS-MPR (Variance-Stabilizing Mid-P Exact Test) value of 5.122e+20. The obtained p-value of < 0.001 indicates a highly significant difference in the occurrence of uncertain results between the two sample

groups. This suggests that the larger-scale test with 141 genes has a higher likelihood of generating uncertain results compared to the smaller-scale test. The VS-MPR value of 5.122e+20 further supports the statistical significance of this difference. (Table 10 and Figure 10).

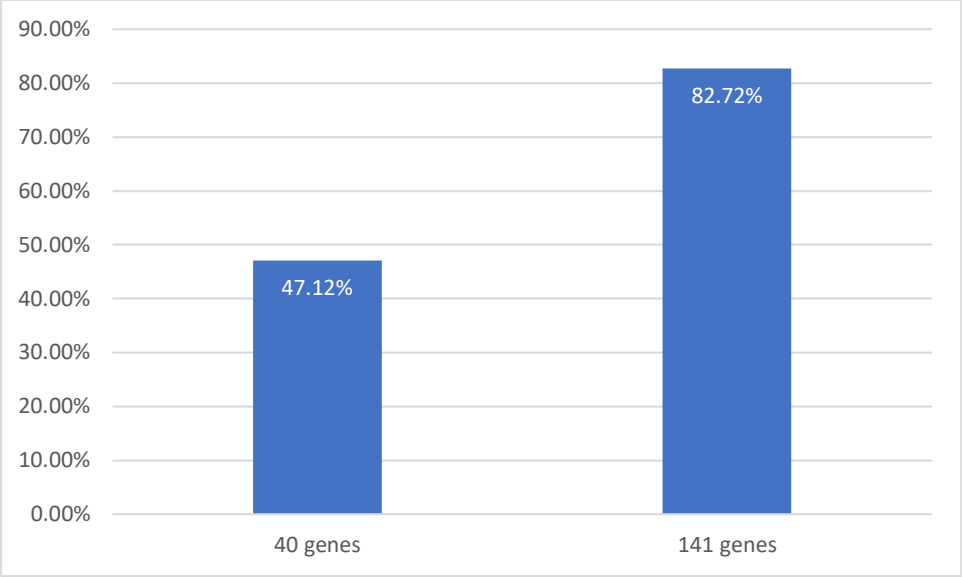
Table 10 – Proportion of variants of uncertain clinical significance according to gene panel

	Number of variants of uncertain clinical significance								
Number of genes	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Total**
141 genes panel	61	100	90	61	30	8	1	2	353
40 genes panel	184	115	40	7	2	0	0	0	348
Total	245	215	130	68	32	8	1	2	701

Chi-Squared Tests

	Value	**df**	**p**
X^2	160.383	7	< .001
N	701		

Figure 10 - Proportion of variants of uncertain clinical significance according to gene panel.



Pathogenic and/or probably pathogenic variants.

A total of 34 genes showed pathogenic or probably pathogenic variants. *BRCA1* variations classified as pathogenic or probably pathogenic were detected in 25 individuals (17,2%), *BRCA2* were detected in 24 (16,6%) individuals, *MUTYH* in 22 (15,2%), *CHEK2* in 12 (8,3%), *ATM* in eight (5,5%), *PALB2* in seven (4,8%), *BRIP1* in five (3,4%), *TP53* in four (2,8%), *RAD51D* and *MITF* in three (2,1%), *APC, FANCG, FANCI, FANCM, MERTK, NTHL1, PMS2, RAD51C* in two each one (1,45%), *BUB1B, CDH1, CTC1, ERCC2, ERCC3, EXT2, FANCL, LZTR1, MSH6, NBN, PRF1, PTEN, RECQL4, SBDS, SDHA* and *SLX4* in one individual each one (0,7%). (Figure 11, Table 11).

Figure 11 – proportion of pathogenic or likely pathogenic variants according to the gene.

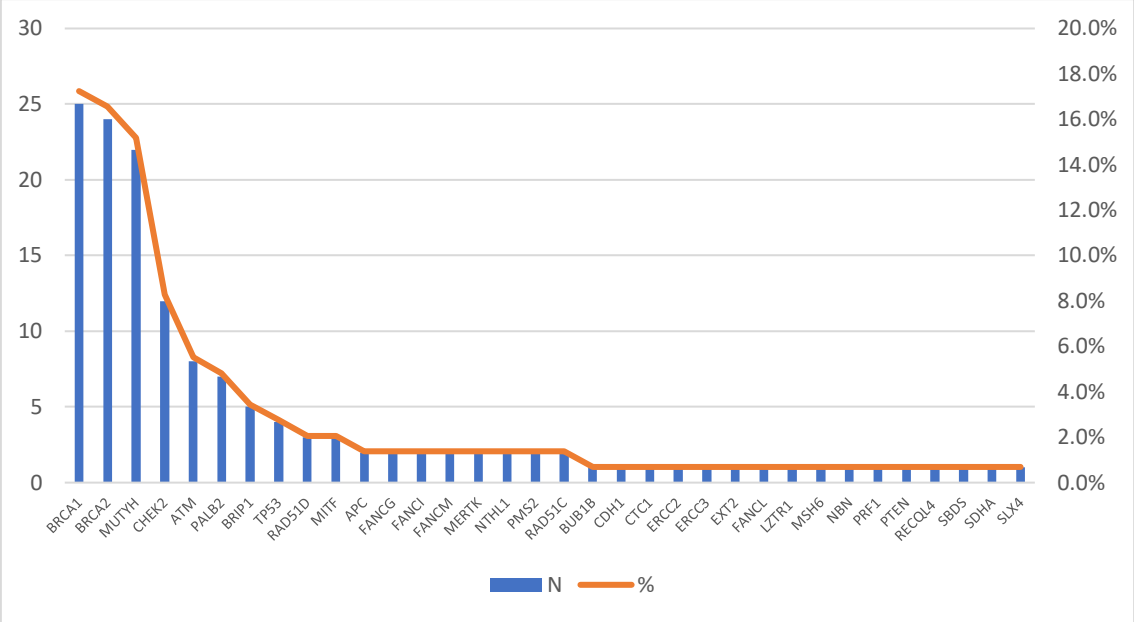


Table 11 – proportion of pathogenic or likely pathogenic variants according to studied genes.

Gene	N	%
BRCA1	25	17,2%
BRCA2	24	16,6%
MUTYH	22	15,2%
ATM	8	5,5%
PALB2	7	4,8%
CHEK2	12	8,3%
BRIP1	5	3,4%
TP53	4	2,8%
APC	2	1,4%
FANCG	2	1,4%
FANCI	2	1,4%

FANCM	2	1,4%
MERTK	2	1,4%
NTHL1	2	1,4%
PMS2	2	1,4%
RAD51C	2	1,4%
RAD51D	3	2,1%
BUB1B	1	0,7%
CDH1	1	0,7%
CTC1	1	0,7%
ERCC2	1	0,7%
ERCC3	1	0,7%
EXT2	1	0,7%
FANCL	1	0,7%
LZTR1	1	0,7%
MITF	3	2,1%
MSH6	1	0,7%
NBN	1	0,7%
PRF1	1	0,7%
PTEN	1	0,7%
RECQL4	1	0,7%
SBDS	1	0,7%
SDHA	1	0,7%
SLX4	1	0,7%

Ninety-seven pathogenic or probably pathogenic variants were detected. (Table 12) Twenty-six pathogenic or probably pathogenic variants were found in *BRCA1* and *BRCA2* (Figure 12).

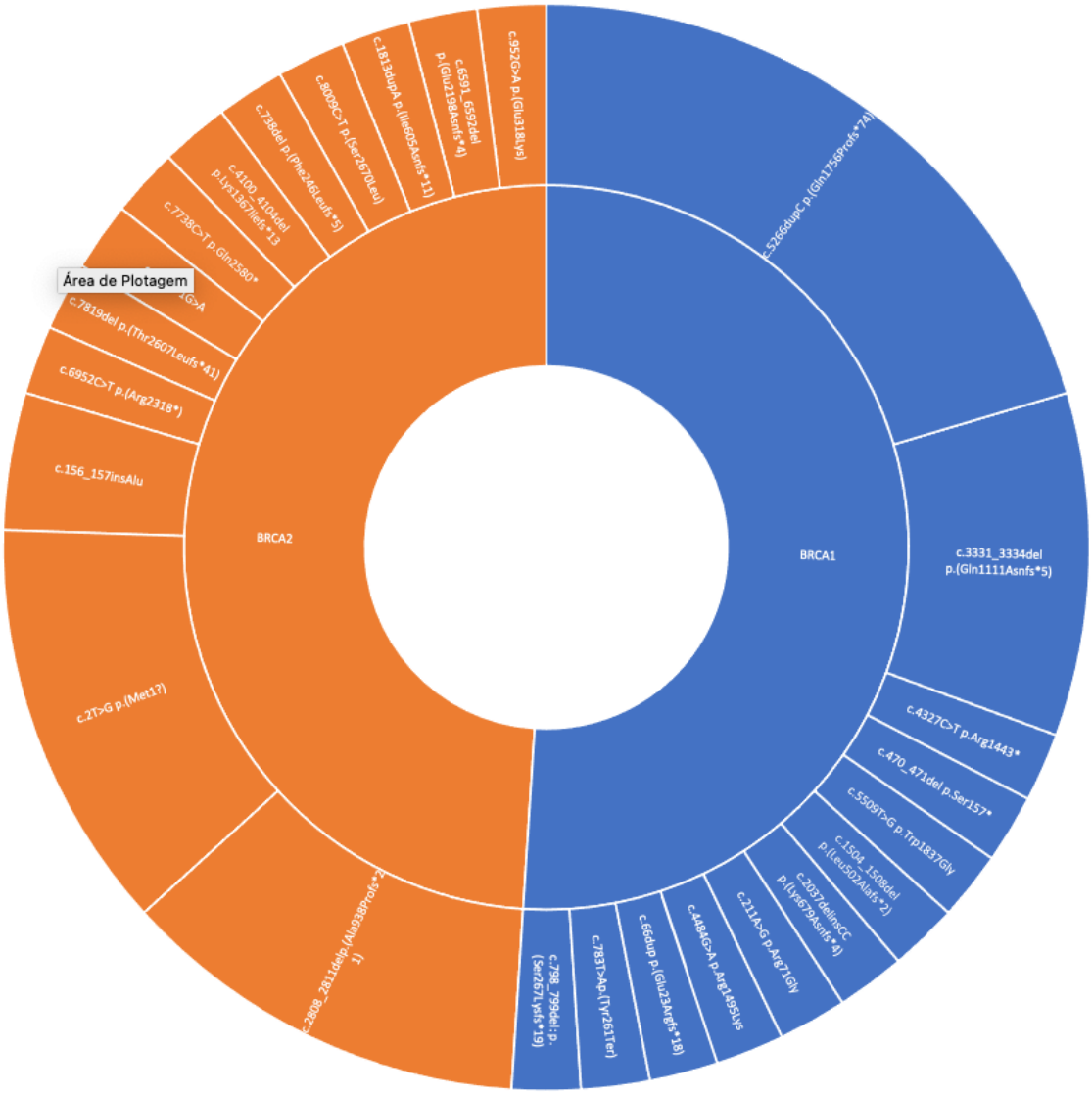
Incidence of pathogenic or likely pathogenic variants

	Gene	Pathogenic or likely pathogenic variant	N
1	*BRCA1*	c.5266dupC, p.(Gln1756Profs*74)	10
2	*MUTYH*	c.1103G>A, p.(Gly368Asp)	10
3	*BRCA2*	c.2T>G, p.(Met1?)	6
4	*BRCA2*	c.2808_2811del, p.(Ala938Profs*21)	6
5	*MUTYH*	c.452A>G, p.(Tyr151Cys)	6
6	*BRCA1*	c.3331_3334del, p.(Gln1111Asnfs*5)	4
7	*CHEK2*	c.349A>G, p.(Arg117Gly)	4
8	*ATM*	c.2921+1G>A	2
9	*BRCA2*	c.156_157insAlu	2
10	*BRIP1*	c.2392C>T, p.(Arg78Ter)	2
11	*CHEK2*	c.1100del, p.(Thr367Metfs*15)	2
12	*CHEK2*	c.409C>T, p.(Arg137Ter)	2
13	*CHEK2*	c.593 -1G>T	2
14	*RAD51D*	c.694C>T, p.(Arg232Ter)	2
15	*APC*	c.3920T>A, p.(Ile1307Lys)	1
16	*APC*	c.5805del, p.(Gln1935Hisfs*35)	1
17	*ATM*	c.1236-2A>G p.?	1
18	*ATM*	c.4575dup, p.(Pro1526Thrfs*5	1
19	*ATM*	c.2413C>T, p.Arg805*	1
20	*ATM*	c.3802del, p.(Val1268*)	1
21	*ATM*	p.(Ser2764Argfs*4)	1
22	*ATM*	c.9047_9057del	1
23	*BRCA1*	c.3477_3480del, p.(Ile1159Metfs*50)	1
24	*BRCA1*	c.783T>A, p.(Tyr261Ter)	1
25	*BRCA1*	c.1504_1508del, p.(Leu502Alafs*2)	1
26	*BRCA1*	c.2037delinsCC, p.(Lys679Asnfs*4)	1
27	*BRCA1*	c.5509T>G, p.Trp1837Gly	1
28	*BRCA1*	c.4327C>T, p.Arg1443*	1
29	*BRCA1*	c.4484G>A, p.Arg1495Lys	1
30	*BRCA1*	c.211A>G, p.Arg71Gly	1
31	*BRCA1*	c.470_471del, p.Ser157*	1
32	*BRCA1*	c.798_799del, p. (Ser267Lysfs*19)	1
33	*BRCA1*	c.66dup, p.(Glu23Argfs*18)	1
34	*BRCA2*	c.952G>A, p.(Glu318Lys)	3
35	*BRCA2*	c.6591_6592del, p.(Glu2198Asnfs*4)	1
36	*BRCA2*	c.1813dupA, p.(Ile605Asnfs*11)	1
37	*BRCA2*	c.8009C>T, p.(Ser2670Leu)	1
38	*BRCA2*	c.738del, p.(Phe246Leufs*5)	1

39	*BRCA2*	c.4100_4104del, p.Lys1367Ilefs*13	1
40	*BRCA2*	c.7738C>T, p.Gln2580*	1
41	*BRCA2*	c.8488- 1G>A	1
42	*BRCA2*	c.7819del, p.(Thr2607Leufs*41)	1
43	*BRCA2*	c.6952C>T, p.(Arg2318*)	1
44	*BRIP1*	c.1741C>T, p.(Arg581Ter)	1
45	*BRIP1*	c.918+1G>A	1
46	*BRIP1*	c.1940G>A, p. (Trp647*)	1
47	*BUB1B*	c.580C>T, p.Arg194*	1
48	*CDH1*	c.790C>T, p.Gln264*	1
49	*CHEK2*	c.264dup, p.(Thr89Tyrfs*19)	1
50	*CHEK2*	c.349A>G, p.(Arg117Gly)	1
51	*CTC1*	c.2959C>T, p.(Arg987Trp)	1
52	*ERCC2*	c.1847G>C, p.Arg616Pro	1
53	*ERCC3*	p.Glu588Glyfs*16	1
54	*EXT2*	c.168G>A, p.Trp56*	1
55	*FANCG*	c.1077-2A>G	1
56	*FANCG*	c.1077- 2A>G	1
57	*FANCI*	c.1583+2T>C	1
58	*FANCI*	c.1583+2T>C	1
59	*FANCL*	c.296_297del, p.(Gln99Argfs*17)	1
60	*FANCM*	c.2586_2589del, p.(Lys863Ilefs*12)	1
61	*FANCM*	c.985del , p. (Leu329*)	1
62	*LZTR1*	c.1360G>T, p. (Glu454*)	1
63	*MERTK*	c.992_993del, p.Ser331Cysfr*5	1
64	*MERTK*	c.992_993del, p.Ser331Cysfs*5	1
65	*MITF*	c.952G>A, p.Glu318Lys	1
66	*MSH6*	c.1519dupA, p.(Arg507Lysfs*9)	1
67	*MUTYH*	c.1063del p.(Ala357Profs*23)	1
68	*MUTYH*	c.452A>G, p.(Tyr151Cus)	1
69	*MUTYH*	c.637C>T, p.(Arg213Trp)	1
70	*MUTYH*	c.649C>T, p.(Arg217Cys)	1
71	*MUTYH*	c.721C>T, p.(Arg241Trp)	1
72	*MUTYH*	c.652G>T, p.(Val218Phe)	1
73	*NBN*	c.1688delinsAC, p.(Leu563Tyrfs*15)	1
74	*NTHL1*	c.115+1G>A	1
75	*NTHL1*	c.526- 1G>A	1
76	*PALB2*	c.3526C>T, p.(arg1086*)	1
77	*PALB2*	c.2730T>A, p.(Tyr910Ter)	1
78	*PALB2*	c.1633G>T, p.(Glu545Ter)	1

79	*PALB2*	c.3350+4A>G	1
80	*PALB2*	c.226del, p.(Ile76Tyrfs*101)	1
81	*PALB2*	c.2964del, p.(Val989*)	1
82	*PALB2*	deleção envolvendo os exons: 1- 10	1
83	*PMS2*	c.1939A>T p.(Lys647*)	1
84	*PMS2*	c.137G>T:p.(Ser46Ile)	1
85	*PRF1*	p. (Leu17Argfs*34)	1
86	*PTEN*	c.564T>G p.(Tyr188Ter)	1
87	*RAD51C*	c.890_899del p.(Leu297Hisfs*)	1
88	*RAD51C*	chr17:56.780.556-56.780.690	1
89	*RAD51D*	c.709C>T p.(Arg237Ter)	1
90	*RECQL4*	c.1573del p.(Cys525Alafs*33)	1
91	*SBDS*	c.183_184delinsCT: p. (Lys62*)	1
92	*SDHA*	c.699del p.(Val234Serfs*6)	1
93	*SLX4*	p. (Gln991Argfs*12)	1
94	*TP53*	c.743G>A p.(Arg248Gln)	1
95	*TP53*	c.1010G>Ap. (Arg337His)	1
96	*TP53*	c.375G>A p.(Thr125Thr)	1
97	*TP53*	c.374C>T p.(Thr125Met)	1

Figure 12 – Proportions of pathogenic and likely pathogenic variants in *BRCA1* and *BRCA2*.


BRCA1
BRCA2
c.5266dupC p.(Gln1756Profs*74)
c.3331_3334del p.(Gln1111Asnfs*5)
c.4327C>T p.Arg1443*
c.470_471del p.Ser157*
c.5509T>G p.Trp1837Gly
c.1504_1508del p.(Leu502Alafs*2)
c.2037delinsCC p.(Lys679Asnfs*4)
c.211A>G p.Arg71Gly
c.448A>G p.Arg149Gly
c.66dup p.(Glu23Argfs*18)
c.7831>Aq.(Tyr261Ter)
c.798_799del p.(Ser267Lysfs*19)
c.2808_2811delp.(Ala938Profs*21)
c.27>G p.(Met17)
c.156_157insAlu
c.6952C>T p.(Arg2318*)
c.7819del p.(Thr2607Leufs*41)
c.7738C>T p.Gln2580*
c.4100_4104del p.Lys1367Ilefs*13
c.7984del p.(Phe2661Leufs*5)
c.8009C>T p.(Ser2670Leu)
c.1813dupA p.(Ile605Asnfs*11)
c.6591_6592del p.(Glu2198Asnfs*4)
c.952G>A p.(Glu318Lys)
Área de Plotagem

More than four different pathogenic or probably pathogenic variants were found in the MUTYH, ATM, PALB2, CHEK2, BRIP1 and TP53 genes (Figure 13).

Figure 13 - Proportion of different pathogenic or probably pathogenic *MUTYH, ATM, PALB2, CHEK2, BRIP1 and TP53*

Management Recommendations according to the genetic result

According to the National Comprehensive Cancer Network version 2.2023 guidelines, among these 34 genes that presented a pathogenic or probably pathogenic, 24 (70.59%) have no recommendation for risk-reducing mastectomy, three (8.82%) recommend management of according to family history and seven (20.59%) recommend risk-reducing surgery discussion with the patient. Regarding risk-reducing salpingo-oophorectomy, 26 (76.47%) have no recommendations, two (5.88%) are indicated from 35 years of age, 4 (11.76%) are indicated from 45 years of age of age, 1 (2.94%) recommended management according to family history and 1 (2.94%) recommended risk-reducing surgery discussion with the patient. Regarding pancreatic cancer screening, 28 (82.35%) do not recommend it, while 6 (17.65%) recommend annual screening based on the presence of a familial pathogenic variant associated with a positive family history.

Among the sample with pathogenic or probably pathogenic results and genes with a risk of at least 40% of developing breast cancer, 49 (35.77%) were patients with localized variants in the *BRCA1/BRCA2* genes while 38 (27.74%) are in the *ATM, CHEK2, RAD51D, RAD51C, CDH1, PALB2, PTEN, TP53* genes.

In the sample of 34 genes that presented pathogenic or probably pathogenic variants and according to data from the Online Mendelian Inheritance in Man (OMIM), thirteen (38.24%) presented autosomal dominant inheritance, fifteen (10.95%) autosomal recessive, three (2.19%) have the two described mechanisms according to the associated phenotype and three (2.19%) have no described inheritance mechanism (Table 13).

Table 13 – Proportion among pathogenic and/or likely pathogenic variants and management recommendations.

NCCN Risk reducing mastectomy	N	Proportion among pathogenics and likely pathogenics genes	Proportion of the total number of patients with pathogenic and likely pathogenic variants	Proportion of the total number of patients evaluated
N/D	24	70,59%	17,52%	3,42%
Manage based on family history	3	8,82%	2,19%	0,43%
Discuss mastectomy	7	20,59%	5,11%	1,00%

NCCN Risk-Reducing Salpingo-Oophorectomy	N	Proportion among pathogenics and likely pathogenics genes	Proportion of the total number of patients with pathogenic and likely pathogenic variants	Proportion of the total number of patients evaluated
N/D	26	76,47%	18,98%	3,71%
> 35 years	2	5,88%	1,46%	0,29%
> 45 years	4	11,76%	2,92%	0,57%
Manage based on family history	1	2,94%	0,73%	0,14%
Discuss Salpingo-Oophorectomy	1	2,94%	0,73%	0,14%

NCCN Pancreatic screening	N	Proportion among pathogenics and likely pathogenics genes	Proportion of the total number of patients with pathogenic and likely pathogenic variants	Proportion of the total number of patients evaluated
N/D	28	82,35%	20,44%	3,99%
Screen if family history	6	17,65%	4,38%	0,86%

Inheritance mechanism (OMIM® database)	**N**	**Proportion among pathogenics and likely pathogenics genes**	**Proportion of the total number of patients with pathogenic and likely pathogenic variants**	**Proportion of the total number of patients evaluated**
AD	13	38,24%	9,49%	9,56%
AR	15	10,95%	10,95%	11,03%
AD/AR	3	2,19%	2,19%	2,21%
N/D	3	2,19%	2,19%	2,21%

High and moderate penetrance genes for breast cancer risk	**N**	**Proportion of the total number of patients with pathogenic and likely pathogenic variants**	**Proportion of the total number of patients evaluated**
BRCA1/BRCA2	49	35,77%	6,99%
ATM, CHEK2, RAD51D, RAD51C, CDH1,PALB2, PTEN, TP53	38	27,74%	5,42%

Of the total sample, 93 patients (13.27%) had pathogenic or probably pathogenic variants with an autosomal dominant inheritance mechanism (AD), 84 (11.98%) of the patients had pathogenic or probably pathogenic variants that result in the possibility of risk-reducing mastectomy (RRM) and 75 (10.70%) on the possibility of risk-reducing salpingo-oophorectomy (RRSO) (Table 14 and 15).

Table 14 - Inheritance mechanism, indication for risk-reducing surgery

	N	**Proportion of the total of patients**
Autosomal dominant	93	13,27%
Risk reducing mastectomy	84	11,98%

Risk-reducing salpingo-oophorectomy	75	10,70%

Table 15 – Autosomal dominant genes and National Comprehensive Cancer Network recommendations.

Gene with variant	**N**	**NCCN Breast Cancer Risk**	**NCCN Risk reducing mastectomy**	**NCCN Ovarian Cancer Risk**	**NCCN Risk-Reducing Salpingo-Oophorectomy**	**NCCN Pancreatic Cancer Risk**	**NCCN Pancreatic screening**
BRCA1	25	>60%	Discuss	39% - 58%	> 35 years	< 5%	With family history
BRCA2	24	>60%	Discuss	13% - 29%	> 35 years	5 - 10%	With family history
CHEK2	12	20% - 40%	Managed based on family history	N/D	N/D	N/D	N/D
ATM	8	20% - 40%	Managed based on family history	2% - 3%	Managed based on family history	5 - 10%	With family history
PALB2	7	41% - 60%	Discuss	3% - 5%	> 45 years	N/D	With family history
BRIP1	5	N/D	N/D	5% - 15%	> 45 years	N/D	N/D
TP53	4	>60%	Discuss	N/D	N/D	5 - 10%	With family history
APC	2	N/D	N/D	N/D	N/D	N/D	N/D
PMS2	2	<15%	Managed based on family history	1.3% - 3%	N/D	N/D	N/D
CDH1	1	41% - 60%	Discuss	N/D	N/D	N/D	N/D
LZTR1	1	N/D	N/D	N/D	N/D	N/D	N/D
MSH6	1	<15%	N/D	1% - 13%	Discuss	5 - 10%	With family history
PTEN	1	>60%	Discuss	N/D	N/D	N/D	N/D

DISCUSSION

DISCUSSION

In this study, we specifically evaluated patients who met the criteria for Hereditary Breast and Ovary Cancer Syndrome described in the Brazilian National Health Agency. These criteria determine the molecular investigation of tens of thousands of people who have health insurance in Brazil and measuring the results allowed us to identify that the pipeline of three investigation stages, through New Generation Sequencing of the *BRCA1* and *BRCA2* genes, MLPA of the genes *BRCA1* and *BRCA2* and the germ panel could be compressed in a single step through the germ panel.

The results were close to others published in the international literature. Garber & Offit, 2005 described that 5 to 10% of the 200,000 annually reported cases of breast cancer in the United States are associated with *BRCA1* and *BRCA2* variants. This data converges with Brazilian data, in which Instituto Nacional de Câncer José Alencar Gomes da Silva & Ministério da Saúde, 2019 also describes about 10% identifiable hereditary risk of the 59,700 incident cases of breast cancer reported for 2019, however attributing it only to the *BRCA1* and *BRCA2* genes. Guindalini *et al*. (2022) evaluated 1682 patients with hereditary cancer criteria and detected an approximate prevalence of 18.1% of pathogenic or probable pathogenic variants. Matta et al. (2022) detected a prevalence of 15.7% of pathogenic and probably pathogenic variants in the BRCA1, BRCA2 and TP53 genes in 257 patients with Hereditary Breast and Ovarian Cancer Syndrome criteria in the Brazilian public health system. Sandoval et al. (2021) retrospectively evaluated the medical records of 224 patients with breast cancer who underwent genetic counseling at an Oncology Center. Eighty-five percent fulfilled the National Comprehensive Cancer Network criteria and in this group the prevalence of pathogenic and likely pathogenic variants was 23.5%. Gifoni et al. (2022) evaluated 355 patients who met the National

Comprehensive Cancer Network 1.2021 criteria for Hereditary Breast Cancer and detected pathogenic and probably pathogenic variants in 27.3%. Paixão et al., (2022) evaluated 321 patients who met the National Comprehensive Cancer Network criteria for hereditary breast cancer and detected a 25.2% prevalence of pathogenic and probably pathogenic variants.

In a cohort of 2984 unselected cancer patients Samadder et al., 2021, found an overall prevalence of 13.3% of patients with germline pathogenic variants. In the same sampling, the percentage rose to 21.8% in patients who had a positive family history for cancer. Analyzing 1040 patients with advanced cancer, Mandelker et al., 2017 found a 17.5% prevalence of variants associated with increased susceptibility to developing cancer. Tsaousis et al. (2019) found a prevalence of 22.1% of pathogenic variants in individuals with a family history of breast and/or ovarian cancer. Dalivandan et al. (2021). suggested an approximate prevalence of 25% of high and moderate penetrance genes in hereditary breast cancer.

The current study identified pathogenic and likely pathogenic variants in 19.54% in individuals with Hereditary Breast and Ovarian Cancer Syndrome criteria described in the Brazilian National Health Agency. Compared to Instituto Nacional de Câncer José Alencar Gomes da Silva data, the present study suggests a 90% gain in identifying individuals with pathogenic or likely pathogenic variants that are associated with clinical criteria for investigating the risk of hereditary breast and ovarian cancer. The data allowed observing different detection rates according to the volume of genes analyzed in each panel. In panels of 40 genes, the detection rate of pathogenic and probably pathogenic variants was 16.4%, while in panels of 141 genes, the detection rate was 22.7%, an approximate gain of 38.34% in detection of pathogenic or probably pathogenic variants

when comparing the panel of 141 genes in relation to the panel of 40 genes. Furthermore, the detection rate of individuals with more than one mutation (transheterozygotes) differed between the panels, with the detection rate of two pathogenic and/or probably pathogenic variants being 0.29% in the panels of 40 genes and of 2, 27% in panels of 141 genes, representing an almost tenfold gain.

This increase in sensitivity has a direct impact on the individual's genetic counseling, as the incidence of possible carrier individuals increases. 9.56% of individuals were identified as carrying deleterious variants in genes with autosomal dominant inheritance and 11.03% in genes with autosomal recessive inheritance. 2.21% of individuals have variants in genes that have described both mechanisms.

Comparing with data from the Brazilian literature cited above, the prevalence of pathogenic and probably pathogenic variants was lower. One of the possible explanations for this would be the different criteria used. The highest prevalence rates were found in studies that used the criteria of the National Comprehensive Cancer Network, which included more patients than the criteria of the Brazilian National Health Agency.

Comparing with the recent recommendations of the National Comprehensive Cancer Network 2.2023, the possibility of risk-reducing mastectomy would cover about 1.43% of the total sample of 701 individuals. Salpingo-oophorectomy would be considered in 1.14% of individuals. Screening for pancreatic cancer in 0.86%.

Identification of variants of uncertain clinical significance varied substantially according to the type of panel used. In the sample analyzed through a panel of 40 genes, the prevalence of variants of uncertain clinical significance was 47.12%, while in the sample analyzed through a panel of 141 it was as high as 82.72%, in which the minority of individuals had totally negative result.

CONCLUSION

CONCLUSION

The present study proposed to use only the germ panel as a molecular investigation method for patients with Hereditary Breast and Ovary Cancer Syndrome criteria according to the National Supplementary Health Agency. The current protocol stipulates a three-step pipeline: next generation sequence and MLPA of the *BRCA1* and *BRCA2* genes and, if the result is inconclusive, performing germ panel.

Our study identified the prevalence of variants compatible with recent studies published both worldwide and in Brazil. We detected pathogenic and probably pathogenic variants in approximately 19% of the patients - 90% above the Instituto Nacional do Câncer José de Alencar Gomes Silva data - 13% in high and medium penetrance genes. Furthermore, we detected that two percent of the patients evaluated through a panel of 141 genes had two distinct deleterious variants.

These data suggest that the adoption of the germ panel as a standardized test for patients with hereditary breast and ovarian cancer criteria can increase the sensitivity of detection by up to 90% in relation to the 10% prevalence currently described in the literature. In addition, findings of the chi-square tests strongly suggest that the use of panels consisting of 141 genes is preferable over panels consisting of 40 genes. The statistical analysis revealed a significant difference in the detection of pathogenic variants, with the larger-scale test consistently outperforming the smaller-scale test in identifying such variants. The obtained p-values and VS-MPR values provide robust evidence to support the hypothesis that the larger gene panel has a greater ability to detect pathogenic variants. These results emphasize the importance of comprehensive genetic testing approaches that encompass a broader range of genes. By utilizing panels with 141 genes, healthcare professionals and researchers can improve the accuracy and reliability

of identifying pathogenic variants, leading to more informed clinical decision-making and potentially better patient outcomes. Therefore, it is recommended to prioritize the use of gene panels with a larger gene count in genetic testing endeavors. Further studies and evaluations are warranted to explore the specific genes included in these panels and to validate their clinical utility in different populations and contexts.

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APPENDIX

Anexo: Diretrizes de Utilização - Resolução Normativa 465/2021 da Agência Nacional de Saúde Suplementar. Disponível em: http://www.ans.gov.br/images/stories/Legislacao/rn/rn465/Anexo_II_-_Diretrizes_de_Utiliza%C3%A7%C3%A3o_-_RN_465.2021.pdf. Acesso em: [03 de julho de 2023].