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Mafalda Melo *, Mariana Ribeiro, Paulo Filipe Silva, Susana Valente, Filipe da Costa Alves, Margarida Venâncio, Jorge Sequeiros, Diana Antunes, João Parente Freixo, Jorge Oliveira *

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*Article*

# Medically Actionable Secondary Findings from Whole Exome Sequencing (WES) Data in a Sample of 3972 Individuals

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**Abstract:** The application of whole-exome sequencing (WES) for diagnostic purposes has the potential to unravel secondary findings unrelated with the primary reason of testing. Some of those might be of high clinical utility and comprise disease-causing variants in genes, related to life-threatening and clinically actionable diseases. Clarifying the allelic frequencies of such variants in specific populations is a crucial step for the large-scale deployment of genomic medicine. We analysed medically relevant variants in the 81 genes from the American College of Medical Genetics and Genomics (ACMG) v3.2 list of actionable *loci*, using WES data from a diagnostic laboratory cohort of 3,972 persons, tentatively resampled to represent the Portuguese population geographic distribution. We identified medically actionable variants in 6.2% of our cohort, distributed across several disease domains: cardiovascular disorders (3.0%), cancer predisposition (2.0%), miscellaneous disorders (1.1%), and metabolic disorders (0.1%). Additionally, we estimated a frequency of heterozygotes for recessive disease alleles of 11.1%. Overall, our results suggest that medically actionable findings can be identified in approximately 6.2% of persons from our population. This is the first study estimating medically actionable findings in Portugal. These results provide valuable insight for patients, healthcare providers, and policymakers involved in advancing genomic medicine at the national and international level.

**Keywords:** whole-exome sequencing; actionable; secondary findings; genomic medicine

## 1. Introduction

Whole-exome sequencing (WES) has become widely used in clinical practice to diagnose hereditary diseases. In addition to this primary purpose, WES holds a strong potential to uncover findings with significant health implications that may be unrelated to the original indication for testing. These findings can be unexpectedly discovered (incidental findings) or intentionally sought (secondary findings).

The American College of Medical Genetics and Genomics (ACMG) published guidelines for reporting secondary findings that could influence medical management of both adults and minors undergoing genomic testing. These guidelines allow for opting-out from receiving such findings. Over time, the ACMG has progressively expanded its list of genes recommended to identify secondary findings. In 2013, the list included 57 genes; these grew to 59 in 2017, 73 in 2021, 78 in 2022, and 81 genes by 2023. They were selected with specific criteria: association with highly penetrant diseases, long asymptomatic or prodromal stages, significant contribution to morbidity and/or mortality, availability of preventive measures or treatments, and potential for early detection to reduce long-term health risks effectively [1–7].

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In contrast, the European Society of Human Genetics (ESHG) recommended a more cautious approach, aligned with the concept of opportunistic screening. The ESHG suggests further research, and does not recommend a specific list of genes [8,9].

Since the introduction of these guidelines, various research groups have reported secondary findings identified in diverse populations. These efforts have contributed to understand the clinical significance and implications of such findings in different genetic groups; they resorted to different sampling strategies [10–39].

In Portugal, however, frequency of pathogenic (PAT) or likely-pathogenic (L-PAT) variants in ACMG-actionable genes remain unknown, and no national policies for reporting secondary findings have been issued. Access to this information is crucial to support informed decision-making by patients, genetics laboratories, healthcare providers, and policymakers. Ultimately, it might support the development of health policies for genomic screening, in each country.

In this study, we aimed at determining the frequency of PAT/L-PAT variants at *loci* listed as medically actionable by the ACMG (*n*=81), and estimating the overall frequency of secondary findings in a diagnostic cohort at the Centre for Predictive and Preventive Genetics of the Institute for Molecular and Cellular Biology (CGPP-IBMC), which was resampled to be potentially representative of the Portuguese population.

## 2. Results

### *2.1. Data Set*

A subset of 3,972 persons was selected from a global dataset of 12,167 WES sequenced, as described in the Material and Methods section, and data were anonymized.

### *2.2. Variant Filtering and Curation*

In the selected cohort, there were 12,336 variants in the 81 ACMG medically actionable genes (Figure 1). To select variants of sufficient quality, we limited the analysis to those with a minimum read depth of 10x and genotype quality of 60. In general, each of the remaining 11,207 variants was then annotated with information available from (i) our internal laboratory database, (ii) ClinVar and HGMD, (iii) gnomAD, and (iv) molecular consequence predictors, by local bioinformatic analysis. After the filtering steps, 164 PAT/L-PAT variants internally validated or present in specific disease databases were included. Then, 5,289 variants required further revision and manual curation using the ACMG/AMP guidelines and gene specific recommendations, when available; of these, additional 95 variants met the inclusion criteria.

Thus, a total of 259 variants were classified as PAT/L-PAT, across 50 genes associated to autosomal dominant (*n*=42), recessive (*n*=7) and X-linked (*n*=1) phenotypes (Supplementary Table S1).

### *2.3. Medically Actionable Findings and Carriers*

After interpretation and classification of each of the 259 variants, we estimated the number of mutated alleles for each variant and corresponding gene. As expected, mutated alleles were more frequent in genes related to recessive (*n*=449), then dominant (*n*=238), and X-linked (*n*=4) diseases. The ACMG recommendations specify reporting only di-allelic (homozygous or compound heterozygous variants at the same *locus*) PAT/L-PAT variants in recessive disease genes. Frequency of potential medically actionable findings was estimated separately from carriers' frequency, based on the zygosity of the variant, number of mutated alleles, and total number of alleles analysed.

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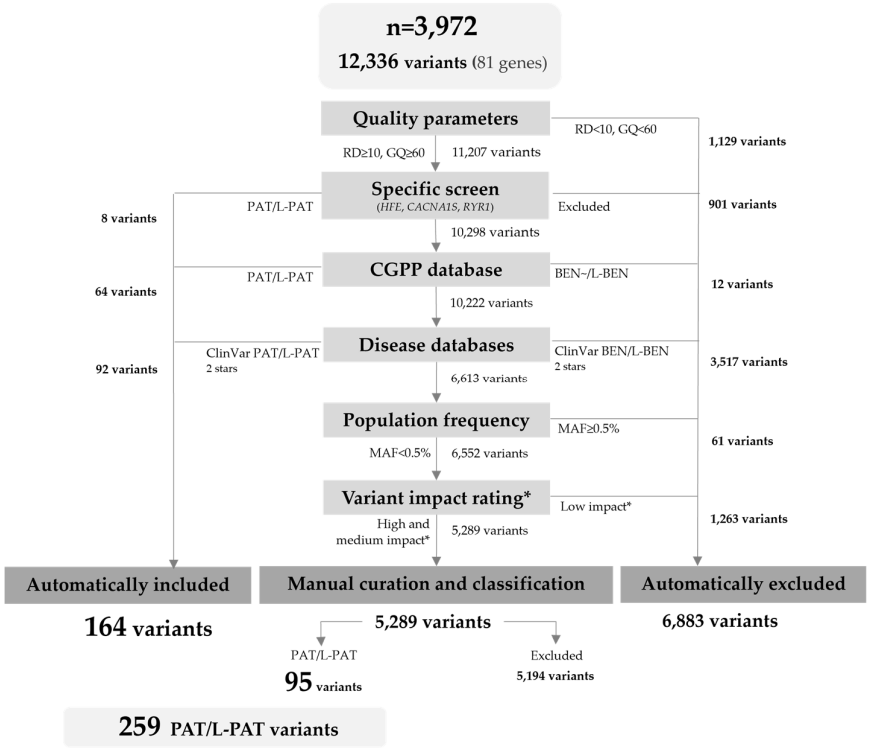


**Figure 1.** Flowchart of the variant filtering and curation process. RD: read depth; GQ: genotype quality; MAF: minor allele frequency; PAT: pathogenic; L-PAT: likely-pathogenic; BEN: benign; L-BEN: likely-benign.

2.3.1. Frequency of Medically Actionable Findings

A total of 246 persons (6.19%) was estimated to have a potential medically actionable finding (Figure 2, Supplementary Tables S2A, S2B, S2C and S2D, Supplementary Tables S3A and S3B) in the cohort, distributed across four disease groups: cardiovascular, cancer, metabolic and miscellaneous disorders. Table 1 provides a summary of the total number of variants per gene and their combined allele counts, distributed across the respective disease group.

Actionable variants in genes for cardiovascular disease were the most frequent, present in 120 individuals, 48.78% of all persons with medically actionable findings and 3.02% of our cohort; these were subdivided in cardiomyopathies, CMP, (*n*=61, 1.54%); dyslipidaemias (*n*=23, 0.58%); rhythm disorders (*n*=18, 0.45%); and hereditary connective tissue disorders, HCTD, (*n*=18, 0.45%), as detailed in Supplementary Table S2A. Among the CMP, variants related to dilated CMP (DCM) were the most frequent (*n*=30, 0.76%), and found in *TTN*, *TNNT2*, *LMNA*, *FLNC*, *DES* and *TNNC1*; followed by hypertrophic CMP (HCM) variants (*n*=22, 0.55%), in *MYH7* and *MYBPC3*. Arrhythmogenic right ventricular CMP (ARVC) variants were the least common (*n*=9, 0.23%), and identified in *PKP2*, *DSP* and *DSC2*. Among the dyslipidaemias, variants in *LDLR* (*n*=19, 0.48%) and *APOB* (*n*=4, 0.10%) were found, associated to familial hypercholesterolemia (FH). Arrhythmia predisposition variants were found in *KCNQ1* (*n*=11, 0.28%), *KCNH2* (*n*=2, 0.05%), and *SCN5A* (*n*=5, 0.13%) genes. Finally, HCTD findings were reported to *FBN1* (*n*=8, 0.20%), *MYH11* (*n*=5, 0.13%), *TGFBR2* (*n*=3, 0.08%), and *COL3A1* (*n*=2, 0.05%).

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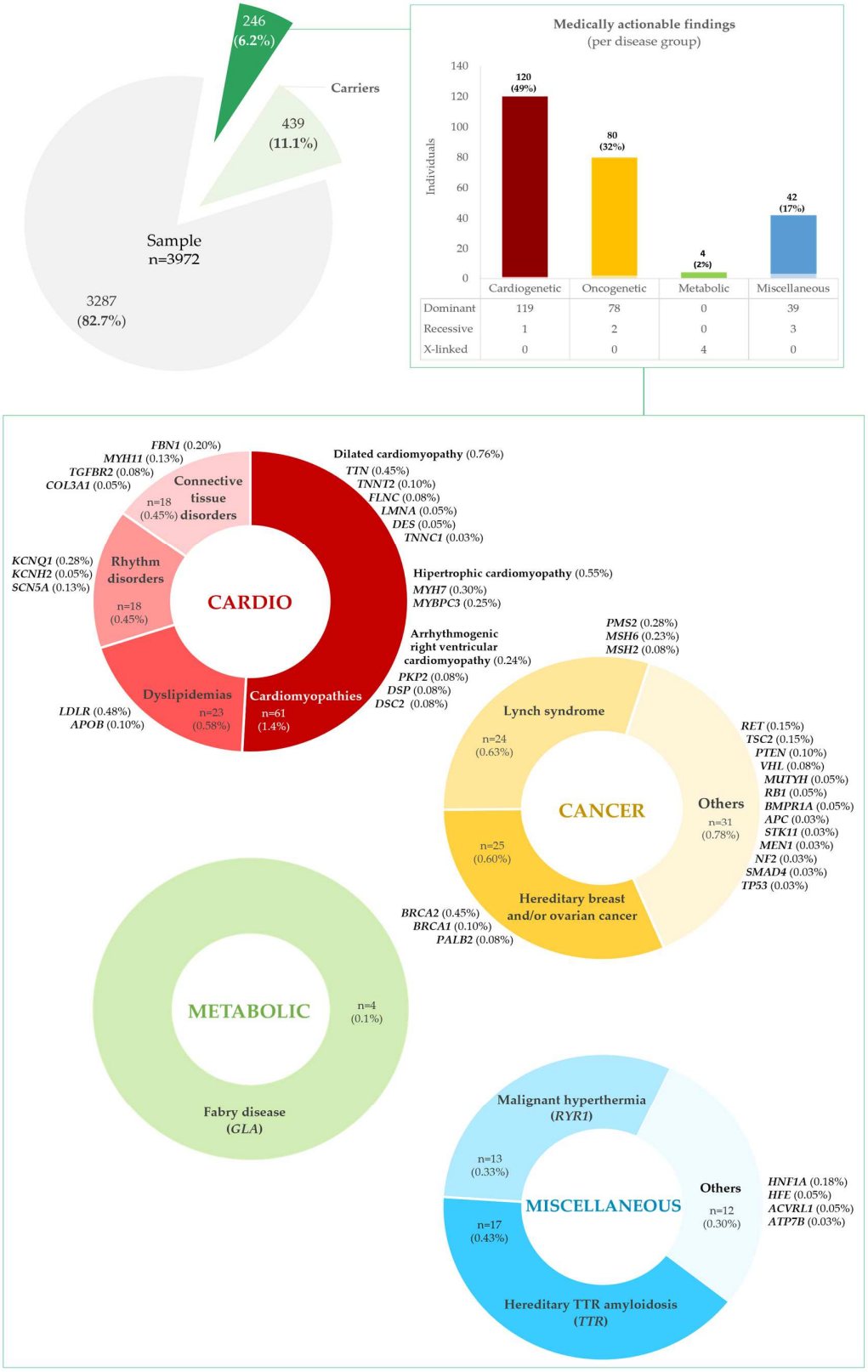


**Figure 2.** Total medically actionable findings and non-medically actionable findings (carrier status) present in our cohort of 3,972 persons, presumed to be representative of the Portuguese population.

Pathogenic variants in genes predisposing to hereditary cancer were present in 80 persons, or 32.52% of all with medically actionable findings, and 2.01% of the cohort (see Supplementary Table

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S2B for details). The most frequent were associated to hereditary breast and ovarian cancer (HBOC) and Lynch syndrome (HNPCC), in 25 (0.63%) and 24 (0.60%) persons, respectively. HBOC-related variants were present in *BRCA2* (*n*=18, 0.45%), *PALB2* (*n*=4, 0.10%), and *BRCA1* (*n*=3, 0.08%); and HNPCC variants in *PMS2* (*n*=11, 0.28%), *MSH6* (*n*=9, 0.23%), and *MSH2* (*n*=4, 0.10%). Less frequent cancer actionable variants were seen in *RET* (*n*=6, 0.15%), *TSC2* (*n*=6, 0.15%), *PTEN* (*n*=4, 0.10%), *VHL* (*n*=3, 0.08%), *BMPR1A* (n=2, 0.05%), *MUTYH* (*n*=2, 0.05%), RB1 (N=2, 0.05%), *APC* (*n*=1, 0.03%), *MEN1* (*n*=1, 0.03%), *NF2* (n=1, 0.03%), *SMAD4* (n=1, 0.03%), *STK11* (*n*=1, 0.03%), *TP53* (*n*=1, 0.03%), genes.

Concerning metabolic disorders, PAT/L-PAT variants were identified in 4 persons (3 females, 1 male), in *GLA*, associated with Fabry disease, 1.63% of all with medically actionable findings and 0.10% of the cohort (refer to Supplementary Table S2C).

In the group of miscellaneous disorders, PAT/L-PAT variants were identified in 42 persons, 17.07% of all with medically actionable findings and 1.03% of the cohort (outlined in Supplementary Table S2D). Most represented variants for *TTR*-related hereditary amyloidosis (*n*=17, 0.43%) and *RYR1*-related malignant hyperthermia (*n*=13, 0.33%). Other, less frequent variants related to *HNF1A* - maturity onset of diabetes of the young, MODY, (*n*=7, 0.18%); *HFE* - hereditary hemochromatosis (*n*=2, 0.05%), *ACVRL1* - hereditary haemorrhagic telangiectasia (*n*=2, 0.05%), and Wilson disease (n=1, 0.03%).

**Table 1**. Summary of genes by phenotype group with the number of pathogenic and likely-pathogenic variants identified in our cohort according to ACMG/AMP criteria.

| Disease | Gene | Disease Inheritance | Nr. of variants per gene | Allele count | | | Allelic frequency (%) |
|---|---|---|---|---|---|---|---|
| | | | | Total | Hom. | Het. | |
| **Cancer phenotype group** | | | | | | | |
| Familial adenomatous polyposis (FAP) | *APC* | AD | 1 | 1 | 0 | 1 | 0.013 |
| Familial medullary thyroid cancer | *RET* | AD | 3 | 6 | 0 | 6 | 0.076 |
| Hereditary breast and/or ovarian cancer | *BRCA1* | AD | 3 | 3 | 0 | 3 | 0.038 |
| | *BRCA2* | AD | 16 | 18 | 0 | 18 | 0.227 |
| | *PALB2* | AD | 2 | 4 | 0 | 4 | 0.050 |
| Hereditary paraganglioma-pheochromocytoma syndrome | *SDHD* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *SDHAF2* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *SDHC* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *SDHB* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *MAX* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *TMEM127* | AD | 0 | 0 | 0 | 0 | 0.000 |
| Juvenile polyposis syndrome (JPS) | *BMPR1A* | AD | 2 | 2 | 0 | 2 | 0.025 |
| Juvenile polyposis syndrome / hereditary hemorrhagic telangiectasia syndrome | *SMAD4* | AD | 1 | 1 | 0 | 1 | 0.013 |
| Li–Fraumeni syndrome | *TP53* | AD | 1 | 1 | 0 | 1 | 0.013 |
| **Disease** | **Gene** | **Disease Inheritance** | **Nr. of variants per gene** | **Allele count** | | | **Allelic frequency (%)** |
| | | | | **Total** | **Hom.** | **Het.** | |
| Lynch syndrome (HNPCC) | *MLH1* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *MSH2* | AD | 3 | 4 | 0 | 4 | 0.050 |
| | *MSH6* | AD | 8 | 9 | 0 | 9 | 0.113 |
| | *PMS2* | AD | 6 | 11 | 0 | 11 | 0.138 |
| Multiple endocrine neoplasia type 1 | *MEN1* | AD | 1 | 1 | 0 | 1 | 0.013 |
| MUTYH-associated polyposis (MAP) | *MUTYH* | AR | 17 | 95 | 4 | 91 | 1.196 |
| Neurofibromatosis type 2 | *NF2* | AD | 1 | 1 | 0 | 1 | 0.013 |
| Peutz-Jeghers syndrome (PJS) | *STK11* | AD | 1 | 1 | 0 | 1 | 0.013 |
| PTEN hamartoma tumor syndrome | *PTEN* | AD | 4 | 4 | 0 | 4 | 0.050 |
| Retinoblastoma | *RB1* | AD | 2 | 2 | 0 | 2 | 0.025 |
| Tuberous sclerosis complex | *TSC1* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *TSC2* | AD | 6 | 6 | 0 | 6 | 0.076 |
| von Hippel-Lindau syndrome | *VHL* | AD | 1 | 3 | 0 | 3 | 0.038 |
| WT1-related Wilms tumor | *WT1* | AD | 0 | 0 | 0 | 0 | 0.000 |
| **Cardiovascular phenotype group** | | | | | | | |
| Aortopathies | *FBN1* | AD | 8 | 8 | 0 | 8 | 0.101 |
| | *TGFBR1* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *TGFBR2* | AD | 2 | 3 | 0 | 3 | 0.038 |
| | *SMAD3* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *ACTA2* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *MYH11* | AD | 3 | 5 | 0 | 5 | 0.063 |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Arrhythmogenic right ventricular cardiomyopathy (a subcategory of ACM) | *PKP2* | AD | 3 | 3 | 0 | 3 | 0.038 |
| | *DSP* | AD | 3 | 3 | 0 | 3 | 0.038 |
| | *DSC2* | AD | 3 | 3 | 0 | 3 | 0.038 |
| | *TMEM43* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *DSG2* | AD | 0 | 0 | 0 | 0 | 0.000 |
| Catecholaminergic polymorphic ventricular tachycardia | *RYR2* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *CASQ2* | AR | 0 | 0 | 0 | 0 | 0.000 |
| | *TRDN* | AR | 6 | 12 | 0 | 12 | 0.151 |
| Dilated cardiomyopathy | *TNNT2* | AD | 3 | 4 | 0 | 4 | 0.050 |
| | *LMNA* | AD | 2 | 2 | 0 | 2 | 0.025 |
| | *FLNC* | AD | 3 | 3 | 0 | 3 | 0.038 |
| | *TTN* | AD | 18 | 18 | 0 | 18 | 0.227 |
| | *BAG3* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *DES* | AD | 2 | 2 | 0 | 2 | 0.025 |
| | *RBM20* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *TNNC1* | AD | 1 | 1 | 0 | 1 | 0.013 |
| **Disease** | **Gene** | **Disease Inheritance** | **Nr. of variants per gene** | **Allele count** | | | **Allelic frequency (%)** |
| | | | | **Total** | **Hom.** | **Het.** | |
| Ehlers-Danlos syndrome. vascular type | *COL3A1* | AD | 2 | 2 | 0 | 2 | 0.025 |
| Familial hypercholesterolemia | *LDLR* | AD | 15 | 19 | 0 | 19 | 0.239 |
| | *APOB* | AD | 4 | 4 | 0 | 4 | 0.050 |
| | *PCSK9* | AD | 0 | 0 | 0 | 0 | 0.000 |
| Hypertrophic cardiomyopathy | *MYH7* | AD | 8 | 12 | 0 | 12 | 0.151 |
| | *MYBPC3* | AD | 10 | 10 | 0 | 10 | 0.126 |
| | *TNNI3* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *TPM1* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *MYL3* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *ACTC1* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *PRKAG2* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *MYL2* | AD | 0 | 0 | 0 | 0 | 0.000 |
| Long QT syndrome types 1 and 2 | *KCNQ1* | AD | 6 | 12 | 2 | 10 | 0.151 |
| | *KCNH2* | AD | 2 | 2 | 0 | 2 | 0.025 |
| Long QT syndrome 3. Brugada syndrome | *SCN5A* | AD | 4 | 5 | 0 | 5 | 0.063 |
| Long QT syndrome types 14-16 | *CALM1* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *CALM2* | AD | 0 | 0 | 0 | 0 | 0.000 |
| | *CALM3* | AD | 0 | 0 | 0 | 0 | 0.000 |
| **Inborn errors of metabolism phenotype group** | | | | | | | |
| Biotinidase deficiency | BTD | AR | 10 | 15 | 0 | 15 | 0.189 |
| Fabry disease | GLA | XL | 3 | 4 | 1 | 3 | 0.063 |
| Pompe disease | GAA | AR | 9 | 20 | 0 | 20 | 0.252 |
| Ornithine transcarbamylase deficiency | OTC | XL | 0 | 0 | 0 | 0 | 0.000 |
| **Miscellaneous phenotype group** | | | | | | | |
| Hereditary hemochromatosis | *HFE* | AR | 1 | 211 | 4 | 207 | 2.656 |
| Hereditary hemorrhagic telangiectasia | *ACVRL1* | AD | 2 | 2 | 0 | 2 | 0.025 |
| | *ENG* | AD | 0 | 0 | 0 | 0 | 0.000 |
| Malignant hyperthermia | *RYR1* | AD | 7 | 13 | 0 | 13 | 0.164 |
| | *CACNA1S* | AD | 0 | 0 | 0 | 0 | 0.000 |
| Maturity-onset of diabetes of the young | *HNF1A* | AD | 3 | 7 | 0 | 7 | 0.088 |
| RPE65-related retinopathy | *RPE65* | AR | 11 | 21 | 0 | 21 | 0.264 |
| Wilson disease | *ATP7B* | AR | 24 | 75 | 2 | 73 | 0.944 |
| Hereditary TTR-related amyloidosis | *TTR* | AD | 2 | 17 | 0 | 17 | 0.214 |

Footnote: Hom.- Homozygous; Het.- Heterozygous. (end of table)

### 2.3.2. Carrier Frequencies

In addition to medically actionable alleles, we have estimated that 439 persons (11.05%) in our cohort were carriers of PAT/L-PAT disease-causing alleles in recessive actionable disease genes (Figure 2, Supplementary Tables S3C). These variants were most frequent in *HFE* (*n*=207, 5.21%), *MUTYH* (*n*=91, 2.29%) and *ATP7B* (*n*=73, 1.84%), associated with hereditary hemochromatosis, *MUTYH*-associated polyposis (MAP), and Wilson disease. Less frequently, variants in heterozygosity were found in *RPE65* (*n*=21, 0.53%), *GAA* (*n*=20, 0.50%), *BTD* (*n*=15, 0.38%), and *TRDN* (*n*=12, 0.30%), associated with *RPE65*-related retinopathy, Pompe disease, biotinidase deficiency, and catecholaminergic polymorphic ventricular tachycardia. Further details are provided for each disorder group—

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cardiovascular, cancer, metabolic, and miscellaneous—in Supplementary Tables S4A, S4B, S4C and S4D, respectively.

2.3.3. Novel Variant Findings

Out of the 259 variants identified, we found 68 novel PAT/L-PAT variants (~26%) that have not been previously reported in the literature (as ascertained in the HGMD2024.4 Pro database and literature review). Detailed information on these novel variants is presented in Table 2. Their proportion varied across phenotype groups, with 30% in cancer-related genes, 38% in cardiovascular genes, and 6% in the miscellaneous group, while no novel findings were identified in the metabolic group.

**Table 2.** Novel pathogenic and likely-pathogenic variants identified in this study that have not been previously reported in the literature.

| Gene | cDNA (HGVS) | Predicted Splicing Impact (Y / N) | Protein change (HGVS) | Freq. (gnomAD 4.1) (%) | ClinVar ID (2025-01-02) |
|---|---|---|---|---|---|
| ***BMPR1A*** | NM_004329.3:c.231-2A>T | Y | - | - | 2866138 |
| | NM_004329.3:c.231-1G>T | Y | - | - | 567998 |
| ***BRCA1*** | NM_007294.4:c.109A>G | N | NP_009225.1:p.Thr37Ala | - | 868146 |
| ***BRCA2*** | NM_000059.4:c.2974A>T | N | NP_000050.3:p.Lys992* | - | - |
| | NM_000059.4:c.4933A>T | N | NP_000050.3:p.Lys1645* | - | 51744 |
| | NM_000059.4:c.7258delG | N | NP_000050.3:p.Glu2420Asnfs*47 | - | - |
| ***MEN1*** | NM_130799.3:c.467G>C | N | NP_570711.2:p.Gly156Ala | - | - |
| ***MSH2*** | NM_000251.3:c.2084T>G | N | NP_000242.1:p.Val695Gly | - | - |
| ***MSH6*** | NM_000179.3:c.195_199delACCGC | N | NP_000170.1:p.Pro66Glnfs*22 | 0.0001 | - |
| | NM_000179.3:c.198_199insTT | N | NP_000170.1:p.Pro67Phefs*15 | - | - |
| | NM_000179.3:c.841G>T | N | NP_000170.1:p.Gly281* | - | 2673649 |
| | NM_000179.3:c.2437A>T | N | NP_000170.1:p.Lys813* | 0.0001 | 1791241 |
| | NM_000179.3:c.3682_3698del | N | NP_000170.1:p.Ala1228Argfs*4 | - | - |
| ***MUTYH*** | NM_001128425.2:c.788+2_788+4delTAG | Y | - | - | - |
| | NM_001128425.2:c.785_786insG | N | NP_001121897.1:p.Trp263Leufs*66 | - | - |
| | NM_001128425.2:c.781delC | N | NP_001121897.1:p.Gln261Serfs*5 | - | - |
| ***PTEN*** | NM_000314.8:c.802-1_805delGGACA | N | NP_000305.3:p.? | - | - |
| | NM_000314.8:c.804_805insTTTTT | N | NP_000305.3:p.Lys269Phefs*9 | - | - |
| ***RB1*** | NM_000321.3:c.1422-2A>T | Y | - | 0.0004 | - |
| ***SMAD4*** | NM_005359.6:c.904+1_904+2ins(45) | Y | - | 0.0053 | - |
| ***TSC2*** | NM_000548.5:c.264_265delGT | N | NP_000539.2:p.Leu89Alafs*36 | 0.0160 | 45485999 |
| | NM_000548.5:c.340G>T | N | NP_000539.2:p.Glu114* | - | 65033 |
| | NM_000548.5:c.775-2A>C | Y | - | - | - |
| | NM_000548.5:c.2340_2341ins(37) | N | NP_000539.2:p.Asp781Phefs*12 | - | - |
| ***APOB*** | NM_000384.3:c.9743_9744insG | N | NP_000375.3:p.Ile3248Metfs*12 | - | - |

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| | | | | | |
|---|---|---|---|---|---|
| | NM_000384.3:c.9735delC | N | NP_000375.3:p.Gln3247Lysfs*19 | - | - |
| | NM_000384.3:c.2297_2298delAA | N | NP_000375.3:p.Lys766Ilefs*25 | - | 1553385715 |
| *COL3A1* | NM_000090.4:c.1429G>A | N | NP_000081.2:p.Gly477Arg | - | - |
| | NM_000090.4:c.2229+1G>A | Y | - | - | 640856 |
| *DES* | NM_001927.4:c.75_76insAG | N | NP_001918.3:p.Leu26Serfs*6 | - | - |
| *DSC2* | NM_024422.6:c.1044_1047dupAAAT | N | NP_077740.1:p.Asp350Lysfs*2 | - | - |
| | NM_024422.6:c.631-1G>A | Y | - | 0.0001 | 2775190 |
| *DSP* | NM_004415.4:c.107delG | N | NP_004406.2:p.Gly36Alafs*12 | - | - |
| | NM_004415.4:c.1258G>T | N | NP_004406.2:p.Glu420* | - | - |
| | NM_004415.4:c.2572delG | N | NP_004406.2:p.Glu858Lysfs*6 | - | - |
| *FBN1* | NM_000138.5:c.4282C>T | N | NP_000129.3:p.Arg1428Cys | 0.0007 | - |
| | NM_000138.5:c.4015_4016insTG | N | NP_000129.3:p.Cys1339Leufs*75 | - | - |
| *FLNC* | NM_001458.5:c.502delT | N | NP_001449.3:p.Trp168Glyfs*84 | - | - |
| | NM_001458.5:c.2550+1G>A | Y | - | - | - |
| *KCNH2* | NM_000238.4:c.1621C>T | N | NP_000229.1:p.Arg541Cys | 0.0004 | 937094 |
| *LDLR* | NM_000527.5:c.1315A>T | N | NP_000518.1:p.Asn439Tyr | - | 375813 |
| *MYBPC3* | NM_000256.3:c.2995-2A>G | Y | - | 0.0001 | - |
| *MYH7* | NM_000257.4:c.1756G>A | N | NP_000248.2:p.Val586Met | 0.0004 | 1172186 |
| *PKP2* | NM_004572.4:c.1489C>T | N | NP_004563.2:p.Arg497* | 0.0003 | 78974 |
| | NM_004572.4:c.328delA | N | NP_004563.2:p.Met110Cysfs*2 | - | - |
| *SCN5A* | NM_198056.3:c.5306C>T | N | NP_932173.1:p.Ala1769Val | 0.0001 | - |
| *TGFBR2* | NM_003242.6:c.760C>T | N | NP_001020018.1:p.Arg279Cys | 0.0001 | 213942 |
| *TNNT2* | NM_001276345.2:c.87_88delGG | N | NP_001263274.1:p.Asp30Argfs*13 | - | - |
| | NM_001276345.2:c.80G>A | N | NP_001263274.1:p.Trp27* | - | - |

| Gene | cDNA (HGVS) | Predicted Splicing Impact (Y / N | Protein change (HGVS) | Freq. (gnomAD 4.1) (%) | ClinVar ID (2025-01-02) |
|---|---|---|---|---|---|
| *TRDN* | NM_006073.4:c.1831+1G>A | Y | - | - | - |
| | NM_006073.4:c.1155delA | N | NP_006064.2:p.Lys385Asnfs*5 | 0.0013 | - |
| | NM_001256021.2:c.601_610delCTGGCGAAAG | N | NP_001242950.1:p.Leu201Asnfs*19 | 0.0029 | - |
| | NM_001256021.2:c.439_440delAA | N | NP_001242950.1:p.Lys147Aspfs*2 | 0.0001 | 2114339116 |
| *TTN* | NM_001267550.2:c.107409_107410insCC | N | NP_001254479.2:p.Leu35804Profs*2 | - | - |
| | NM_001267550.2:c.97573_97574insTC | N | NP_001254479.2:p.Asp32525Valfs*8 | - | - |
| | NM_001267550.2:c.95576_95577delAA | N | NP_001254479.2:p.Lys31859Argfs*6 | - | - |
| | NM_001267550.2:c.93623_93626dupAGCC | N | NP_001254479.2:p.Gln31210Alafs*8 | - | - |
| | NM_001267550.2:c.84525G>A | N | NP_001254479.2:p.Trp28175* | - | - |
| | NM_001267550.2:c.79811dupT | N | NP_001254479.2:p.Arg26605Lysfs*19 | - | - |
| | NM_001267550.2:c.70971_70972insT | N | NP_001254479.2:p.Leu23658Serfs*18 | - | - |
| | NM_001267550.2:c.64266delA | N | NP_001254479.2:p.Asp21423Ilefs*2 | 0.0001 | - |
| | NM_001267550.2:c.58709C>G | N | NP_001254479.2:p.Ser19570* | - | - |
| | NM_001267550.2:c.52975_52976delCA | N | NP_001254479.2:p.Gln17659Thrfs*6 | 0.0001 | - |
| | NM_001267550.2:c.41845dupA | N | NP_001254479.2:p.Ile13949Asnfs*2 | - | - |
| | NM_001267550.2:c.13184delT | N | NP_001254479.2:p.Leu4395Argfs*25 | - | - |
| *ACVRL1* | NM_000020.3:c.830C>T | N | NP_000011.2:p.Thr277Met | 0.0004 | 2731545 |
| *ATP7B* | NM_000053.4:c.3959G>C | N | NP_000044.2:p.Arg1320Thr | 0.0010 | 1479012 |

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| | | | | | |
|---|---|---|---|---|---|
| ***RPE65*** | NM_000329.3:c.1544G>A | N | NP_000320.1:p.Arg515Gln | 0.0015 | 1052287 |

Footnote: Freq.- Frequency; Y- Yes; N- No. (end of table).

## 3. Discussion

This research developed a strategy to obtain a potentially representative sample of the Portuguese population using WES-derived data from a diagnostic setting. The final purpose was to determine the frequency of PAT/L-PAT variants at ACMG´s medically actionable *loci*. Such data, however, were previous unavailable for our Portuguese population.

We could infer that 6.19% of healthy persons had potentially actionable findings. This is in line with previous reports, ranging 0.59% to 12.64% [10–39]. Several factors may explain this variation, including differences in populations, sample size, study design, number of genes screened, sequencing technology, and variant filtering, interpretation and classification; in addition to actionability criteria, and the increasing number of validated PAT/L-PAT variants in disease databases over time.

This study may also stand out from others for several reasons: (i) unlike most other, our cohort was resampled to be as representative as possible of the general population; (ii) sample size was larger and more significant considering our population (of about 10.5 million people); (iii) the most recent ACMG list (v. 3.2, 2023) of 81 genes was analysed; and (iv) variant filtering and interpretation followed the most recent guidelines and gene specifications, and included a manual curation step.

In line with previous studies, PAT/L-PAT variants identified associated with "silent" but life-threatening diseases within four groups: cardiovascular, cancer, miscellaneous and metabolic disorders. Variants associated to cardiovascular diseases were the most frequent, and included cardiomyopathy, arrhythmias, dyslipidaemia, and connective tissue disorders.

CMP-related variant frequencies aligned with similar studies or disease prevalence (DCM: 1:500, HCM: 1:500, ARVC: 1:1,000–5,000), considering penetrance (DCM: 45%, HCM: 40%, ARVC: 30–75%) [40–42]. Likewise, primary arrhythmia variants, notably LQTS (0.45%), matched reported prevalence (1:2,500) and penetrance (75%) [43]. Both CMP and LQTS pose sudden death risks, warranting regular clinical surveillance, pharmacotherapy, and potentially implantable cardioverter defibrillators.

Concerning dyslipidaemia, frequency of FH variants (0.58%) is in accordance with previously estimated disease frequency (1:200-250) in a meta-analysis [44]. FH leads to coronary heart disease events and death, if untreated. It has been estimated that only less than 10% of persons with FH are diagnosed, and even less receive treatment. Thus, early detection and possible treatment with lipid-lowering drugs are crucial.

On the other hand, HCTD related findings, including Marfan syndrome (MS, 0.20%), familial thoracic aortic aneurism and dissection (FTAAD, 0.13%), Loeys-Dietz syndrome (LDS, 0.08%), and vascular type of Ehlers-Danlos syndrome (vEDS, 0.05%) were more frequent than expected, according to disease frequency in the literature (1:3,300-1:20,000 for MS; 1:5,000-1:4,000,000 for FTAAD; unknown for LDS; and 1:50,000 for vEDS) [45–48]. Due to an increased risk of rupture of the aorta, early diagnosis is life-saving, in order to proceed with surveillance, therapy, and eventually prosthetic surgery.

The second most frequent group of findings is related with cancer, being HBOC and HNPCC the most common. Frequency of reportable HBOC-related variants was estimated as 0.63%. This is 2-5-fold higher, when compared to the described frequency of *BRCA* pathogenic variants (1:400 to 1:800) [49]. However, this rate may be underestimate in our cohort, given the limitation to detect the NM_000059.4(*BRCA2*):c.156_157insAlu (a known founder variant in the Portuguese population) using WES data [50]. Mismatch-repair variants showed a frequency of 0.60%, similarly to reported prevalence of HNPCC of 1:279 in the general population, and incomplete penetrance (>90% for colonic adenomas) [51]. All these findings support the relevance of early diagnosis of hereditary cancer syndromes, since these persons may be offered clinical surveillance, following family history and knowing genetic status.

In the metabolic disorders group, we highlight the findings related to Fabry disease. Our screening showed a frequency of 0.10% for variants in *GLA*, corroborating recent data on prevalence of Fabry disease (1:1,250 to 1:5,732) [58–62]. Specifically, NM_000169.3:c.337T>C, p.(Phe113Leu), a founder variant in the region of Guimarães, Portugal and associated to the late-onset form, was found in 0.03% [63]. This higher prevalence of Fabry disease is related to knowledge of a broader phenotypic spectrum for *GLA*, encompasses the classical and a late-onset form. Persons with this late-onset phenotype may have significant diagnostic delay [64]. Genetic testing may allow for earlier diagnosis,

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critical for efficiency of enzyme replacement or chaperone therapy, recommended to be initiated as early as possible.

*TTR*-related hereditary amyloidosis has a significant cluster in northern Portugal, the largest worldwide [52,53]. In our data the frequency of *TTR* pathogenic variants was 0.43%. The p.Val50Met variant (NM_000371.4:c.148G>A) showed a frequency of 0.28%, comparable to that reported in northern Portugal (1:538) [54]. This variant is predominantly associated to *TTR*-related amyloid neuropathy [55]. Surprisingly, the p.Val142Ile variant (NM_000371.4:c.424G>A), known to be associated to the *TTR*-related cardiac amyloidosis, particularly prevalent in persons of African ancestry, was also present in 0.16% [55]. The higher prevalence of *TTR* variants identified supports the greater awareness for *TTR* amyloidosis in Portugal, allowing for early diagnosis, follow-up and treatment, including liver transplant, pharmacotherapy, and ICD implant when indicated.

We also identified variants in the *RYR1* gene with an overall frequency of 0.33%. The exact worldwide prevalence of malignant hyperthermia susceptibility (MHS) has been difficult to clarify given disparities in clinical diagnosis methods and criteria. Using population genomics data, estimated prevalence of an MHS-related pathogenic variants was 1:1450-1:1556 [56,57]. Here, we report a frequency 10-times higher, after having restricted to variants functionally validated and curated by the European Malignant Hyperthermia Group. Assessment of risk for MHS is relevant so that effective measures, such as avoidance of certain anaesthetics upon surgery.

In addition, we identified heterozygous variants in genes associated with autosomal recessive diseases. In total, 11.05% of persons were carriers for any high-risk ACMG actionable genes linked to recessive diseases, including *HFE*, *MUTYH*, *ATP7B*, *RPE65*, *GAA*, *BTD* and *TRDN*. Frequency, in our cohort, of heterozygotes at *HFE* (5.21%), *MUTYH* (2.29%), *ATP7B* (1.83%), *GAA* (0.50%) and *BTD* (0.38%) was comparable to previous estimates in the general population (1:10 for *HFE*, 1:50 for *MUTYH*, 1:50-90 for *ATP7B*, 1:70 for *GAA*, and 1:120 for *BTD*) [51,65–71]. For comparison, the American College of Obstetricians and Gynecologists endorsed carrier screening for cystic fibrosis and spinal muscular atrophy, as well as other diseases with a carrier frequency ≥1/100 [72]. Our data may provide support to which genes should be selected for preconception carrier screening based on population carrier frequency, to address together with considerations on penetrance, severity, and predictable genotype-phenotype correlation.

Our work shows some limitations, most of which are transversal to similar published studies:

- *Lab cohort bias*. Our sample was derived from cases ascertained for genetic diagnosis of various Mendelian disorders; therefore, a few persons in the cohort may already be affected by a disease attributable to one of the genes in the ACMG list. Despite this, when excluding L-PAT/PAT variants listed as primary diagnosis in the genetic test reports of these patients, the overall frequency did not differ significantly (only 0.6%);
- *Gene list*. We limited our analysis to the current set of the ACMG genes. We did not consider other clinically relevant genes, as those curated by the ClinGen Actionability Working Group, for instance. Inclusion of additional conditions, some of specific impact in the Portuguese population, should be considered in future studies, what might increase the overall frequency of actionable findings;
- *Study design*. In order to minimize impact of data used, our project protocol prevented us from including individual-level information regarding ethnic background, age, gender, reason for referral for WES, or phenotype. Additionally, genotypes obtained were related to the whole cohort, not the patient. Consequently, we were not able to estimate compound heterozygosity, or the number of findings per individual;
- *Technical limitations*. Methodologies used may have led to missed variants due to: (i) intrinsic WES limitation to detect deep intronic, triplet repeats expansion, and structural variants; (ii) use of different capture kits along time within this cohort; (iii) incomplete coverage in some regions; (iv) not considering structural variants, including copy number variants (CNVs); and (v) MAF cut-off;
- *Potential for false-positive interpretation of variants*. Variants accurately classified as PAT/L-PAT, based on available evidence, may not be in fact disease causing, due to incomplete penetrance or variable expressivity. This is exacerbated when genetic testing is performed in the context of population screening;

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- *Actionability*. The term "actionable" is highly subjective and its application may fluctuate. The ClinGen Actionability Working Group is addressing this issue by curating the actionability of several gene-disease groups, including those listed by the ACMG. We took this into consideration; however, some gene-disease groups are not yet curated and others are classified as *actionable* depending on individual-level information, such as age and sex, which were not considered due to our study protocol.

Future studies, including more persons, are needed to determine more accurately the frequency of ultra-rare disease-causing alleles in our study population. Our methodology could also be used to evaluate the allelic frequency for non-ACMG actionable *loci*. This could include other diseases known to have impact in Portugal, such as haemoglobinopathies, congenital adrenal hyperplasia, and cystic fibrosis; some of those genes may be of interest for designing of preconceptional screening programmes. Optimizing guidelines for variant interpretation and reporting criteria in asymptomatic persons is also suggested, as well as research on long-term phenotypic effects of presumed PAT/L-PAT variants in the general population. Finally, it is recommended to perform responsible research on genomic screening, before considering its eventual implementation within healthcare services.

## 4. Materials and Methods

### *4.1. Study Design and Data Set of Exomes*

This is a cross-sectional observational study with a quantitative approach. We analysed the WES data obtained from patients tested at CGPP-IBMC (12,167 samples, retrieved in 19-05-2023), in an anonymized and aggregated form. All patients had been clinically diagnosed or had a diagnostic suspicion of a disease for which the molecular test requested by their physician was either a virtual gene-panel based on WES or a complete WES analysis.

### *4.2. Resampling from CGPP-IBMC Clinical Database*

The approach used to generate a sample approaching representativeness of the Portuguese population is detailed elsewhere [74]. Briefly, to remove possible population biases, the CGPP-IBMC cohort was resampled to i) include only one sample per family; ii) include one partner per consanguineous couple; iii) remove pre-natal or foetal samples. The final subset used was corrected for geographic distribution of cases and population size, by using data from Census 2021 [75]; also, distinct datasets were made according to the known distribution of the population, by municipalities both in mainland Portugal, and Madeira and the Azores archipelagos.

### *4.3. Selection of Genes for Which Reporting of Secondary Findings Are Recommended*

The list of genes in the present analysis covered the 81 genes that are part of the revised guidelines on secondary findings from the ACMG, version 3.2 [7]. This list encompasses genes related to cancer ($n$=28), cardiovascular ($n$=40), inborn errors of metabolism ($n$=4) and miscellaneous ($n$=7) phenotypes (Supplementary Table S5).

### *4.4. Data Processing*

BED files, containing genomic coordinates corresponding to all 81 ACMG genes (exonic coding regions plus 20bp intronic flanks), were intersected with the full WES VCF files from each sample, to select variants in those genes. Variants with <10x coverage, <30% heterozygous allele ratio and base-call quality score ≤Q20 were excluded. For each gene, the obtained individual VCF files, containing the gene's variants, were processed into aggregated, multi-sample VCF files. These multi-sample VCFs were then further processed by calculating allele count, allele number, allele frequency, number of homozygotes, heterozygotes and hemizygotes, and removing individual genotype information to ensure irreversible anonymization of patients' data. This aggregation and anonymization were performed for (i) all samples and (ii) resampled data, grouped by municipalities, as described previously.

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*4.5. Variant Annotation*

Variants were systematically annotated with information available from (i) our in-house database, (ii) disease databases, namely ClinVar (retrieved 2023-09-07) and HGMD (version 2023.2), (iii) population database gnomAD (versions 2.1.1, 3.1.2 and 4.1), and (iv) molecular consequence predictors.

*4.6. Variant Filtering*

The overall filtering and manual curation workflow are explained in Figure 1 and Supplementary Tables S6A, 6B and 6C. First, variants were filtered based on confidence metrics including genotype quality (GQ, ≥60) and read depth (RD, minimum 10x). Second, variants were prioritized according to prior classification in our in-house laboratory database, being automatically included if classified as PAT/L-PAT. Third, variants were filtered based in previous ClinVar classification, being included if classified as PAT/L-PAT 2 stars and excluded if BEN/L-BEN 2 stars. Fourth, variants were filtered based on their minor allele frequency (MAF), with exclusion of variants with MAF≥0.5% in gnomAD, except for the *HFE* variant NM_000410.4:c.845G>A (p.Cys282Tyr). Finally, variants were filtered according to effect prediction (VEP); variants with low predicted impact were excluded.

*4.7. Manual Variant Curation, Classification, and Actionability*

We further curated manually the remaining variants, following the guidelines for interpretation and classification of sequence variants from the ACMG/AMP [76]. Additionally, specifications for variant classification of the Association for Clinical Genomic Science (ACGS), Clinical Genome Resource (ClinGen), Cancer Variant Interpretation Group UK (CanVIG-UK), and the European Molecular Genetics Quality Network (EMQN) were considered, when applicable (Supplementary Tables S6A, S6B and S6C) [77–80]. All variants classified as PAT/L-PAT, referring to the potential of the variant to cause a relevant phenotype according to the ACMG v3.2 recommendations, were considered to be medically actionable.

*4.8. Frequency of Actionable Findings Calculation*

The overall frequency of actionable findings was then calculated considering the total of size of the WES cohort and the number of genes.

## 5. Conclusions

To the best of our knowledge, this is the first evaluation of medically actionable findings in the Portuguese population, making this study a pioneering effort. We demonstrate that this population, as others, is expected to harbour medically actionable variants that can be identified through WES in routine diagnosis; however, while our study provides important insight, additional evidence is needed to take its findings to the population level.

This foundational research establishes a basis for future investigations into genomic screening and highlights the potential for data from WES-based genetic testing to significantly impact public health.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1- Medically ACMG actionable findings and carrier status identified in our study. Tables S2: Frequency of medically actionable findings per gene [S2A- Cardiogenetic; S2B- Oncogenetic; S2C- Metabolic; S2D- Miscellaneous]. Tables S3: Overall frequencies, actionable findings and carrier frequencies per disease [S3A- Overall frequency of ACMG medically actionable findings and non-medically actionable findings (carriers); S3B- Frequency of ACMG medically actionable findings (per disease group); S3C- Frequency of carrier findings (per disease group)]. Tables S4: Carrier frequencies for recessive disease findings per gene [S4A- Cardiogenetic carrier; S4B- Oncogenetic carrier; S4C- Metabolic carrier; S4D- Miscellaneous carrier]. Table S5- List of genes for which reporting incidental findings are recommended by the ACMG. Tables S6: Manual curation and variant classification [S6A- Gene specifications; S6B- Standard manual curation - evidence analysis; S6C- Standard manual curation - variant class calculation]. Supplementary data S1. References for Table S6A Gene specifications - variant interpretation details.

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