

A Collaborative Effort to Define Classification Criteria for *ATM* Variants in Hereditary Cancer Patients

Lidia Feliubadaló,^{a,b,c,*} Alejandro Moles-Fernández,^{d,†} Marta Santamariña-Pena,^{e,f,g,†} Alysson T. Sánchez,^{a,b,†} Anael López-Novo,^{e,f} Luz-Marina Porras,^h Ana Blanco,^{e,f,g} Gabriel Capellá,^{a,b,c} Miguel de la Hoya,^{c,i} Ignacio J. Molina,^j Ana Osorio,^{g,k} Marta Pineda,^{a,b,c} Daniel Rueda,^l Xavier de la Cruz,^{h,m} Orland Diez,^{d,n} Clara Ruiz-Ponte,^{e,f,g} Sara Gutiérrez-Enríquez,^d Ana Vega,^{e,f,g} and Conxi Lázaro^{a,b,c,*}

BACKGROUND: Gene panel testing by massive parallel sequencing has increased the diagnostic yield but also the number of variants of uncertain significance. Clinical interpretation of genomic data requires expertise for each gene and disease. Heterozygous *ATM* pathogenic variants increase the risk of cancer, particularly breast cancer. For this reason, *ATM* is included in most hereditary cancer panels. It is a large gene, showing a high number of variants, most of them of uncertain significance. Hence, we initiated a collaborative effort to improve and standardize variant classification for the *ATM* gene.

METHODS: Six independent laboratories collected information from 766 *ATM* variant carriers harboring 283 different variants. Data were submitted in a consensus template form, variant nomenclature and clinical information were curated, and monthly team conferences were established to review and adapt American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) criteria to *ATM*, which were used to classify 50 representative variants.

RESULTS: Amid 283 different variants, 99 appeared more than once, 35 had differences in classification among laboratories. Refinement of ACMG/AMP criteria to *ATM* involved specification for twenty-one criteria and adjustment of strength for fourteen others. Afterwards, 50 variants carried by 254 index cases were

classified with the established framework resulting in a consensus classification for all of them and a reduction in the number of variants of uncertain significance from 58% to 42%.

CONCLUSIONS: Our results highlight the relevance of data sharing and data curation by multidisciplinary experts to achieve improved variant classification that will eventually improve clinical management.

Introduction

Genetic diagnosis for hereditary cancers (HC) has changed over the past decade thanks to the introduction of massive parallel sequencing (MPS) technologies which allow the screening of multiple genes outright. MPS diagnostic panels increase sensitivity but also the number of variants of uncertain clinical significance (VUS) identified; application of MPS panels poses a significant challenge in the clinical management of patients and evidences the need for standardization in variant classification. The American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) have provided a general framework for classification of genetic variants (1). However, these universal guidelines need to be tuned according to the disease and the specific gene by a consensus of experts. Currently ACMG/AMP guidelines

^aHereditary Cancer Program, Catalan Institute of Oncology, IDIBELL, Hospitalet de Llobregat, Barcelona, Spain; ^bOncobell Program, IDIBELL, Hospitalet de Llobregat, Barcelona, Spain; ^cCentro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain; ^dHereditary Cancer Genetics Group, Vall d'Hebron Institute of Oncology (VHIO), Barcelona, Spain; ^eFundación Pública Galega Medicina Xenómica (FPGMX), SERGAS, Santiago de Compostela, Spain; ^fInstituto de Investigación Sanitaria de Santiago de Compostela (IDIS), Santiago de Compostela, Spain; ^gCentro de Investigación en Red de Enfermedades Raras (CIBERER), Madrid, Spain; ^hResearch Unit in Clinical and Translational Bioinformatics, Vall d'Hebron Institute of Research (VHIR), Universitat Autònoma de Barcelona, Barcelona, Spain; ⁱMolecular Oncology Laboratory, Hospital Clínico San Carlos, IdISSC (Instituto de Investigación Sanitaria del Hospital Clínico San Carlos), Madrid, Spain; ^jInstitute of Biopathology and Regenerative Medicine, Center for Biomedical Research, Health Sciences Technology Park, University of Granada, Granada, Spain; ^kHuman Genetics Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain; ^lHereditary Cancer Laboratory, Doce de Octubre University

Hospital, i+12 Research Institute, Madrid, Spain; ^mInstitució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain; ⁿClinical and Molecular Genetics Area, University Hospital Vall d'Hebron, Barcelona, Spain.

* Address correspondence to: L.F. at Hereditary Cancer Program, Catalan Institute of Oncology, IDIBELL, Hospitalet de Llobregat, Av. Gran Via 199-203, 08908 Barcelona, Spain. Fax +34-932607466; e-mail lfeliubadalo@iconcologia.net. C.L. at Hereditary Cancer Program, Catalan Institute of Oncology, IDIBELL, Hospitalet de Llobregat, Av. Gran Via 199-203, 08908 Barcelona, Spain. E-mail clazaro@iconcologia.net.

[†]These authors contributed equally to this work and should be considered second co-authors

[‡]These authors should be considered corresponding co-authors

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have been adapted for some hereditary cancer genes such as *PTEN* (2), *CDH1* (3), and *TP53* (4).

Most of the currently used HC panels include the *ATM* gene, mainly because heterozygous *ATM* mutations increase the risk of cancer, particularly breast cancer (BC) (5), and have also been associated with colorectal, prostate, and pancreatic cancer predisposition (6–8). A moderate breast cancer risk of about 2.4-fold was estimated from breast cancer families with *ATM* pathogenic variants (9). In this sense, *ATM* loss-of-function variants confer an increase in breast cancer risk 10 times greater than that of missense variants (10); however, the p.(Val2424Gly) missense variant seems to confer a higher risk, comparable to that of *BRCA2* variants (11). For other cancers, an overall risk of 2.23 (94% CI 1.26–4.28) has been suggested to increase to 4.94 (95% CI = 1.90 to 12.9) in carriers under 50 years of age (8).

ATM is also responsible for the autosomal recessive genetic disorder ataxia telangiectasia (AT) (MIM# 208900) (12). AT is a pleiotropic neurodegenerative disease whose symptoms include malignancy and genome instability, often accompanied by immunodeficiencies, premature aging, insulin resistance, and infertility (13, 14). Most AT patients bear compound heterozygous pathogenic variants from over 800 currently registered in the Human Genome Mutation Database (15). A recent study in Spanish AT patients identified disease-causing mutations in 96% of the alleles studied, frameshift being the most common type of variant (16). The *ATM* protein is a member of the phosphatidylinositol-3' kinase-related protein kinase (PIKK) family, which phosphorylates hundreds of targets containing Ser/Thr-Gln motifs, and plays critical roles in double-strand break (DSB) DNA repair and cell cycle (14). DNA breaks recruit inactive *ATM* dimers through the Mre11-Rad50-NBS1 (MRN) sensor complex, which allows *ATM* dissociation into Ser1981-autophosphorylated active monomers, able to act upon a number of direct substrates such as TP53 or indirect such as histone H2AX (14). These events are key indicators of *ATM* functional activity, and coordinated activity of phosphorylated downstream targets determines whether the genomic instability resulting from DNA damage can be prevented (17).

With the aim of improving and standardizing variant classification for HC genes in Spain, 6 independent molecular laboratories using MPS panels agreed to create a common variant database. *ATM* was chosen for the pilot study because it is a large gene included in the majority of HC panels and shows a remarkable number of VUS (18). After adapting ACMG/AMP classification guidelines to *ATM*, 50 variants were designated for classification with the established consensus.

Materials and Methods

A detailed description of the methodology used can be found in the Supplemental Patients and Methods. Briefly, a multidisciplinary group was built with complementary expertise. Most of the members are molecular geneticists with experience in hereditary cancer and RNA splicing. Some are members of gene-specific international endeavors such as Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA, <https://enigmaconsortium.org/>), International Society for Gastrointestinal Hereditary Tumours (InSiGHT, <https://www.insight-group.org/>), and Clinical Genome Resource (ClinGen, <https://clinicalgenome.org/>). In addition, the team had a Spanish expert in AT and *ATM* functional assays and 2 experts in computational biology and bioinformatics. Patients included in this study were seen in the different genetic counseling units of each reference laboratory. All patients had a clinical suspicion of HC and were tested by gene panel sequencing. All variants detected in the *ATM* coding sequence and 20-bp surrounding regions with minor allele frequency lower than 1% were collected in the Spanish Hereditary Cancer Variant Database (DB hereinafter) created for this purpose.

Cut-offs for allele frequency calculations, as well as the selection of different splicing and protein prediction assessment tools and the adjustment of the corresponding threshold values, are described in the [Supplemental Methods](#). This section also details the process of functional study type selection and the strategy for variant classification of 50 pilot variants from our DB.

Results

ATM VARIANT DATABASE

In total, we collected information from 769 individuals carrying 283 different *ATM* variants; 104 index cases carried more than one *ATM* variant. Hereditary breast and/or ovarian cancer was the most common clinical indication in the whole cohort (67%) ([Supplemental Fig. 1](#)), being women 85% of individuals ([Supplemental Table 1](#)). Ninety-nine of the 283 different variants collected appeared in more than one family; 78 were found in more than one laboratory, and 20 appeared in 10 or more families ([Supplemental Fig. 1](#)). The 5-tier pathogenicity classification given by each laboratory was recorded, and 35 of the 78 variants detected by more than one laboratory had discordant classifications (45%). Thirty of these discordances were due to the variant being classified as VUS vs. likely benign (LB); the remaining 5 discordances were as follows: 3 from likely pathogenic (LP) vs. pathogenic (P), 1 from VUS vs. benign (B), and 1 from VUS vs. LP.

ATM-SPECIFIC REFINEMENT OF ACMG-AMP CLASSIFICATION GUIDELINES

Based on previous studies of other HC genes (2, 3), we decided to adapt the widely used ACMG/AMP classification guidelines (1) to the characteristics of the *ATM* gene and its associated phenotypes. From the 28 criteria listed in the guidelines, several have been modified, restricted, rejected as non-applicable or expanded to diverse strengths. The resulting criteria proposed are detailed in Table 1. Criteria where modifications were based on *ATM*-specific data and unpublished modifications are justified in the following sections. Some criteria are not applied because they overlap with others (PP4), ClinGen itself has discarded them (PP5 and BP6) (22) or are not applicable to *ATM* (PP2, BP1, BP3 BP5, see Table 1 footnotes). Combination rules are kept from Richards et al. (1).

POPULATION EVIDENCE

Since allelic frequencies in general populations are powerful tools for identifying common benign variants, we used the statistical framework defined by Whiffin et al. (23) to calculate the maximum credible population allele frequency (MCPAF) for *ATM* pathogenic variants with AT data. We obtained a cut-off allele frequency in the general population of 0.005 for BA1 and 0.0005 for BS1. We translated the threshold to population datasets as the lower boundary of their 99% confidence interval and propose to use any of the nonfounder GnomAD v2.1.1 non-cancer populations (24). Due to the low penetrance of *ATM* pathogenic variants for breast cancer, we cannot apply BS2 to healthy heterozygous variant carriers. BS2 is met if we find one homozygous carrier without AT affection. BS2_Supporting will be applied to 2 homozygous observations with no clinical data provided. As the main manifestations of AT are neurologic, we propose to use the GnomAD v2.1.1 non-neuro dataset.

PREDICTIVE EVIDENCE

Regarding splicing alterations, our performance assessment of *in silico* predictors supports the election of the predictor SPiCE (25) for variants affecting the canonical donor splice site, applying PP3 when they exceed the threshold of 0.240 (100% sensitivity), and BP4 when they are below it (with a sensitivity of 89.9% to identify variants not affecting splicing). For variants affecting the canonical acceptor splice site, PP3 is assigned when exceeding the threshold of 0.789 (sensitivity 87.6%) and BP4 when they are under 0.282 (with a sensitivity of 86.3% to identify variants not affecting splicing), no evidence is considered for acceptor variants with scores between 0.282 and 0.789. No called variants account for 6.2% of splicing altering and 10.8% of splicing neutral variants in our dataset (Supplemental Fig. 2). For

activation or creation of splicing sites, we used a combination of predictors such as SpliceSiteFinder-like, MaxEntScan and GeneSplicer, as detailed in Table 1.

In relation to protein predictors for missense variants, we performed a comparative analysis of different tools for the two *ATM* halves (see Supplemental Methods and Supplemental Table 2). Our results sustain the use of the following combinations of two predictors: REVEL plus VEST4 for the N-terminal half (residues 1–1959) and REVEL plus PROVEAN for the C-terminal half (residues 1960–3056). We proposed the same procedure for both halves: PP3 or BP4 is awarded when the 2 predictors assigned to the protein half agree on a damaging effect or an absence of effect, respectively; otherwise, the contribution of *in silico* evidence is not considered.

FUNCTIONAL EVIDENCE

Spliceogenic variants are usually confirmed by the study of the RNA of carriers or by mini-gene assays. Splicing analysis in RNA from a carrier, if well designed and performed quantitatively with the appropriate controls, can demonstrate that a variant produces only aberrant transcripts with premature termination codons undergoing nonsense-mediated decay (NMD). We consider that such cases deserve to be very strong pathogenic evidence, PS3_VeryStrong, analogous to the strength bestowed in ClinGen's PVS1 decision tree (19). We propose a gradual decrease of PS3 strength when the damaging effect is less certain or less severe (Tables 1 and 2).

Protein function assays are quite specific to the gene and associated conditions. AT-patient cells show hypersensitivity to ionizing radiation and other DSB-DNA-inducing agents manifesting as absence of *ATM* serine 1981 phosphorylation (26), decrease in cell survival, an increased rate of chromosomal aberrations and defects in cell cycle checkpoints (14, 27, 28). Null variants that result in the absence or loss of *ATM* expression or prevent the Ser1981-mediated activation of *ATM*, reduce the phosphorylation of numerous substrates and increase the sensitivity to DNA damaging agents have been associated with classical AT phenotypes. On the other hand, missense and splicing variants allowing some *ATM* expression, thus presenting residual kinase activity and/or intermediate sensitivity to agents that damage DNA, have been associated with AT patients with milder or atypical phenotypes (16, 29). Consequently, we consider that these 3 functional assays are useful for investigating the pathogenicity of *ATM* variants for the 2 phenotypes (Fig. 1, A). We propose to confer different strengths to PS3 depending on how many of the 3 assays are found to be altered, and the extent of the alteration. Thereby, PS3 will be met when the 3 assays are completely altered,

Table 1. ATM variant classification proposed criteria.		
PATHOGENIC CRITERIA		
Criteria	Criteria description	Specification
STAND-ALONE CRITERION		
PVS1_StandAlone	For a full gene deletion, a pathogenic classification is warranted (in the absence of conflicting data). ^a	None
VERY-STRONG CRITERIA		
PVS1	-Null variant (nonsense, frameshift, canonical ± 1 or 2 splice sites, single or multi-exon deletion or tandem duplication) predicted to undergo NMD. ^a OR -Variants disrupting the initiation codon. ^b	None
PS2_VeryStrong or PM6_VeryStrong	AT patients with de novo score ≥ 4.0 as per ClinGen SVI Recommendation for de novo Criteria (PS2 & PM6) - Version 1.0. ^c	Strength
PS3_VeryStrong	Splicing analysis in RNA from a carrier quantitatively proves that the variant produces a splicing alteration predicted to undergo NMD, and the variant allele does not produce any full-length transcript. See text and Table 2 for details.	Strength
PS4_VeryStrong	Sixteen AT families. ^d It can only be applied to AT families and NOT in: breast cancer families, breast cancer case-control studies, variants that meet BA1 or BS1, nor together with PM3 at any strength.	Strength
PM3_VeryStrong	AT probands with <i>in trans</i> score ≥ 4.0 as per ClinGen SVI Recommendation for <i>in trans</i> Criterion (PM3) - Version 1.0. ^e It cannot be applied to variants that meet BA1 or BS1, nor together with PS4 at any strength.	Strength
STRONG CRITERIA		
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change (none of the variants affect splicing according to predictors).	None
PS2 or PM6_Strong	AT patients with a de novo score 2.0-3.75 as per ClinGen SVI Recommendation for de novo Criteria (PS2 & PM6) - Version 1.0. ^c	Strength
PS3	-SPlicing analysis in carrier RNA quantitatively proves that: · the variant alters splicing resulting in a deletion or insertion NOT predicted to undergo NMD but to alter/truncate a region critical to protein function or remove >10% of protein, and the variant allele does not produce any full-length transcript. See text and Table 2 for details. OR - The three following PROTEIN studies performed in AT patients or transfected cells show a strong alteration: · levels of ATM protein (or ATM phosphorylated in Ser1981) · levels of phosphorylation of two ATM substrates · sensitivity to DNA damaging agents. See text and Fig. 1 for details.	ATM-specific
PS4	Four to 15 AT probands. ^d It can only be applied to AT families and NOT in: breast cancer families, breast cancer case-control studies, variants that meet BA1 or BS1, nor together with PM3 at any strength.	Strength
PVS1_Strong	-Nonsense, frameshift, canonical ± 1 or 2 splice sites, single or multi-exon deletion NOT predicted to undergo NMD but to alter/truncate a region critical to protein function or remove >10% of protein. ^a -Also single or multi-exon duplication presumed in tandem with prediction of NMD. ^a	None
PM3_Strong	AT probands with <i>in trans</i> score 2.0-3.75 as per ClinGen SVI Recommendation for <i>in trans</i> Criterion (PM3) - Version 1.0. ^e It cannot be applied to variants that meet BA1 or BS1, nor together with PS4 at any strength.	Strength
<i>Continued</i>		

Table 1. (continued)		
PATHOGENIC CRITERIA		
Criteria	Criteria description	Specification
PP1_Strong	Co-segregation with AT in multiple affected family members, with ≥ 7 meioses observed across at least two families. ^{d,f}	Strength
MODERATE CRITERIA		
PM1	Variant affecting the mutational hotspot codon p. R3008 (NP_000042.3; see Results section) or the autophosphorylation codon p. S1981. See text for reasoning.	ATM-specific
PM2	Absent, or present at <0.00001 (0.001%) allele frequency in gnomAD or another large sequenced population. If multiple alleles are present within any subpopulation, allele frequency in that subpopulation must be <0.00002 (0.002%). ^f	ATM-specific
PM3	AT probands with <i>in trans</i> score 1.0-1.75 as per ClinGen SVI Recommendation for <i>in trans</i> Criterion (PM3) - Version 1.0. ^e It cannot be applied to variants that meet BA1 or BS1, nor together with PS4 at any strength.	Strength
PM4	Protein length changes as a result of in-frame deletions/insertions impacting at least one residue in a critical functional region (see PM1)	ATM-specific
PM5	Missense change at an amino acid residue where a different missense change determined to be pathogenic or likely pathogenic has been seen before. In addition, variant being interrogated must have a BLOSUM62 score equal to or less than the known variant. ^{f,g}	Restrictive
PM6 or PS2_Moderate	AT patients with de novo score 1.0-1.75 as per ClinGen SVI Recommendation for de novo Criteria (PS2 & PM6) - Version 1.0. ^c	Strength
PVS1_Moderate	Nonsense, frameshift, canonical ± 1 or 2 splice sites, single or multi-exon deletion NEITHER predicted to result in NMD NOR to alter/truncate a region critical to protein function, removing $<10\%$ of protein. ^a	None
PS3_Moderate	- SPLICING analysis: <ul style="list-style-type: none"> · in patient RNA quantitatively proves that the variant alters splicing resulting in a deletion or insertion NOT predicted to result in NMD but to remove $<10\%$ of protein, and the variant allele does not produce any full-length transcript; OR · in patient RNA quantitatively proves that the variant produces 90%-99% of altered transcript predicted to undergo NMD; OR · with a mini-gene quantitatively proves that the variant alters splicing resulting in NMD, and the variant allele does not produce any full-length transcript; OR · in patient RNA with NMD inhibition, semi-quantitatively shows with similar band intensity that the variant alters splicing resulting in NMD, without evidence that the variant allele produces any full-length transcript. See text and Table 2 for details. OR -Two of the following PROTEIN studies in AT patients or transfected cells show a strong alteration and the other one shows an intermediate alteration or has not been performed: <ul style="list-style-type: none"> · levels of ATM protein (or ATM phosphorylated in Ser1981) · levels of phosphorylation of two ATM substrates · sensitivity to DNA damaging agents. See text and Fig. 1 for details. 	Strength; ATM-specific
PS4_Moderate	Two to three AT probands. ^d It can only be applied to AT families and NOT in: breast cancer families, breast cancer case-control studies, variants that meet BA1 or BS1, nor together with PM3 at any strength.	Strength
Continued		

Table 1. (continued)		
PATHOGENIC CRITERIA		
Criteria	Criteria description	Specification
PP1_Moderate	Co-segregation with AT in multiple affected family members, with 5-6 meioses observed. ^{d,f}	Strength
SUPPORTING CRITERIA		
PP1	Co-segregation with AT in multiple affected family members, with 3-4 meioses observed. ^{d,f}	ATM-specific
PP2	Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease.	N/A ^h
PP3	-Probability of splicing alteration of the closest natural site predicted with SPiCE 2.1 is ≥ 0.240 for donor sites or ≥ 0.789 for acceptor sites, OR a splicing site is created/activated according to at least 2 splicing predictors of the set SpliceSiteFinderlike-MaxEntScan-NNSplice, with a score higher than the score of the natural site in the mutated allele. ⁱ OR -Only for missense variants, when the above splicing predictors indicate no impact, but protein predictors do. For variants affecting codons 1-1959, PP3 is met when VEST4 and REVEL predict damaging effects (scores >0.5). For variants affecting codons 1960-3056, PP3 is met when PROVEAN (score <-2.5) and REVEL (score >0.5) predict damaging effects. ^j	ATM-specific
PP4	Patient's phenotype or family history is highly specific for a disease with a single genetic etiology.	N/A (use PS4 instead) ^d
PP5	Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation.	N/A ^k
PS1_Supporting	Different variant at same nucleotide position as a pathogenic SPLICING variant, where <i>in silico</i> models predict impact equal to or greater than the known pathogenic variant.	ATM-specific
PS2_Supporting or PM6_Supporting	AT patients with <i>de novo</i> score 0.5-0.75 as per ClinGen SVI Recommendation for <i>de novo</i> Criteria (PS2 & PM6) - Version 1.0. ^c	ATM-specific
PS3_Supporting	- SPLICING analysis: · with NMD inhibition in carrier RNA shows by visual inspection that the altered and wild-type electrophoretic bands have similar intensity, and the altered transcript is predicted to undergo NMD; OR · found in peer-reviewed article(s), without gel shown or quantitation mentioned, where authors declare that the variant produces a splicing alteration predicted to undergo NMD. See text and Table 2 for details. OR -One of the following PROTEIN studies in AT patients or transfected cells shows a strong alteration and the other two show an intermediate alteration or have not been performed: · levels of ATM protein (or ATM phosphorylated in Ser1981) · levels of phosphorylation of two ATM substrates · sensitivity to DNA damaging agents. See text and Fig. 1 for details.	Strength; ATM-specific
PS4_Supporting	One AT proband. ^d It can only be applied to AT families and NOT in: breast cancer families, breast cancer case-control studies, variants that meet BA1 or BS1, nor together with PM3 at any strength.	ATM-specific
PM1_supporting	Missense or small in-frame deletion or insertion located in the kinase (residues 2712-2962) or FATC (residues 3024-3056) functional domains (NP_000042.3; see results section).	ATM-specific
PM3_Supporting	AT probands with <i>in trans</i> score 0.5-0.75 as per ClinGen SVI Recommendation for <i>in trans</i> Criterion (PM3) - Version 1.0. ^e It cannot be applied to variants that meet BA1 or BS1, nor together with PS4 at any strength.	None
<i>Continued</i>		

Table 1. (continued)		
PATHOGENIC CRITERIA		
Criteria	Criteria description	Specification
BENIGN CRITERIA		
Criteria	Criteria description	Specification
STAND-ALONE CRITERION		
BA1	99% confidence interval of the variant allele frequency in any of the NFE, AFR, LAT, EAS, SAS GnomAD v2.1 (non-cancer) populations is > 0.5%. ^l	ATM-specific
STRONG CRITERIA		
BS1	99% confidence interval of the variant allele frequency in any of the NFE, AFR, LAT, EAS, SAS GnomAD v2.1 (non-cancer) populations is > 0.05%. ^l	ATM-specific
BS2	Observed in the homozygous state in a healthy or AT-unaffected individual. One observation if homozygous status confirmed; two if not confirmed. Note that if BS1 is applied, BS2 must be downgraded to BS2_Supporting. ^f	ATM-specific
BS3	- SPLICING analysis in carrier RNA demonstrate (by Sanger sequencing or a quantitative technique) biallelic expression of the full-length transcript by an exonic SNV. See text and Table 2 for details. OR -In a variant not predicted or proven to alter RNA splicing, the three following PROTEIN studies in AT patients or transfected cells show results similar to a wild-type control: · levels of ATM protein phosphorylated in Ser1981 · levels of phosphorylation of 2ATM substrates · sensitivity to DNA damaging agents. See text and Fig. 1 for details.	ATM-specific
BS4	Lack of segregation in affected members of 2 or more AT families. ^f	None
SUPPORTING CRITERIA		
BP1	Missense variant in a gene for which primarily truncating variants are known to cause disease.	N/A ^h
BP2	Co-occurrence in trans of the variant with a pathogenic or likely pathogenic ATM variant in well phenotyped AT-unaffected individual from internal cohort or the literature.	ATM-specific
BP3	In-frame deletions/insertions in a repetitive region without a known function	N/A ^m
BP4	-For synonymous and intronic variants, probability of splicing alteration of the closest natural site predicted with Splice 2.1 is < 0.240 for donor sites or < 0.282 for acceptor sites, AND no splicing site is created/activated according to at least 2 splicing predictors of the set SpliceSiteFinderlike-MaxEntScan-NNSplice (if a site is recognized, the score is lower than the score of the natural site in the variant allele). ⁱ -For coding non-synonymous variants, NEITHER splicing predictors as above NOR protein predictors predict any impact. The latter is established for variants affecting codons 1–1959 when both VEST4 and REVEL (scores <0.5) predict NO alteration, and for variants affecting codons 1960–3056 when both PROVEAN (score >-2.5) and REVEL (score <0.5) predict NO alteration. ^j	ATM-specific
BP5	Variant found in a case with an alternate molecular basis for disease.	N/A ⁿ
BP6	Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation.	N/A ^k
BP7	Synonymous variant where nucleotide is not highly conserved (100 vertebrates basewise conservation PhyloP score < 6.66, available at the UCSC Browser). ^o This evidence can be used with BP4, as appropriate, to classify variants meeting both criteria as likely benign.	ATM-specific
<i>Continued</i>		

Table 1. (continued)		
PATHOGENIC CRITERIA		
Criteria	Criteria description	Specification
BS2_Supporting	Two homozygous observations with no clinical data provided, or meets criteria for BS2 but BS1 is also applied. ^f Observations without clinical data provided can be retrieved from the GnomAD non-neuro dataset.	<i>ATM</i> -specific
BS3_Supporting	- SPLICING analysis in carrier RNA with NMD inhibition and proper controls, shows only the wild-type transcript although do not demonstrate biallelic expression by an exonic SNV. See text and Table 2 for details. OR - In a variant not predicted or proven to alter RNA splicing, two of the following PROTEIN studies in AT patients or transfected cells show results similar to a wild-type control and the other one shows an intermediate alteration or has not been performed: · levels of ATM protein phosphorylated in Ser1981 · levels of phosphorylation of two ATM substrates · sensitivity to DNA damaging agents. See text and Fig. 1 for details.	<i>ATM</i> -specific
BS4_Supporting	Lack of segregation in affected members of one AT family. ^f	<i>ATM</i> -specific

NMD, nonsense-mediated decay; AT, ataxia-telangiectasia; **N/A, Not applicable to *ATM*.**
^aFollowing Tayoun et al., decision tree (19, 20).
^bInitiation codon variants have been shown to cause (classic or atypical) AT and absence of ATM kinase (21). Expression studies performed in these patients show a shorter underexpressed protein probably starting at the next in-frame methionine at codon 94 (21).
^cPoint-based system to determine the strength of de novo evidence based upon confirmed versus assumed status, phenotypic consistency and number of de novo observations, available at <https://clinicalgenome.org/working-groups/sequence-variant-interpretation/> (20).
^dApplied analogously to *CDH1* ClinGen Specifications (3, 20).
^ePoint-based system to determine the strength of homozygous and *in trans* observations based upon variant phasing and classification of the variant occurring on the other allele, available at <https://clinicalgenome.org/working-groups/sequence-variant-interpretation/>.
^fApplied analogously to *PTEN* ClinGen Specifications (2, 20).
^gIf the other missense change is determined to be likely pathogenic, the variant being classified should not reach pathogenic classification.
^hBoth missense and frameshift variants contribute with comparable frequency to *ATM*-related diseases.
ⁱSplicing predictor assessment is detailed in the text. SPiCE 2.1 predictions can be found at <https://sourceforge.net/projects/spicev2-1/#>.
^jProtein predictor assessment is detailed in the text. VEST4 predictions can be found at <http://cravat.us/CRAVAT/>, REVEL predictions at <https://sites.google.com/site/jpoggen/dbNSFP> and PROVEAN predictions at http://provean.jcvi.org/genome_submit_2.php?species=human.
^kFollowing Biesecker et al., recommendations (20, 22).
^lThe 99% confidence intervals can be calculated in the INVERSE AF tab of the website <http://cardiodb.org/allelefrequencyapp/>; see Materials and methods and Results sections for details on cut-offs.
^mA repetitive region without a known function has not been found in *ATM*.
ⁿThe frequency of pathogenic variants in *ATM* and other breast cancer predisposing genes is high enough to allow such combinations and a lethal or strikingly stronger phenotype is not anticipated.
^oThe 100 vertebrates basewise conservation PhyloP score can be seen as a graphic track at the UCSC Genome Browser (<https://genome.ucsc.edu/>), and the scores can be downloaded for each position.

PS3_Moderate when 2 are altered and 1 has not been performed or gives intermediate results, and PS3_Supporting when 1 is altered and the other 2 have not been performed or give intermediate results, as depicted in Fig. 1, B.

In cases where experimental data from RNA and protein support the same damaging effect, the evidence of higher strength will be used. When RNA data do not support an effect in splicing, the protein data prevail to reflect other defects in protein function.

Benign criterion BS3 has a similar approach (Tables 1 and 2). If assay(s) in carrier RNA demonstrate biallelic expression of the variant or an exonic single

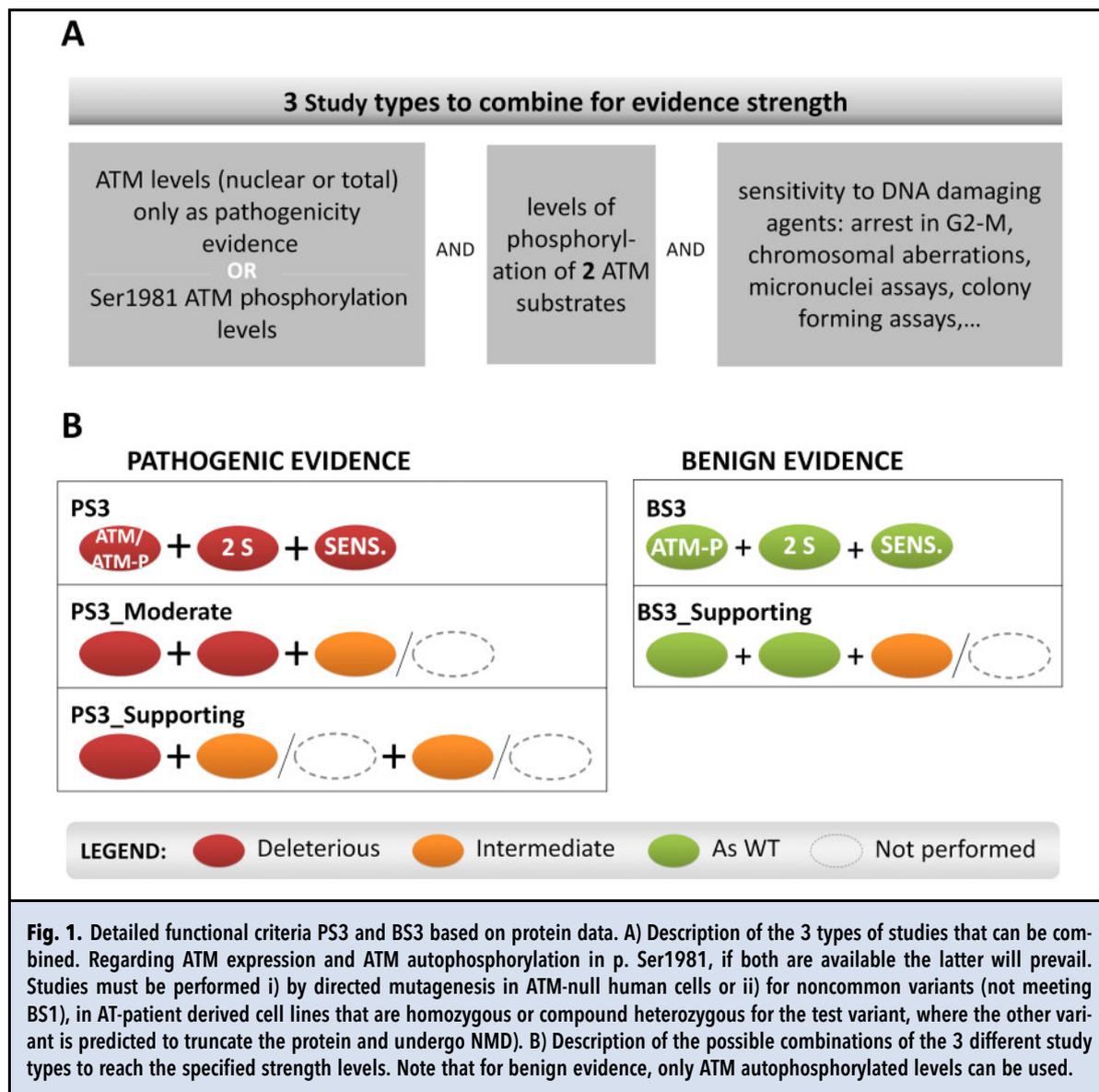
nucleotide variation (SNV), quantitatively or with similar peak height by Sanger sequencing, BS3 will be met. BS3_Supporting is achieved when no additional band to wild-type is detected in electrophoresis of carrier RNA, although biallelic expression cannot be demonstrated by an exonic variant. BS3 can be achieved by protein assays when the 3 assays yield the same results as the wild-type control (Fig. 1, B). BS3_Supporting is met when 2 assays give the same results as wild-type and the other one gives intermediate results or has not been performed.

We have found germline deleterious missense variants in AT patients, located throughout the ATM

Table 2. Detailed criteria for functional evidences PS3 and BS3 based on RNA splicing studies.

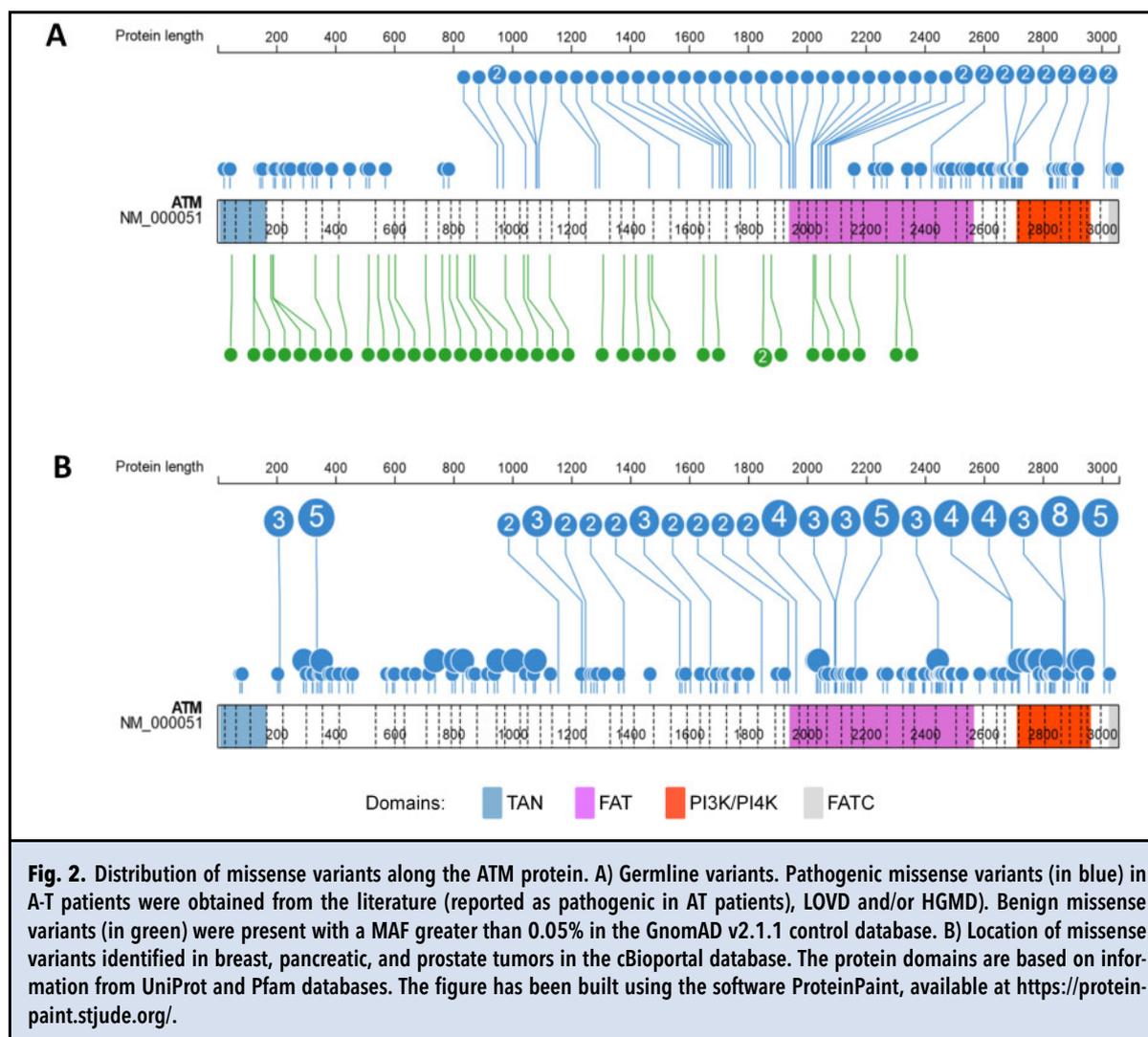
criteria	Assay	NMD	Protein effect / experimental evidence	Transcript effect	Qualitative / quantitative method
PS3_Very Strong	carrier RNA	NMD predicted		variant allele does not produce full-length transcript	quantitative allele specific expression
PS3	carrier RNA	frame-shift or in frame; NMD not predicted	truncates OR alters critical region OR removes > 10% protein	variant allele does not produce full-length transcript	quantitative allele specific expression
PS3_Moderate^a	carrier RNA minigene carrier RNA carrier RNA	frame-shift or in frame; NMD not predicted NMD predicted NMD predicted NMD predicted	removes <10% protein (no critical regions) variant band with similar intensity as WT	variant allele does not produce full-length transcript variant allele does not produce full-length transcript NO evidence that the variant allele produces or not full-length transcript variant allele produces 1%-10% full-length transcript	quantitative allele specific expression quantitative expression semi-quantitative transcript specific expression quantitative allele specific expression
PS3_Supporting^a	carrier RNA carrier RNA minigene	NMD predicted NMD predicted NMD predicted	variant band with similar intensity as WT no electrophoresis result or quantitation shown in peer-reviewed article	NO evidence that the variant allele produces or not full-length transcript	visual inspection (gel + Sanger seq)
BS3	carrier RNA	NMD predicted	no additional band is seen / similar peaks by Sanger seq	variant allele does not produce full-length transcript	visual inspection (gel + Sanger seq)
BS3_Supporting	carrier RNA		no additional band is seen	biallelic expression is demonstrated by an exonic SNV	quantitative / visual inspection
				biallelic expression is NOT demonstrated by an exonic SNV	visual inspection

^aThis criterion is met when any of the options represented by the corresponding rows are true.



protein and a similar distribution was observed in breast, pancreatic, and prostate tumors (Fig. 2, A and B). In contrast, missense variants with a MAF \geq 0.05% (gnomAD 2.1 controls) are distributed throughout the ATM regions except for phosphoinositide 3-kinase (PI3K) and FRAP, ATM, TRRAP C-terminal (FATC) domains (Fig. 2, A). The absence of germline frequent variants in PI3K and FATC C-terminal domains suggests their critical role. For this, we propose applying PM1_supporting to variants located in these specific domains. We have also found some candidate codons for a PM1 hotspot, according to the ClinGen Germline/Somatic Variant Curation Subcommittee (30). Two *ATM* codons accumulate >10 somatic

missense occurrences in cancerhotspots.org (v2) (Supplemental Fig. 3). Codon 337 has 31 observations of p.(Arg337His) and 9 of p.(Arg337Cys); codon 3008 has 15 observations, distributed between p.(Arg3008Cys), p.(Arg3008His), and p.(Arg3008Leu). In GnomAD v2.1.1 (noncancer) variants c.1009C>T p.(Arg337Cys) and c.1010G>A p.(Arg337His) have 26 and 20 counts, respectively, whereas only variants c.9022C>T p.(Arg3008Cys) and c.9023G>A p.(Arg3008His) have been detected, with 3 and 2 counts, respectively. For this reason, we only consider codon 3008 as a hotspot. In addition, we propose applying PM1 to codon p. Ser1981, since autophosphorylation of this residue has been found to be



required for sustained retention of ATM at DSBs. Furthermore, its directed mutagenesis affects the ability of ATM to phosphorylate its downstream targets after DNA damage and correct the radiosensitivity of an AT cell line (31).

DE NOVO, ALLELIC AND SEGREGATION EVIDENCE

De novo criteria (PS2, PM6) and allelic evidence PM3 are applied following the ClinGen SVI recommendations (32, 33); segregation criteria (PP1, BS4) are formulated as in published guidelines (2) only for AT families, whereas the benign allelic evidence BP2 has been simplified (Table 1).

PILOT CLASSIFICATION OF 50 ATM VARIANTS

We performed a pilot classification of 50 ATM variants from our database which were selected to represent the variant type proportions of the whole set. The evidence

assigned to each variant, the data and publications on which they are based and the resulting pathogenicity classes are displayed in Table 3 and Supplemental Table 3. All this information together with the clinical information (Supplemental Table 4) will be submitted to ClinVar database (34) to be made publicly available to the whole community.

The pilot reclassification of 50 variants with the adapted criteria allowed us to reassign 18 cases from VUS to a more clinically meaningful class; of the remaining cases, 4 were moved to class 3 and 28 were left unchanged (Table 3, Supplemental Fig. 1). Of note, establishing ATM-adjusted cut-offs for BA1 and BS1 favored the classification of several recurrent variants as class 1 or 2. The BS2-supporting criterion, applied to variants with at least 2 appearances in the GnomAD non-neuro dataset in the homozygous state, supported by the high penetrance and young age of onset observed

Table 3. Result of the 50-variant pilot classification.

cDNA name	Protein name	Nr carriers	Initial Submitted Classification	Consensus classification	Evidence combination ^a
c.61A>G	p.(Thr21Ala)	1	3	3	PM2 + BP4
c.162T>C	p.(Tyr54=)	9	2, 3	2	BS1 + BP4 + BP7
c.496 + 4T>C	p.?	3	3	2	BP4 + BS3_P
c.609C>T	p.(Asp203=)	14	2, 3	2	BS1 + BP4 + BP7
c.826A>G	p.(Lys276Glu)	1	3	3	PM2
c.998C>T	p.(Ser333Phe)	60	2, 3	2	BS1 + BS2_P
c.1380G>C	p.(Thr460=)	2	2, 3	2	BS1 + BP4 + BP7
c.1463G>A	p.(Trp488*)	1	5	5	PVS1 + PM2 + PM3 + PS3_P
c.1564_1565del	p.(Glu522Ilefs*43)	2	5	5	PVS1 + PM3_VS
c.1810C>T	p.(Pro604Ser)	48	2, 3	1	BA1 (+ BS1 + BS2_P)
c.1899T>G	p.(Cys633Trp)	1	3	3	PM2
c.2012T>A	p.(Ile671Lys)	1	3	3	BP4
c.2250G>A	p.(Lys750=)	1	4	5	PP3 + PS3_M + PM3_VS + BP7
c.2362A>C	p.(Ser788Arg)	1	2	1	BA1 (+ BS1 + BS2_P)
c.2386A>C	p.(Asn796His)	1	3	3	PM2 + BP4
c.2839-2A>G	p.?	1	4	4	PVS1 + PM2
c.2921 + 1G>A	p.?	2	4, 5	5	PVS1 + PM3_VS + PS3_P
c.2921 + 1G>T	p.?	1	5	5	PVS1 + PM2 + PS1_P
c.3747-1G>C	p.?	2	4, 5	5	PVS1 + PS4_P + PM2 + PS3_M
c.3802del	p.(Val1268*)	3	4, 5	5	PVS1 + PM3_VS + PS3
c.4060C>A	p.(Pro1354Thr)	2	3	3	BP4
c.4110-9C>G	p.?	1	3	3	PS3_P + PM3_P + PP3 + PM2
c.4396C>G	p.(Arg1466Gly)	4	3	3	PM2 + PP3
c.4802G>A	p.(Ser1601Asn)	2	2, 3	3	BP4
c.4852C>T	p.(Arg1618*)	1	4	5	PVS1 + PS4_M + PM2
c.5071A>C	p.(Ser1691Arg)	9	2, 3	1	BS1 + BS2_P + BS3
c.5373T>C	p.(Asp1791=)	1	2	3	PM2 + BP4 + BP7
c.5558A>T	p.(Asp1853Val)	32	2, 3	1	BA1 (+ BS1 + BS2_P + PP3)
c.5623C>T	p.(Arg1875*)	2	5	5	PVS1 + PM3_S + PS3_M
c.6067G>A	p.(Gly2023Arg)	19	2, 3	3	BS1 + PP3
c.6115G>A	p.(Glu2039Lys)	1	3	3	PS4_P + PM2 + PP3
c.6203T>C	p.(Leu2068Ser)	1	3	4	PM2 + PS4_M + PS3_M + PP3
c.6315G>C	p.(Arg2105Ser)	1	3	3	PP3
c.6679C>T	p.(Arg2227Cys)	1	4	5	PM2 + PS4 + PP3 + PS3_M + PP1
c.6848C>T	p.(Ser2283Leu)	2	3	3	PM2 + BP4
c.6860G>C	p.(Gly2287Ala)	1	3	3	BP4
c.7135C>G	p.(Leu2379Val)	1	4	3	PS3_M + PP3
c.7191A>G	p.(Gln2397=)	2	3	2	BP4 + BP7
c.7375C>G	p.(Arg2459Gly)	10	3	3	PP3
c.7381C>T	p.(Arg2461Cys)	1	3	3	PP3

Continued

Table 3. (continued)

cDNA name	Protein name	Nr carriers	Initial Submitted Classification	Consensus classification	Evidence combination ^a
c.7390T>C	p.(Cys2464Arg)	1	3	3	BS1 + PP3
c.7788 + 3A>G	p.?	1	4	4	PM2 + PM3 + PP3 + PS3_P
c.8122G>A	p.(Asp2708Asn)	1	4	4	PM2 + PP3 + PS3_M + PM3_S
c.8269-5T>G	p.?	1	3	3	PM2
c.8734A>G	p.(Arg2912Gly)	4	3	3	PP3 + PM1_P
c.8786 + 1G>T	p.?	1	5	5	PVS1 + PS3_M + PM2
c.8876_8879del	p.(Asp2959Glyfs*3)	2	5	5	PVS1 + PS3_P + PM3
c.9007_9034del	p.(Asn3003Aspfs*6)	2	4	5	PVS1_S + PS3 + PM2 + PM3
c.9023G>A	p.(Arg3008His)	1	4	4	PM1 + PM2 + PS4_M
c.9079dup	p.(Ser3027Lysfs*36)	1	4	4	PVS1_S + PM2 + PS4_M

See evidence details in [Supplemental Table 3](#).
^acode for evidence strength modifications: VS, Very Strong; S, Strong; M, Moderate; P, Supporting.

in AT patients, allowed the classification of 5 variants as likely benign by its combination with BS1, without any other evidence needed. Similarly, our DB recorded the appearance of 3 variants in homozygosis in well-phenotyped individuals not presenting AT. This information allowed us to classify variant c.998C>T as likely benign; this variant was present in 60 patients in our DB but did not reach BS2_P requirements with GnomAD data.

Nineteen out of 50 variants of the pilot study were classified as class 4 or 5 being present in 27 patients from our Spanish cohort. Most of these patients had breast/ovarian cancer although there were cases of other tumors ([Supplemental Table 5](#)). Cosegregation data was available in a few of these families and, as expected for a moderate penetrance cancer risk gene, was not very informative ([Supplemental Table 5](#)).

Discussion

Variant classification is one of the main clinical challenges in the MPS era, being an enormous bottle neck in most genetic testing laboratories. In this article, we present the seed for a Spanish database of hereditary cancer variants, beginning with 6 laboratories and 1 gene, *ATM*. We identified *ATM* as a good candidate since it is one of the genes with more identified VUS (18) and it has been associated with different cancer syndromes (6–8, 10), making it well worth the joint effort to refine variant classification. Since there were no specific criteria for *ATM* variant classification, we also made an effort to adapt the ACMG/AMP guidelines (1) to *ATM*.

In our pilot classification study, the use of *ATM*-specific guidelines and data sharing amongst experts and

clinical laboratories led to a decrease in VUS from 58% to 42%, with the identification of 27 carriers of *ATM* (likely) pathogenic variants. Because pathogenic *ATM* variants predispose to potentially lethal cancers for which there are clinical management recommendations (35), these findings are clearly clinically actionable for carrier individuals and their relatives.

Since the ACMG/AMP classification guidelines were proposed for high-penetrance genes in classical Mendelian disorders (1), their adaptation to moderate/low-penetrance genes, such as *ATM*, is challenging and requires collaborative efforts. In this respect, we analyzed every ACMG/AMP classification criterion in the context of reported knowledge about the *ATM* gene, *ATM* protein function and *ATM* related phenotypes, with the aim of better adjusting each criterion and eventually facilitating variant classification in routine clinical laboratories. In this process, we took advantage of the fact that biallelic *ATM* variant carriers present the highly penetrant AT disease, allowing the use of criteria for recessive phenotypes. Our adjusted cut-offs for population variant frequency enabled the classification of a large quantity of recurrent variants as (likely) benign that would have been classified as VUS with the original general ACMG/AMP thresholds (1). In this sense, although our DB only contains variants below the common population frequency cut-off of 1%, 39 out of 283 unique variants meet the adjusted BS1 and 12 of these also meet the adjusted BA1. The 39 BS1 variants account for 51% of the individual entries in (448 out of 882, data not shown).

We found it especially challenging to establish functional evidence for or against pathogenicity. At the protein level, the selected assays were based on relevant functional characteristics of *ATM* that are altered in AT patients and are involved in oncogenic mechanisms, such as double-

strand break signaling (presence and activation of *ATM*, phosphorylation of its substrates), and mitosis checkpoints and chromosomal stability. We set the splicing-related criteria at the RNA level on the basis of previous ENIGMA and InSiGHT recommendations and the authors' own experience. Additional considerations will be required if *ATM* naturally occurring in-frame transcripts are described that would rescue the variant allele effect. Functional evidence has helped us to classify 18 out of 50 variants. Luckily, splicing data from 5 of the 8 variants came from our own laboratories. Unfortunately, protein functional studies are not available in our teams. In this sense, the development of calibrated high-throughput *ATM* functional assays, similar to the saturation genome editing study published for *BRCA1* (36) will provide more power to PS3 and BS3 criteria.

The presence of a rare variant in AT families or *in trans* with a (likely) pathogenic variant in an AT patient has allowed us to classify 12 of 50 variants, while co-segregation AT data has turned out to be scarce in the literature. Conversely, *ATM* variant classification is most frequently requested for breast cancer risk assessment, but the great heterogeneity and numerous phenocopies of breast cancer impairs its use in co-segregation or family counting evidence. Large case-control studies by international hereditary cancer consortia like BRIDGES (37) in Europe and CARRIERS (38) in US will hopefully help to classify some of these variants.

An underlying assumption of this and other studies in the field is that the very same spectrum of *ATM* variants causing autosomal recessive AT disease when present in both alleles, cause increased BC risk when present in one allele. Overall, the assumption is probably true, and as far as we know, it holds true for premature termination codon variants expected to cause NMD. Nevertheless, some evidence suggests that subtle differences might also exist. For instance, variant p.(Val2424Gly) is associated with a 6-fold increased BC risk, much higher than average truncating variants. Conversely, the same variant does not cause classical AT, but an attenuated form (11, 39). Another study suggested that the risk of malignancies is higher in individuals with mild *ATM* missense variants producing proteins with residual kinase activity (40).

In summary, by pooling variant information currently stored in individual clinical laboratories, we have developed a general framework for homogeneous and clinically useful variant interpretation in our country. It will also serve for the identification of Spanish founder/recurrent variants and analysis of their associated cancer risk. Moreover, it will facilitate sharing of curated data to international databases. In recent years, similar initiatives focused on the generation of clinical-grade genetic variant databases have been conducted in other countries (41–44). In our case, we have started by adjusting

general ACMG/AMP guidelines to a single gene, *ATM*, with the aim of using them within the framework of molecular diagnostics for HC. In our joint effort we performed a pilot study and classified 50 *ATM* variants carried by 257 index cases. Our results highlight the relevance of data sharing and data curation by multidisciplinary experts to achieve improved variant classification that will eventually improve clinical management.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: HC, hereditary cancer; MPS, massive parallel sequencing; VUS, variants of uncertain clinical significance; ACMG, American College of Medical Genetics and Genomics; AMP, Association for Molecular Pathology; BC, breast cancer; AT, ataxia telangiectasia; PIKK, phosphatidylinositol-3' kinase-related protein kinase; DSB, double-strand break; MRN, Mre11-Rad50-NBS1; DB, Spanish Hereditary Cancer Variant Database; LB, likely benign; LP, likely pathogenic; P, pathogenic; B, benign; NMD, nonsense mediated decay; SNV, single nucleotide variation; PI3K, phosphoinositide 3-kinase; FATC, FRAP, *ATM*, TRRAP C-terminal (domain)

Human Genes: *ATM*, *ATM* serine/threonine kinase; *BRCA1*, *BRCA1* DNA repair associated; *BRCA2*, *BRCA2* DNA repair associated; *PTEN*, phosphatase and tensin homolog; *CDH1*, cadherin 1; *TP53*, tumor protein p53

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