Approach to the patient with a variant of uncertain significance on genetic testing

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Clinical Question

Title: Approach to the patient with a variant of uncertain significance on genetic testing

Short Title: Approach to the patient with a VUS

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Summary

Establishing a genetic diagnosis may lead to major health benefits for the patient and their wider family but is dependent on the accurate interpretation of test results. The processes of variant interpretation are by their nature imprecise such that the

potential for uncertain test results (i.e. variant(s) of uncertain significance (VUS)) are an inevitable consequence of genomic testing. With an increased responsibility for diagnostic testing in the hands of the specialty physician (e.g. endocrinologist) rather than clinical geneticist, it is essential that they are familiar with the possible outcomes of testing including an understanding of the VUS category. Whilst uncertainty is endemic to many aspects of clinical medicine, receiving a VUS result may pose a considerable challenge to both the clinician and patient. In this article, a framework to support decision-making when confronted with a VUS variant is provided, focusing on the key components of the genetic testing pathway. This highlights the importance of assessing the VUS result in the context of the clinical presentation and genetic testing strategy, the value of multidisciplinary team working, and ensuring good communication with the patient.

Introduction

Diagnostic genetic testing is an integral part of the clinical work up of a wide spectrum of endocrine disorders and may result in major health benefits for the patient and their wider family.[1] A rapidly expanding portfolio of genetic tests are increasingly accessed directly by the endocrinologist who requires sufficient knowledge to deploy such testing safely.[2] The utility of genetic testing is not only dependent on ensuring that the right patients are selected for the right tests, but also on obtaining accurate test results. Thus, it is important to have an awareness of the potential limitations of variant interpretation and to integrate any test result into the overall clinical assessment of the patient (i.e. a genetic test result does not equate to a clinical diagnosis). It is also important to acknowledge that variant interpretation is not absolute and static but is based on imprecise methods and dependent on the evidence available at the point of assessment, which may change over time. Indeed, these imprecise methods may result in variant misclassification, which can result in significant harm.[3] For example, incorrectly ascribing pathogenic status to a benign variant may not only lead to an incorrect diagnosis and inappropriate treatment for

the patient, but impact family members (e.g. erroneously identifying relatives at risk of disease (i.e. variant carriers)), as well as wider society (i.e. if the misclassified variant is reported in a public database).[3] Thus, it can be argued that it is better to be 'uncertain' than it is to be wrong, with the VUS category offering a suitable 'holding' area for variants failing to reach the appropriate thresholds for benign or pathogenic classifications.[3] However, understanding how best to manage the patient with a VUS requires an appreciation of several components of the genetic testing workflow.

Variants of Uncertain Significance: Uncertainty is Certain

With the exception of chromosome analysis for Turner and Klinefelter syndrome, genetic testing in the endocrine clinic is predominantly performed to diagnose monogenic endocrine disorders (e.g. endocrine tumour syndromes, hereditary phaeochromocytoma/paraganglioma.[1] These monogenic disorders typically result from small alterations in DNA sequence (i.e. single nucleotide variants (SNVs) or small insertions or deletions (i.e. 'indels')) within the coding region (or splice sites) of the respective gene, but may also arise from larger genetic abnormalities (i.e. 'structural variants' (SVs)) that disrupt gene function (e.g. whole or partial gene deletions). Diagnostic genetic testing (most frequently with DNA sequencing) aims to detect these disease-associated variants and is dependent on an ability to differentiate such genuine 'pathogenic' variants from 'neutral' or benign variants.[4-7] However, this is frequently a major challenge, not least due to the great diversity of genetic variation present at both an individual and population level.[6,8-10] For example, any given individual is estimated to harbour ~3-4 million variants amongst their ~3 billion nucleotides, of which ~25,000 occur within protein-coding regions, including many in monogenic disease genes, of which at least a proportion will be rare and of uncertain significance.[7] Furthermore, in the current GnomAD cohort (representing ~140,000 individuals), each participant has an average of 27 novel coding variants not shared with any other individual,[8,9] and of the total ~6.5 million different missense variants (i.e. variants resulting in a single non-synonymous amino acid substitution)

identified in the cohort, the functional consequence of the overwhelming majority are unknown, even within well-characterised monogenic disease genes.[8,11] The difficulty distinguishing disease-causing variants amongst the 'sea' of rare background variation is further highlighted by the burden of VUSs in the ClinVar database (~45% of ~1 million variants),[5] with the majority represented by rare missense SNVs. Indeed, the interpretation of rare missense variants poses a major challenge for several monogenic endocrine disease genes (i.e. *CASR, MEN1, SDHB, VHL*) accounting for ~50-85% of VUSs in each of the respective genes.[1,12]

VUSs and the Genetic Testing Workflow

Pre-Test Considerations

Clinical Phenotype & Family History

Paradoxically, the potential for uncertain test results increases well before genetic testing is undertaken, if the clinical information provided is insufficient, incomplete or inaccurate. Not only may incomplete clinical assessment result in inappropriate genetic testing it may also influence variant interpretation by preventing the appropriate application of criteria based on the patient's phenotype and family history. Thus, detailed phenotype data should be provided (e.g. clinical, biochemical, radiological, pathological), alongside as detailed a family history as feasible (i.e. ideally at least three-generations).[2] Such information not only helps establish a 'pre-test' probability of identifying a given disorder (and most appropriate testing strategy), it provides important context when receiving test results.

Genetic/Genomic Testing Strategy

The likelihood of identifying variants in the VUS category is dependent on the genetic testing strategy employed. As the content of the test increases, the likelihood of identifying rare 'background' variants increases. Thus, the use of large disease-targeted gene panels, whilst providing a cost-effective and efficient approach to diagnostic testing, increase the likelihood of identifying VUSs compared with single/low number gene tests (Figure 1). Although rarely adopted for adult endocrine

phenotypes, whole genome sequencing will inevitably reveal high numbers of VUSs if the analysis is not restricted to specific groups of genes. Given the wide-adoption of disease-targeted gene panels, there should be an 'expectation' to observe VUS results, with the frequency influenced by the size and content of the panel. For example, recent studies indicate a VUS rate of 10-15% when undertaking multi-gene testing for phaeochromocytoma/paraganglioma,[12,13] and similarly high frequencies should be anticipated for other large gene panels (e.g. those used for hypogonadotrophic hypogonadism or monogenic obesity). Pre-test discussions with the patient should highlight the potential for such results.

Variant interpretation

Variants identified during genetic/genomic testing (i.e. SNVs, indels, SVs) require assessment to determine if they are likely to be disease-causing. This process requires an understanding of the disease phenotype, family history, mode of inheritance, mechanism of disease (e.g. loss or gain of gene function), variant allele frequencies, and insight into the gene-protein 'structure- function'. Most testing laboratories employ approaches based on American College of Medical Genetics & Genomics (ACMG) guidelines,[14,15] although there may be variation in how these are applied (e.g. Association of Clinical Genomic Science (ACGS) guidelines in the United Kingdom).[16] The ACMG guidelines provide a framework to evaluate multiple domains of evidence (i.e. supporting pathogenic or benign interpretations), which are graded and combined in a scoring system to facilitate classification into one of 5 groups; pathogenic (P), likely pathogenic (LP), VUS, likely benign (LB), or benign (B).[16,17] The VUS category is used when the thresholds for LP/P or LB/B classifications are not met or in specific settings where apparently contradictory evidence is observed.

Although the clinician does not require a detailed knowledge of the variant interpretation pipeline, an appreciation of the limitations of the methodology provides insight into the circumstances in which uncertain test results may arise. Notably,

variant interpretation is dependent on the information available at the time of assessment, which may be inaccurate, absent or lack specificity. For example, evidence supporting pathogenicity may be derived from information in existing literature or databases which may be inaccurate; functional assays *or in silico* predictive tools that may lack sensitivity and specificity; family segregation data which may be unavailable or unreliable (e.g. identification of 'affected' and 'unaffected' individuals confounded by reduced penetrance); and population-level genetic data, which may not reflect the population under study (e.g. reduced reliability for under-represented groups).[8]

In the ideal setting, variant interpretation would be sufficiently precise to allow accurate classification without the need to account for the patient's phenotype. However, frequently this is not feasible, and achieving the 'best' interpretation relies on integrating the patient phenotype with the genetic test result, aided by multidisciplinary working (i.e. between endocrinology and genetics teams).[16] Although ACMG guidelines limit the strength of support for variant pathogenicity based on the patient's phenotype, current UK-based ACGS guidelines allow strengthening of such evidence in specific settings, which may help facilitate a 'clinical-genetic' diagnosis.[16] Indeed, modifications to existing ACMG guidelines, incorporating more clinical information, have been proposed for several conditions, including MEN1.[18]

The VUS category and spectrum of risk - not all VUSs are equal

Although the VUS category is an artificial construct (variants are either associated with disease or not), it provides a repository for variants for which there is insufficient evidence to support a definitive interpretation.[16,19] Indeed, the thresholds to achieve P/LP (and B/LB) status are strict (Figure 1), such that a VUS may arise in many different settings; for example, in one scenario all available evidence may indicate a pathogenic classification, yet it may be insufficient to reach the LP threshold; in another, a variant may have little (if any) evidence to support pathogenicity, but

cannot be classified as LB. Thus, the VUS category represents a broad continuum of 'risk' (Figure 1).[16] To address this, many genetic testing laboratories 'internally' stratify VUSs according to strengths and combinations of evidence. For example, current ACGS guidelines utilise a 6-point 'informal' grading system (spanning a temperature gradient from 'ice cold' to 'hot') (Figure 1).[16] This type of approach, underpinned by a Bayesian classification framework, allows a 'risk' value to be generated for individual variants and is increasingly employed by variant repositories (e.g. ClinGen, DECIPHER).[20]

Although there are different views on whether to report all VUS variants, current UK-based ACGS guidelines typically recommend only reporting VUSs where further genetic testing or clinical investigation could result in re-classification to LP/P (i.e. typically 'warm' and 'hot' VUSs) although in some instances it may be challenging to obtain sufficient additional evidence to facilitate reclassification to 'likely pathogenic' status (see Figure 1). In rare instances (and following multidisciplinary team discussion), the reporting of a VUS may still be considered appropriate even when re-classification is unlikely to be possible, if all existing evidence supports a causal association (Figures 1&2).[3,16]

Receiving a VUS Result: The Do's and Don'ts

A VUS is frequently perceived as the least 'helpful' outcome of genetic testing in directing the future management of the patient. However, it is important that the clinician neither 'ignores' the result, nor jumps to any immediate conclusions over its significance. Instead, the clinician should consider the result in the overall context of the clinical presentation and testing strategy (Figure 2).[19,21] For example, the finding of a VUS in a patient with a highly specific phenotype following single gene testing has a different context to that of a variant in a patient investigated with a large gene panel for a condition with marked genetic heterogeneity (Figure 1). The genetic test report should provide clear information on the evidence supporting the current interpretation and the further actions that could facilitate a more definitive

classification (Figure 2). This may involve genetic testing of family members (e.g. co-segregation analysis of affected family members, testing of parental samples to ascertain a *de novo* variant), the acquisition of additional clinical data (e.g. analysis of tumour samples for loss of heterozygosity (LOH) or immunohistochemical assessment), and/or further laboratory based methods to assess the functional impact of the variant (e.g. use of validated *in vitro* assays, mRNA assessment for potential splice variants) (Figure 2). Multidisciplinary discussion may not only guide this further investigation but can identify national/international experts who may have access to additional resources to facilitate a revised variant classification. Discussion with the clinical genetics team may also highlight additional genetic testing strategies that may be appropriate if other possible causes of the phenotype remain.

If further evaluation of the VUS is not feasible or does not facilitate a revised classification, (i.e. VUS status persists) additional multidisciplinary discussion may help guide future management of the patient. Whilst clinical decision-making should not be based on the finding of a VUS, a case-level assessment based on all available clinical and genetic information can establish the most appropriate management plan. In settings where a VUS is still considered of possible relevance to the clinical presentation, periodic review of the patient (e.g. to ascertain any new clinical information/family history), together with interval variant re-evaluation (e.g. assessment for new gene- or variant-level information) may be appropriate. In certain circumstances, depending on the clinical phenotype of the patient, periodic clinical assessment of family members may also be appropriate to the determine emergence of relevant clinical features.

Communication with the Patient

A VUS result has the potential to cause patient (and clinician) anxiety. For example, the finding of a VUS in a hereditary cancer gene (e.g. *MEN1*, *VHL*, *SDHB, RET*) may raise questions regarding the need for interval clinical surveillance and/or intervention, as well as concerns for family members. Therefore, it is important that

the clinician is able to provide appropriate counselling at each stage of the genetic testing workflow. For example, it is important to discuss the possibility of a VUS during the consent process (i.e. included in the pre-test 'Record of Discussion'), the likelihood of which is dependent on the genetic testing strategy (see above). Preparing the patient for such an outcome is likely to be beneficial, as there is evidence that both patients and research subjects have a good tolerance for genomic uncertainty if provided with appropriate information.[22,23] It is also important to outline to the patient what future steps can be taken to help resolve the uncertainty (e.g. additional testing of affected family members), and where resolution is not possible, to provide a clear plan for future care (i.e. establishing whether there is a need for interval clinical assessment and what action should be taken if new information becomes available that facilitates reclassification) (Figure 2). For example, it may be appropriate to recall the patient (or invite the patient to make re-contact) after an appropriate interval (e.g. 3-5 years) to undertake variant re-assessment. In addition, the clinical team should consider recording details of a next of kin in case the proband dies prior to variant re-classification.

Future Developments

The huge wealth of rare background genetic variation coupled with imprecise methods of variant interpretation ensure that the VUS category will continue to present a barrier to the successful implementation of clinical genetic testing. However, a number of initiatives are underway to address this challenge. For example, national/international variant repositories are in ongoing development (e.g. ClinGen, DECIPHER), which not only encourage data-sharing but provide expert-curated variant resources.[16,24-26] In parallel, ongoing large scale genomic sequencing projects continue to define the spectrum of rare genetic variation present across diverse populations (i.e. GnomAD v4 release anticipated to report on ~500,000 individuals).[8] Advances in high-throughput laboratory techniques are facilitating the functional evaluation of variants at a previously unfeasible scale (e.g. >4000 *BRCA1*

missense variants assessed by saturation gene-editing).[27] Likewise, improved high-throughput bioinformatic prediction tools, typically based on machine learning, are in continual development and report improved classification of missense SNVs (e.g. EVE, VARITY).[11,28] In parallel, recent structure-based prediction algorithms offer novel approaches to aid variant interpretation,[29] with resources such as the 'Alphafold' protein structure database offering new opportunities to predict the impact of variants in previously unresolved proteins.[30] Looking forward, variant interpretation processes will continue to be refined incorporating new information as it becomes available, and for many disorders disease-specific variant pathogenicity prediction methods have been developed, which can improve resolution of variants in the VUS category.[31,32] For example, recent studies of rare missense *SDHB* and *SDHD* variants in phaeochromocytoma/paraganglioma and control cohorts, illustrate statistical methods that can be used to strengthen variant pathogenicity scoring.[33] Alongside these advances. it is important to have robust policies facilitating periodic variant re-interpretation, and when reclassification occurs, to ensure appropriate dissemination (i.e. to reach the patient).[34-36]

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BOX 1

Clinical presentation and genetic testing: A patient was referred for *MEN1* genetic testing on the basis of multiple pancreatic neuroendocrine tumours at young age (<40-years). There was no family history relevant to MEN1 reported. Genetic testing revealed a 9-nucleotide deletion in exon 4 of the *MEN1* gene predicting an in-frame 3 amino acid deletion affecting a central region of Menin protein.

Variant classification: Initial classification of the MEN1 variant was a 'Hot' VUS on the following basis: absence of variant from existing population databases (PM2*); predicting a protein length change as a result of in-frame deletion in a non-repeat region of the protein (PM4*); and being associated with a phenotype specific for the condition (PP4_supporting*).

Options for resolving uncertainty: 1. Clinical evaluation of parents/first-degree relatives to determine if they manifest a relevant MEN1 phenotype (MEN1 has high penetrance such that almost all individuals with a pathogenic *MEN1* variant will manifest clinical/biochemical manifestations by age 50-years) 2. If parents do not have a relevant phenotype, determine whether the *MEN1* variant occurred *de novo.* 3. Evaluate relevant tumor samples for loss of heterozygosity (LOH) at the *MEN1* locus.

Resolution: Clinical assessment revealed a parent to have a phenotype consistent with MEN1 (previously unknown) who was subsequently shown to harbour the *MEN1* variant. This facilitated the evidence arising from the specificity of the clinical phenotype to be used at a moderate level of evidence (PP4_moderate*) and the overall assessment of variant to be upgraded to 'likely pathogenic' (following ACGS recommendations) thereby confirming a genetic diagnosis of MEN1.

*evidence supporting pathogenicity as per ACGS guidance

BOX 2

Clinical presentation and Genetic testing: A patient was referred for *VHL* genetic testing on the basis of a diagnosis of renal cell carcinoma and a retinal angioma at young age (<40-years of age). There was no relevant family history reported at the time of testing. Genetic testing revealed a single nucleotide deletion affecting the exon 3 of the *VHL* gene and predicted a frameshift and premature stop codon truncating the C-terminus of the VHL protein.

Variant Classification: Initial classification of the variant gave a 'warm' VUS interpretation based on: absence of variant from existing population databases (PM2*); and the presence of a null variant in a gene where loss of function (LOF) is a known mechanism of disease, but used at only moderate strength as the variant occurred at the 3' end of the gene (PVS1_moderate*).

Options to resolve uncertainty: 1. Given that VHL has high penetrance, determining whether the *VHL* variant occurred *de novo* may provide moderate or strong evidence supporting pathogenicity dependent on the extent of testing of parental samples.

Outcome: The parents were not found to harbor the *VHL* variant. This facilitated a further moderate piece of evidence for pathogenicity (i.e. assumed *de novo* variant (PM6*)) allowing the variant to be upgraded to 'likely pathogenic' and confirming a clinical-genetic diagnosis of VHL.

*evidence supporting pathogenicity as per ACGS guidance

BOX 3

Clinical presentation and genetic testing: A patient was referred for *MEN1* genetic testing with a history of a small intestinal midgut neuroendocrine tumour and presumed primary hyperparathyroidism (raised serum corrected calcium and raised parathyroid hormone (PTH)). No relevant family history was noted at the time of referral. Genetic testing identified a rare heterozygous germline *MEN1* single nucleotide variant (SNV) in the 5' untranslated region (UTR) close to the ATG start codon.

Variant interpretation: At the time of initial classification (prior to current ACMG/ACGS guidelines) the variant was categorised as a VUS on the basis of absence or very low frequency in control populations in existing databases.

Options to resolve uncertainty: 1. Clinical phenotype review – is phenotype consistent with MEN1? 2. Clinical evaluation of parents/first degree relatives to determine if they manifest a relevant clinical phenotype.

Outcome of testing: Small intestinal midgut NETs are not a typical manifestation of MEN1, whilst further clinical work-up demonstrated hypocalciuria (calcium clearance:creatinine clearance excretion index <0.01) not typical of primary hyperparathyroidism. Biochemical screening of the proband's parents identified the father to have hypercalcaemia and inappropriately normal PTH but he did not harbor the *MEN1* variant. Based on this additional information a genetic diagnosis of MEN1 in the proband was considered unlikely, but raised the possibility of an alternate hereditary hypercalcaemic disorder such as Familial Hypocalciuric Hypercalcaemia (FHH).

Further genetic testing of the proband identified a novel missense *CASR* variant. This was classified as a 'warm' VUS based on: absence from population databases (PM2*); predicted deleterious by multiple *in silico* tools (PP3*); missense variant in a gene that has a low rate of benign missense variation (PP2*). Although the variant was also observed in the proband's father it currently remains a 'warm' VUS. Future

attempts to resolve uncertainty could include; identification of additional affected family members for co-segregation analysis or demonstration in a validated functional assay that the *CASR* variant results in a loss of function comparable to other known FHH-associated pathogenic *CASR* variants (not routinely available for clinical use).

*evidence supporting pathogenicity as per ACGS guidance

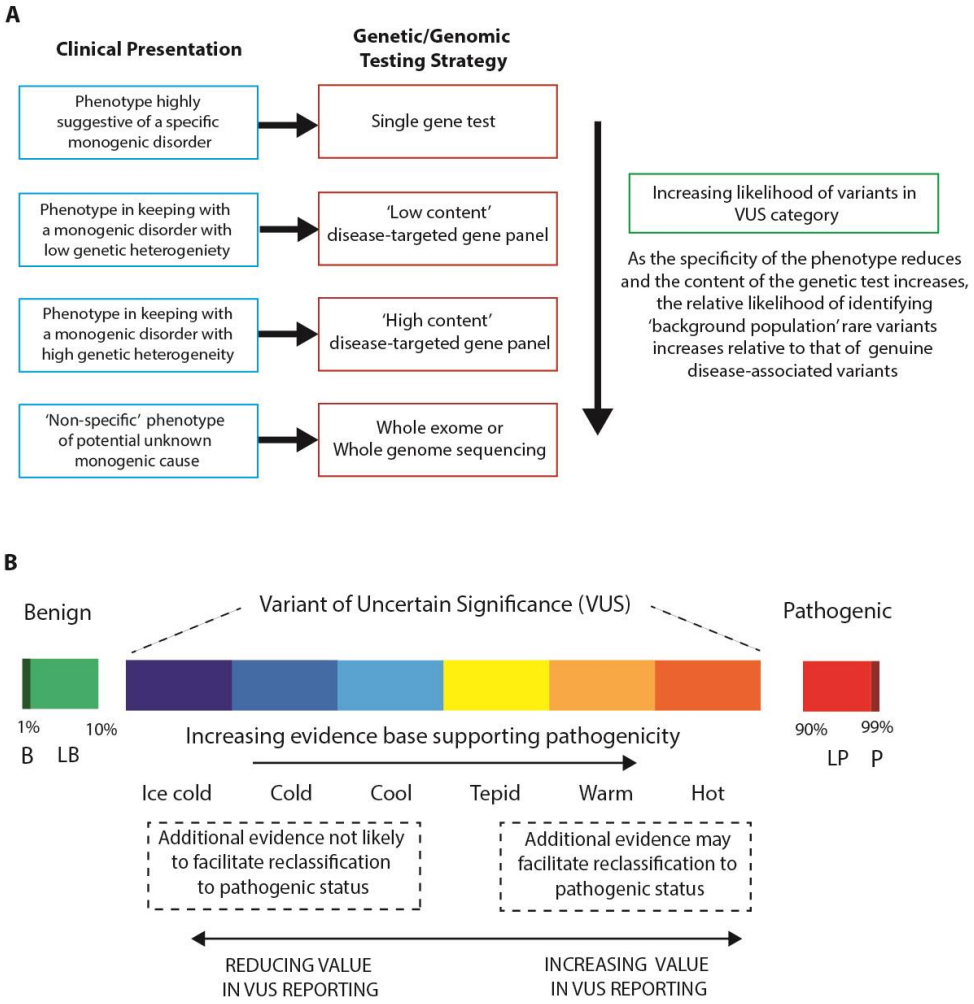

Figure 1. A. Pre-test probability of VUS depends on clinical presentation and genetic/genomic testing strategy. Variants in the VUS category will comprise genuine disease-associated variants for which there is insufficient evidence to reach the thresholds for likely pathogenic/pathogenic (LP/P) status, and rare 'neutral' variants incidentally discovered during the genetic testing process. Whilst the likelihood of identifying genuine disease-associated variants increases with the

specificity of the clinical presentation (comprising clinical phenotype and relevant family history), the likelihood of identifying rare 'incidental' background variants typically increases with the genetic content of the test. Thus, both the clinical presentation and genetic testing strategy influences the respective pre-test 'risk' of identifying each type of variant (i.e. disease-associated allele vs incidental background variant). For the majority of dominantly inherited endocrine disorders rare or novel missense SNV constitute the majority of VUSs, with current variant interpretation methods often insufficiently specific to differentiate disease-associated and rare background alleles.

B. The VUS category represents a continuum of risk. The VUS category provides a repository for variants with insufficient evidence to facilitate pathogenic or benign classifications. Current ACMG guidelines set strict probability thresholds to achieve likely pathogenic (LP) (>90%) and pathogenic (P) (>99%) status. The probability thresholds for achieving likely benign (LB) (<10%) and benign (B) (<1%) are also restrictive, such that the VUS category spans a very broad range of 'risk', ranging from variants with an absent/minimal evidence base supporting pathogenicity (e.g. 'ice cold', 'cold' variants) to those just below the threshold of LP status (e.g. 'warm', 'hot' variants). Thus, stratification of VUS variants based on quantifying the evidence supporting pathogenicity is increasingly used by genetic testing laboratories to guide downstream reporting. For example, the UK-based ACGS guidelines typically recommend reporting only those VUSs where additional testing may facilitate upgrade to LP status (e.g. most commonly 'warm' or 'hot' VUSs) or in situations where all currently available evidence is consistent with a causal association but where reclassification may not be feasible (e.g. due to lack of available family members for co-segregation analysis). Such processes can help reduce the burden of VUS reporting by removing variants with a very low likelihood of being disease-associated. Indeed, Bayesian modelling of the ACMG guidelines are increasingly used to provide risk estimates during variant interpretation thereby facilitating VUS

stratification. However, it should be noted that depending on the clinical scenario and available evidence at the time of testing, there may be instances in which the 'upgrading' of a 'warm' or 'hot' VUS to 'likely pathogenic' is not possible as upgrading in this setting most frequently involves identifying additional new pieces of strong, moderate or supporting evidence (or the strengthening of existing evidence). Information in this figure is based on information and concepts reported in the ACGS guidelines.[16]

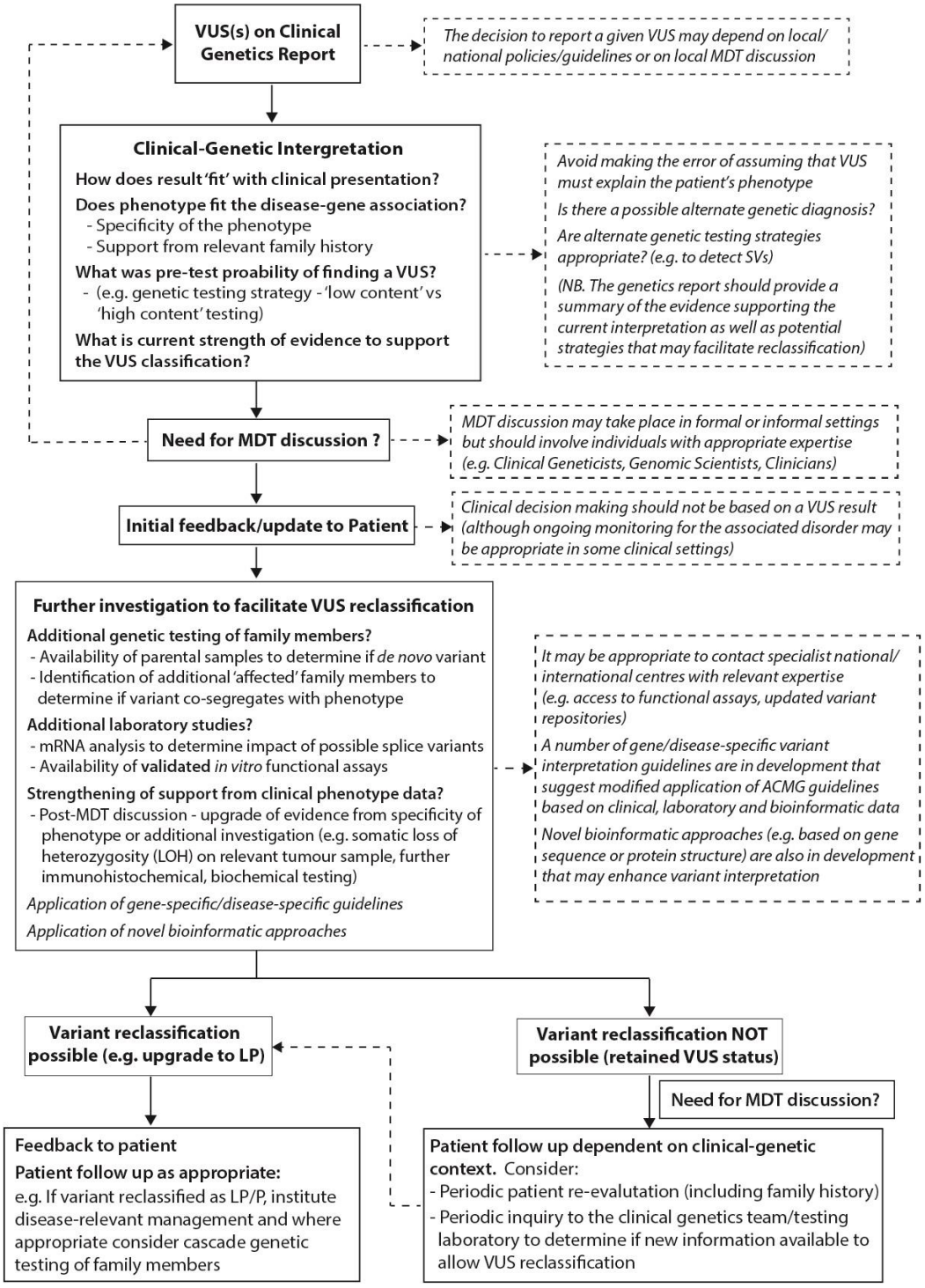

Figure 2. Clinical approach to receiving a VUS result and 'decision-support' workflow.

The decision to report a VUS in a gene related to the primary indication for testing will be determined by the policy framework under which the genetic laboratory operates. It is essential that the clinician considers the VUS in the overall context of the phenotype and testing strategy, neither assuming the result is responsible for the

patient's presentation or is 'uninterpretable'. Instead, the clinician should study the report to understand the evidence base supporting the current interpretation and to identify potential strategies to resolve the uncertainty. At this stage multidisciplinary discussion may offer a valuable opportunity to review the case-level clinical-genetic information and define a future management strategy, which in turn may provide a useful framework for discussions with the patient. Steps to facilitate VUS reclassification may involve additional genetic testing of family members, further clinical investigation, and/or *in vitro* or *in silico* laboratory-based methods. In specific settings the level of evidence supporting pathogenicity based on the patient's phenotype may be strengthened after multidisciplinary discussion. In addition, although not widely employed to date, several current initiatives are establishing disease-specific modifications to the ACMG guidelines (based on clinical and/or bioinformatic parameters) and/or generation of novel gene-specific metrics that aim to improve the specificity of variant interpretation. If initial attempts to resolve the VUS classification are unsuccessful, further multidisciplinary discussion may help determine an appropriate follow up plan, which may include interval clinical review and periodic enquiry to the genetics team and/or testing laboratory to establish if any new information has emerged facilitating a revised classification. Abbreviations; MDT, multidisciplinary team; SV, structural variant; LP, likely pathogenic; P, pathogenic; VUS, variant of uncertain significance.