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Genetic Modifiers and Non-Mendelian Aspects of CMT

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Abstract

Charcot-Marie-Tooth (CMT) neuropathies are amongst the most common inherited diseases in neurology. While great strides have been made to identify the genesis of these diseases, a diagnostic gap of 30–60% remains. Classic models of genetic causation may be limited to fully close this gap and, thus, we review the current state and future role of alternative, non-Mendelian forms of genetics in CMT. Promising synergies exist to further define the full genetic architecture of inherited neuropathies, including affordable whole-genome sequencing, increased data aggregation and clinical collaboration, improved bioinformatics and statistical methodology, and vastly improved computational resources. Given the recent advances in genetic therapies for rare diseases, it becomes a matter of urgency to diagnose CMT patients with great fidelity. Otherwise, they will not be able to benefit from such therapeutic options, or worse, suffer harm when pathogenicity of genetic variation is falsely evaluated. In addition, the newly identified modifier and risk genes may offer alternative targets for pharmacotherapy of inherited and, potentially, even acquired forms of neuropathies.

Genetics of Charcot-Marie-Tooth Disease

As with many other Mendelian diseases, the introduction of next generation sequencing (NGS) revolutionized the genetic diagnosis of Charcot-Marie-Tooth disease with now over 90 known genes causing CMT.[1,2] Though CMT can be caused by an overwhelming amount of genetic defects, it is noteworthy that a handful of genes are responsible for the majority of cases. More than half of all CMT cases are caused by five genetic mutations: *PMP22* duplication (39.5%), *PMP22* point mutation (1.4%), *GJB1* (10.8%), *MFN2* (2.8%), and *MPZ* (3.1%).[3] For autosomal dominant (AD) demyelinating CMT (CMT1), the most commonly mutated genes are: *GJB1, PMP22, MPZ, EGR2, LITAF, NEFL,* or *PMP2*.[3] Unlike CMT1, AD axonal CMT (CMT2) and autosomal recessive (AR) axonal and/or demyelinating forms (CMT4) are caused by many, individually rare, genes that typically

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affect only a handful of families.[3,4] The most common cause of CMT2 (~20%) is mutations within the outer mitochondrial membrane protein, *MFN2*[5], while the most common CMT4 genes are *GDAP1*[6] and *SH3TC2*.[4,7]

In the past 5 years alone, >20 CMT genes have been reported. However, the number of families identified in the initial and follow-on papers is typically low; for example, Tey *et al* recently identified *AHNAK2* to be the causative gene for AR CMT in a single family, and analysis of whole exome sequencing (WES) from 115 unsolved families led to one additional family demonstrating segregation of a different variant in the same gene.[8] In 2013, Gonzalez *et al* reported a single family with a mutation in *MARS* causative for CMT2 with no additional evidence in 400 additional families. Three additional families have since been identified harboring likely pathogenic variants in this gene.[9,10] This has led to the concern that the field is in an asymptotic phase where, even with many new genes, the diagnostic yield may not reach 100%. This potential diagnostic gap in heritability is observed in other rare disorders as well and might be referred to as “dark matter” of clinical genomics.

The “Dark Matter” of Clinical Genomics

It is usually assumed that CMT is caused by Mendelian mechanisms and that eventually nearly all patients will receive a single-gene diagnosis. However, this is not necessarily true. Related motorneuron disorders presently illustrate a diverse situation: inherited ataxias and spastic paraplegias are also highly heterogeneous Mendelian disorders whereas amyotrophic lateral sclerosis (ALS) is largely not explained by Mendelian genes. The proportion of Mendelian genes is even lower in late onset neurodegenerative diseases, such as Parkinson and Alzheimer disease.

The reported diagnostic yield for exome sequencing in the general clinical setting ranges from 25 to 50%.[11,12] In CMT, Fridman *et al* reported a 60.4% (997/1652 patients) genetic diagnosis rate: while 91.2% of CMT1 patients received a genetic diagnosis, only 42% of CMT2 cases received genetic confirmation.[13] Bacquet *et al* report an overall diagnosis rate of 40% (49/123 patients), with 87% of CMT1 cases and 34% of CMT2 cases being solved.[14] Finally, genetic testing of 1,206 patients at a laboratory in Aachen, Germany revealed a genetic diagnosis in 56% of demyelinating CMT cases and 17% of axonal CMT cases.[15]

Several valid pro-Mendelian hypotheses exist as to how to close the diagnostic gap, and the coming years will allow us to test these ideas (Fig. 1). These include non-coding regions of the genome, unorthodox types of mutations (such as repeat expansions) and digenic inheritance models (Fig. 2). However, while the genetics of certain neurodegenerative diseases are deemed ‘complex’, similar models of inheritance are largely unexplored in CMT. Although it is too early to tell the extent of non-Mendelian effects in CMT, increasing observations of non-Mendelian inheritance support further consideration and investigation.

Beyond Mendelian Inheritance

Based on the assumption of fully penetrant alleles, traditional Mendelian disease analysis focuses on the rare DNA variation that segregates within a family. However, these locus-

specific family studies treat Mendelian traits as distinct entities and disregard a more comprehensive genetic model for human disease in which variants of varying effect size as well as environmental influences contribute to disease.[16] The challenge is the unexpected large amount of variation in the human genome on a population level, where >99% of all variants show a minor allele frequency of <1%.[17] While many of these variants are without phenotypic consequence, some certainly are very harmful, and a considerable number must have effect sizes that are below the threshold of a Mendelian gene, but contribute significantly to phenotypic expression. Identification of strong effect sizes in the background of mostly minor effects is a rising challenge in human genetics. Recent method developments in statistical genetics allow for unbiased genome-wide screens for non-Mendelian alleles, and surprisingly, are able to re-identify *bona fide* Mendelian genes as well.[18] The application of such methods to CMT genomics will eventually generate a more complete genetic architecture of the disease.

Reduced penetrant and risk alleles

Contrary to general expectations for CMT families, asymptomatic carriers are not infrequent, in which case, the genotype is said to be incompletely penetrant (Fig. 2).[19,20] Reduced and age-dependent penetrance is a diagnostically challenging situation observed in autosomal dominant CMT, which can lead to misinterpretation of inheritance patterns due to asymptomatic carriers and exclusion of the disease-causing allele.[21] Incomplete penetrance can also manifest in autosomal recessive disorders when the primary mutation leads to varying phenotypic effects depending on the secondary mutation.[19] For example, recessive mutations in *SCO2* typically result in fatal infantile cardioencephalomyopathy; however, Rebelo *et al* reported a less severe CMT phenotype resulting from the specific location and compound combination of observed biallelic pathogenic variants.[22,23]

Risk alleles are another form of variation that does not conform to standard Mendelian inheritance (Fig. 2). Risk alleles have been defined as variants with smaller effect sizes that are part of a multifactorial model of disease causation.[19] However, since the possibility of risk alleles is only recently recognized in rare Mendelian disease, the line between penetrance and risk is often blurred. In this context, risk alleles more broadly refer to rare variants that may lead to a less severe, later-onset form of disease or contribute to an individual's susceptibility to disease, likely through an oligogenic or gene-environment model. For example, heterozygous mutations in *MME* were recently shown to predispose carriers to late-onset axonal neuropathy.[24] Late-onset axonal neuropathy is an autosomal dominant disorder with an age-of-onset in the second half of life.[24] In *MME*, the 'rare variant load' of missense and loss of function changes in late-onset CMT compared to the general population showed a significant enrichment of such variation, indicating *MME* may act as a risk gene.[24] This gene-wide statistical measure, however, does not easily translate to the assessment of pathogenicity of individual *MME* alleles. A recent manuscript by Senderek *et al* aimed at exploring individual allelic pathogenicity in *MME* (*Lancet Neurology, under review*). In addition, specific heterozygous variants in MME demonstrated penetrance in central European families, but non-penetrance in Spanish and Japanese families. Further work remains to explain this diversity in penetrance amongst different ethnic groups, which could possibly be due to genetic modifiers or environmental effects.[47]

Systematic identification of rare variant associations are usually limited by low statistical power unless sample sizes or variant effect sizes are very large.[25] To illustrate, >60,000 cases (and an equal number of controls) would be necessary to detect a disease association for an individual rare variant (0.1% frequency) with an odds ratio of 2.0 for a disease with a 5% population prevalence.[25] Fortunately, powerful study designs can alleviate the sample size requirement to more reasonable numbers.[26] One approach that can be explored in CMT is the gene-based variant burden test, which collapses the number of minor alleles into one genetic score per gene, thus reducing multiple testing and increasing power.[27] One successful example of this approach was the identification of a new ALS gene, *TBK1*, in 2,869 sporadic ALS patients.[18] Remarkably, other known ALS genes showed strong associations, indicating that additional variation in known familial ALS genes also contribute to sporadic ALS forms.[18] This exome-wide study design was recently applied in a cohort of 343 CMT cases and resulted in the identification of a strong association with *EXOC4* (p-value = 6.9×10^{-6}, OR = 2.1).[28] *EXOC4* is involved in vesicle transport and membrane tethering in polarized cells, is expressed in Schwann cells, and is involved in myelination in a CMT4B1 mouse model.[29] Similar to the ALS study, *bona fide* CMT genes were also nominally associated despite an effort to exclude patients with common forms of CMT from exome analysis.[28] The gene-based burden tests is not without limitations. Systematic evaluations have shown that they are sensitive to several variables including analysis unit (e.g. exon versus whole gene), the number and functional class of variants tested within an aggregation, and the magnitude of linkage disequilibrium between variants.

Modifier alleles

An increasing number of exceptions to the fundamental “one gene, one phenotype” paradigm are being published across Mendelian phenotypes.[30] The oversimplified view that phenotypic expression, even for classically monogenic disorders, is driven exclusively by mutations at a single locus is being replaced by the concept of genetic modification (Fig. 2).[31] Though several types of genetic modification are possible, the simple definition is the effect of one allele on the phenotypic outcome of a second allele.[31] If the primary allele is sufficient to cause disease, then the secondary allele is a “modifier” that modulates phenotypic expression, such as disease severity or progression. Modifier alleles can interact both directly or indirectly with the primary gene to exacerbate or reduce the phenotype in a non-additive manner. The resulting phenotype is caused by the primary gene, modifier gene, environmental factors, and their interactions.

Given the high clinical variability among CMT cases with the same genetic subtype, genetic modification of the primary allele was anticipated and has been observed in several instances. For example, additional copies of the *PMP22* gene act as a genetic modifier in several CMT1A cases, resulting in a severe phenotype.[32–34] Additionally, missense variants in the *LITAF* gene contribute to a more severe and earlier onset form of CMT1A.[35,36] About 80% of newly formed PMP22 is degraded due to incorrect folding, and LITAF is thought to play a role in protein degradation, which could explain the exacerbation of the CMT1A phenotype in patients with defective LITAF.[35,36] Nam *et al* reported a polymorphism in microRNA 149 was associated with onset age and severity in CMT1A.[37] Finally, variants in

JPH1 lead to a more severe clinical expression of CMT2K, as the modifier and GDAP1 share a common cellular pathway.[38]

Most of these examples were observed in individual families and thus represent a small portion of phenotypic variability. Systematic identification of genetic modifiers in rare disease is limited by the challenges of collecting a large enough sample for genomic studies. Study designs that include more informative individuals can improve the power of genomic studies within rare disease. One study design that increases the statistical power for association testing of rare variants is the extreme phenotype sampling (EPS) approach.[26] Based on the assumption that rare causal variants are more likely found in the extremes of a quantitative trait such as age of onset or severity of a symptom, EPS can increase the power to detect rare variants over random sampling.[26] For example, Tao *et al* identified *SIPA1L2* as a genetic modifier of muscle strength impairment in CMT1A.[39] *In vitro* knock down of *SIPA1L2* in Schwannoma cells lead to a significant reduction in *PMP22* expression, offering a potential pathway for therapeutic strategies.[39] However, simulations have shown that EPS is not necessarily more powerful than a random sample when other environmental covariates have a strong impact on the phenotype, so it is important to consider the phenotype of interest when using this study design.[64]

Animal model studies also have large potential in contributing to the discovery of modifier genes in CMT and circumvent the challenge of requiring detailed phenotypic data for human genetic studies. Yeast genetic screens have already been successful in identifying modifiers in Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS). For example, *PBP1*, the yeast ortholog of human *ATXN2* (a polyglutamine protein mutated in spinocerebellar ataxia type 2), was identified as a dose-sensitive modulator of TDP-43 toxicity (a major cause of ALS).[40] This enhanced toxicity was next confirmed in transgenic *Drosophila* lines expressing mutant TDP-43, where *ATXN2* overexpression in motor neurons resulted in loss of motility.[40] Treatment of mice with ATXN-2 anti-sense oligonucleotides dramatically increased survival, indicating that modifiers can be important therapeutic targets for human disease.[51] Genetic modifiers of CMT have been suggested in several mouse models. In a CMT2D mouse model, mutated glycyl-transfer RNA synthetase led to aberrant binding of the neuropilin 1 (Nrp1) receptor, an essential receptor for motor neuron axon guidance and cell body migration. Genetic reduction of *Nrp1* worsened the CMT phenotype within the model.[41] In both CMT2D and CMT4C mouse models, homozygous neuronal cell adhesion molecule (*NRCAM*) or heterozygous sodium voltage gate channel 8A (*SCN8A*) mutations exacerbated the neuropathy phenotype. Both proteins are found at the nodes of Ranvier, and although these specific genes may not translate as modifiers in human disease, Morelli *et al* argue that any variants that affect the physiology at nodes could potentially affect the severity of CMT in humans.[42] Nicholson *et al* report that individuals with identical genotypes in *FIG4* causing CMT4J display vast differences in age of onset as well as in disease severity, suggesting a role of modifier alleles.[62] Studies in Drosophila recently identified *Hippo (hpo)*, the drosophila ortholog of *MST1*, as a modifier of CMT4J. Knockdown of *dFIG4* in this model resulted in aberrant motor neuron morphology, a phenotype that was improved through the downregulation of hpo.[63]

Multilocus inheritance

Multilocus inheritance refers to instances when one primary allele is insufficient to cause disease, instead requiring the combined consequence of multiple alleles at multiple loci (Fig. 2).[30] Multilocus inheritance is common in complex genetic diseases in which many genes with small effect sizes contribute to disease (polygenic), whereas occurrence of disease-causing variants in two distinct genes (digenic) is well-documented in Mendelian disease.[43] Oligogenic inheritance affects more than two genes – but less than a polygenic disease – thus serving as a bridge between Mendelian and complex disease etiologies.

Digenic inheritance has been documented in CMT. In a Japanese cohort, digenic variants were identified in five cases: *SETX* and *ARHGEF10*, *SH3TC2* and *SACS*, *LRSAM1* and *MARS*, *HARS* and *ARHGEF10*, and *MFN2* and *PMP22*.[44] Chung *et al* reported a patient with a severe clinical presentation of CMT resulting from digenic inheritance of mutations in two known CMT genes: GJB1 and EGR2.[45] Brusse *et al* described a three generation distal hereditary motor neuropathy (dHMN) family with both a *BSCL2* mutation and a second disease locus on chromosome 16p.[46] Affected individuals carried both alleles while one individual with sub-clinical motor neuron damage carried only the 16p locus. Lastly, possible digenic inheritance of dHMN and CMT2 was identified in one family. One parent transmitted a purely motor phenotype caused by a novel gene mutation while the other parent transmitted a mild CMT2 caused by a *MFN2* mutation. The four affected offspring carrying both mutations were more severely affected with an earlier age-at-onset.[47]

Demonstrating oligogenic inheritance from family studies is challenging without experimental models; however, evidence of oligogenic inheritance has emerged in several neurological disorders. One trending approach to assessing oligogenic inheritance – which has been explored in Parkinson, ALS, Frontotemporal Dementia, Congenital Hypothyroidism, Inherited Neuropathy, and more – is to evaluate the mutational burden across known disease genes through Fisher's exact test or logistic regression.[48–52] For example, in both sporadic and familial ALS cases, patients harboring two or more rare variants had lower survival or earlier age at onset, suggesting that the combined effect of rare variants affects ALS development and progression.[53–55] Similarly, over 30% of PD patients carried additional rare variants in Mendelian PD genes and had younger ages at onset.[48] In CMT, an increased rare variant burden was observed in two cohorts of inherited neuropathy cases, which was followed up with *in vivo* zebrafish experiments.[49] In zebrafish, more severe phenotypic outcomes were observed as a consequence of increased mutational burden in neuropathy genes, consistent with a positive genetic interaction mechanism of oligogenic inheritance.[49] It is important to note that caution should be used with the 'mutational burden' approach as systematic bias can lead to the apparent enrichment of 'oligogenic' variants in familial cases, and control of such bias is essential for investigating an oligogenic role in neurodegenerative diseases.[56]

Multilocus variation has been shown to generate unusually severe phenotypes and apparent phenotypic expansion (clinical features beyond those typically reported with a known disease gene).[57,58] In 2006, Hodapp *et al* identified three families with multiple neuromuscular diseases.[57] In addition to *PMP22* duplications/deletions, each family harbored mutations in other neuromuscular disease-related genes: (1) missense mutations in

GJB1 causing a severe demyelinating CMT, (2) trinucleotide repeat expansion of *DMPK* causing myotonic muscular dystrophy and a more severe neuropathy, and (3) a mutation in *ABCD1* causing adrenomyeloneuropathy and severe peripheral neuropathy. The authors termed this augmentative digenic effect as “double trouble” due to the unique phenotypic manifestations and concluded that individuals with mutations in multiple neuromuscular disease-related genes may develop more severe phenotypes.[57] Høyer *et al* also observed this type of dual pathology in a sporadic case with CMT2 and spasticity who carried ‘likely pathogenic’ variants in *SETX* and *REEP1*.[59] As phenotypic expansion and multiple molecular diagnoses have become more frequent, Karaca *et al* explored whether apparent phenotypic expansion at one known disease locus was actually the result of blended phenotypes from different loci.[58] In a cohort of well-characterized neurodevelopmental phenotypes, the authors identified multilocus variation in 31.6% of families with phenotypic expansion and 2.3% of families without phenotypic expansion, emphasizing the importance of considering multilocus inheritance in apparent phenotypic expansion cases.

Concluding Remarks

Traditional family studies in Mendelian diseases have been very successful in identifying highly penetrant and medically actionable alleles, and next generation sequencing (NGS) has led to an explosion of novel disease genes. Despite great overall advancement, the diagnostic yield for particular genetic subtypes (*e.g.* simplex axonal CMT) remains surprisingly low. With the increasingly low numbers of additional affected families following a novel gene discovery, we advocate for the exploration of non-Mendelian contributors to the CMT genetic etiology in order to close the diagnostic gap. In this review, we summarized examples of reduced penetrance, risk alleles, modifier alleles, and multilocus inheritance in CMT and related disorders. Observation of such non-Mendelian factors in monogenic disorders provides further support for a “unified genetic model for human disease” to coalesce previously distinct disease entities as part of a continuum of genetic disease.[16] Unbiased genomic interrogation has revealed how truly personal each genome is – containing common variants within the population, rare variants from more recent population substructure, new combinations of variants from both parents, and novel *de novo* variation in each individual.[16] Though it may seem apparent that interactions between variants at multiple loci will impact phenotypic expression, these inheritance models still remain largely unexplored in many monogenic disorders. Recent efforts for data aggregation[60] and collaboration[13] are removing the common limitation of small sample size, which will allow the community to apply statistical approaches to CMT and rare disease overall. Though the extent to which non-Mendelian elements will contribute to the CMT genetic architecture remains unclear – and other possibilities exist to close the diagnostic gap not covered in this review, including the non-coding space and structural variation – the reviewed examples demonstrate that non-Mendelian inheritance will likely continue to emerge as a relevant factor.

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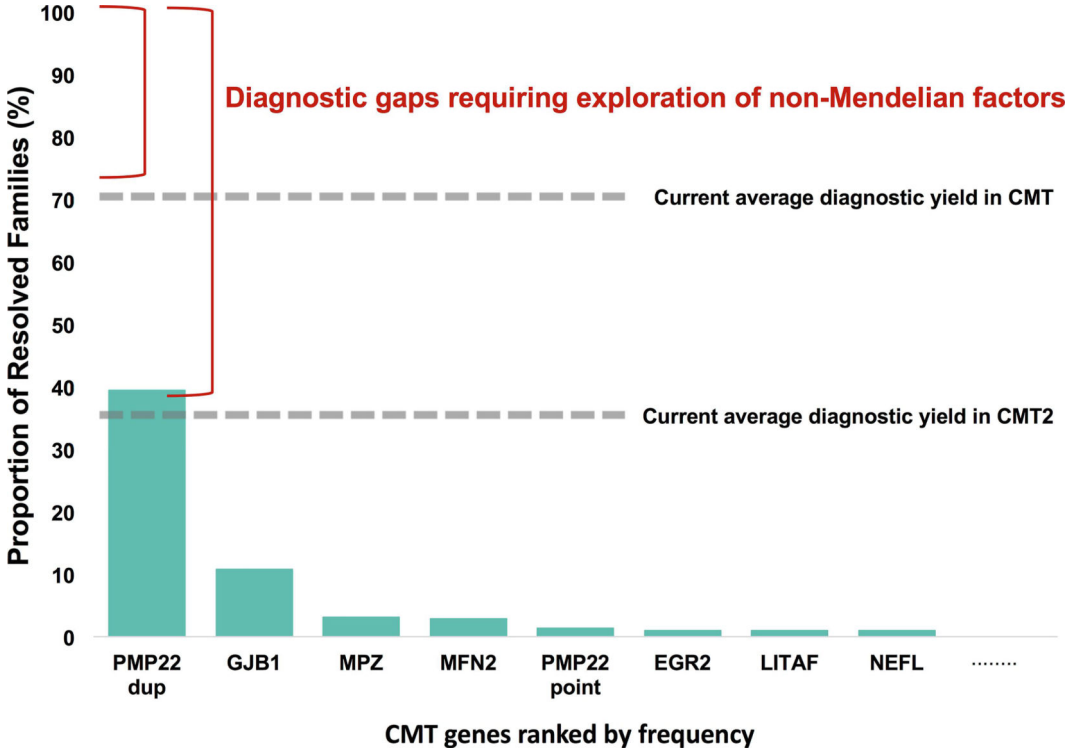

Figure 1.

The gaps in genetic diagnosis in CMT. While novel gene discovery for CMT has been very successful over the past decade, the average diagnostic yield remains under 40% for CMT2 and under 75% for all CMT. Close to 60% of genetically resolved CMT families can be attributed to less than 10 genes, yet over 90 loci are associated with the disease. Many of these loci resolve a small percentage of CMT – sometimes as few as one or two families. Since additional Mendelian disease genes alone have not closed these diagnostic gaps, exploration of non-Mendelian factors is necessary.

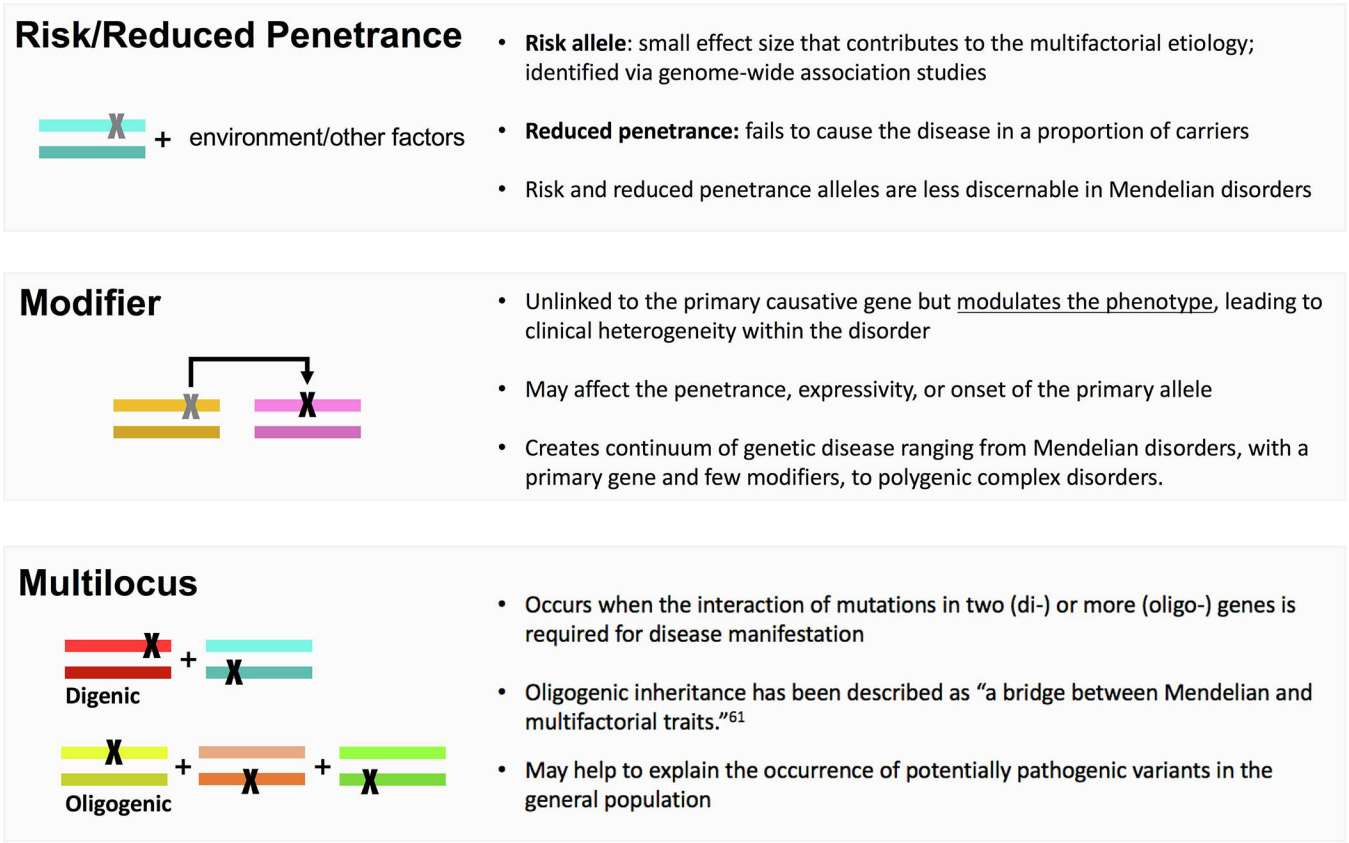

Figure 2.

Each box defines a non-Mendelian phenomenon that is currently investigated in CMT and covered within this review, including risk/reduced penetrance alleles, modifier alleles, and mutlilocus inheritance. Colored rectangles represent diploid genes and X's represent pathogenic variation.