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CONTENTS

• Review Articles

The Role and Prospects of the *Drosophila* Model System in Human Rare Disease Research 15
Sung Dae Kim, Andrew Kim, Sarita Palacio, Hyung-Lok Chung

Preimplantation Genetic Testing for Rare Monogenic Disease: A Female Reproductive Medicine Perspective on the Clinical Pathway 29
Dongsoo Park, Haeryung Choi

Genes Associated with Short Stature: An Update 36
Jung-Eun Moon

Beyond Copy-Number and Coding-Region Analysis: Resolving the Molecularly Unresolved Dystrophinopathy 45
Dae-Hyun Jang

Monogenic Causes of Short Stature in Children Initially Diagnosed with Idiopathic Short Stature 54
Kyungmi Jang

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The Role and Prospects of the *Drosophila* Model System in Human Rare Disease Research

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Drosophila melanogaster has served as one of the most powerful and versatile model organisms in biomedical research for over a century. Its high degree of genetic conservation with humans, with approximately 75% of human disease-associated genes having functional orthologs in the fly, combined with sophisticated genetic tools and a short generation time, makes it uniquely suited for investigating the mechanisms underlying human diseases. This review provides a comprehensive overview of the utility of *Drosophila* in rare disease research, highlighting its contributions across multiple disease categories, including neurological and neurodevelopmental disorders, metabolic and mitochondrial diseases, lysosomal storage disorders, sensory disorders, immunological conditions, and cancer. We discuss the technical advantages that make the fruit fly an ideal first-line model for functional genomics, including the GAL4/UAS system, CRISPR/Cas9 gene editing, and humanization strategies. Furthermore, we examine the inherent limitations of the fly model and the innovative approaches developed to overcome them, such as the integration of complementary model systems and the expression of human disease genes in flies. As rare diseases continue to pose significant diagnostic and therapeutic challenges, *Drosophila* remains an indispensable platform for elucidating disease mechanisms, validating patient-derived genetic variants, and identifying potential therapeutic targets.

Key words: *Drosophila melanogaster*, Rare diseases, Disease models (Animal), Genetic research, Neurodegenerative diseases, Lysosomal storage diseases, Neoplasms, Drug discovery, Functional genomics

REVIEW ARTICLE

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INTRODUCTION

Rare diseases are generally defined as those with a low prevalence in the population, although the specific criteria vary across countries and organizations [1]. For instance, the criteria include a prevalence of 1 or fewer per 1,000 people according to the World Health Organization, affecting fewer than 200,000 people in the United States, fewer than 1 in 2,000 in the European Union, fewer than 50,000 or 1 in 2,500 in Japan, and fewer than 20,000 or being difficult to diagnose in South Korea [1,2]. Rare diseases can be classified based on various factors, such as genetic origin, age of onset, affected organ systems, and prevalence. Many of these conditions have a genetic basis and are characterized by patient-specific mutations [2]. Understanding their classification is crucial for accurate diagnosis, guiding research efforts, and developing targeted therapies. Although individually rare, these diseases collectively represent a significant public health challenge, affecting a substantial portion of the global population (estimated at 3.5%-5.9%, or 263-446 million people) [2]. The estimated number of known rare diseases ranges from 5,000 to over 8,000 [3], and these diseases exhibit highly diverse characteristics. As a result, patients, families, and healthcare systems face significant challenges, including diagnostic delays, a lack of treatments, high medical costs, and psychosocial impacts [2].

Advances in science and technology, particularly genomics, have brought a sig-

nificant turning point to rare disease research. However, effective treatments remain unavailable for most of these conditions, leaving substantial unmet medical needs. While attention has been focused on developing orphan drugs, these efforts only cover a limited number of rare diseases [1]. Furthermore, the small quantity of patients presenting with these diseases gives rise to logistical issues for researchers, like limited funding and insufficient patient cohorts. Restricted resources to fund standard clinical trials lead to a limited understanding of disease progression, which slows the development of adequate treatments and tests. Multifaceted solutions are needed to increase the scope of rare disease research, as well as the resources available to researchers. Using model systems, designing better clinical trials, sharing data between researchers, and having helpful policies in place could provide further advancements in rare disease research [4]. Because human patient data is limited in rare disease research, the use of model organisms to elucidate disease mechanisms and to test therapies is indispensable [5]. Researchers can analyze complex, evolutionary conserved biological processes in simpler systems like nematodes, zebrafish, mice, and fruit flies. Model organisms have emerged as valuable tools for rare disease research, and each one offers distinct advantages that facilitate the investigation of the root causes of diseases, underlying mechanisms and potential therapeutic approaches [6].

Among these systems, *Drosophila melanogaster* has been widely used in genetic research. This species has played a pivotal role in genetics for over a century, beginning with Thomas Hunt Morgan's groundbreaking work linking genes to chromosomes [7]. Subsequent key discoveries include gene mapping by Sturtevant; radiation-induced mutagenesis by H. J. Muller; the genetic control of development by Christiane Nusslein-Volhard, Eric Wieschaus, and Edward B. Lewis; behavioral genetics research by Seymour Benzer; the molecular mechanisms of circadian rhythm regulation by Jeffrey C. Hall, Michael Rosbash and Michael W. Young; and the discovery of conserved pathways of innate immune activation through Jules A. Hoffmann's research on the Toll receptor [7-10]. Many of these studies received Nobel Prizes, highlighting the profound and enduring scientific contributions made through this model organism. *Drosophila*'s utility stems from its short generation time, low maintenance costs, and robust genetic tools [5,6].

The fruit fly exhibits significant genetic similarities with humans; about 75% of human disease-related genes have homologs in *Drosophila*. It also possesses major conserved cellular pathways, such as Notch, Wnt, and JAK/STAT. This strong genetic conservation, paired with low gene redundancy, allows for accurate functional studies and disease modeling. The fly's fully sequenced genome and reduced complexity also make it ideal to investigate biological mechanisms relevant to human health. Consequently, *Drosophila* remains a crucial model for studying genetic diseases, development, immunity, and behavior, providing insights directly translatable to understanding human biology and pathology [5,6,9,11].

In this review, we provide a comprehensive overview of the technical advantages of the fruit fly model and recent utilization trends in rare disease research. We discuss the genetic mechanisms underlying these diseases, explore how *Drosophila* studies have advanced our understanding of disease pathogenesis, and examine the translational potential of these findings for therapy, as well as the limitations and strategies to address them.

TECHNICAL ADVANTAGES OF *DROSOPHILA* FOR MODELING RARE DISEASE

The value of the fruit fly as a model organism lies not only in its individual advantages, but also the powerful synergy between its high genetic conservation with humans, advanced genetic tools, and experimental practicality. For instance, its genetic similarity to humans ensures biological relevance, while its well-developed tools allow precise gene manipulation. Additionally, features like its short generation time and low cost facilitate large-scale experiments. This integration is advantageous for rare disease research, where resources are limited, and candidate variants are few. The convergence of these attributes enables effective translation of human genomic findings, especially single-gene mutations common in rare diseases, into a meaningful understanding of biological function and therapeutic evaluation. This synergistic interplay of its attributes gives the fruit fly a distinct advantage over many other model systems [5,12].

Well-Established genetic tools and resources

Drosophila has an extensive set of genetic tools, developed and refined over decades, which are very useful for complex studies of gene function and for modeling human diseases. Foundational to this are potent binary expression approaches, such as the GAL4/UAS method (Fig. 1) [13]. These systems offer precise spatiotemporal control over when and where genes are active. These instruments allow investigators to activate

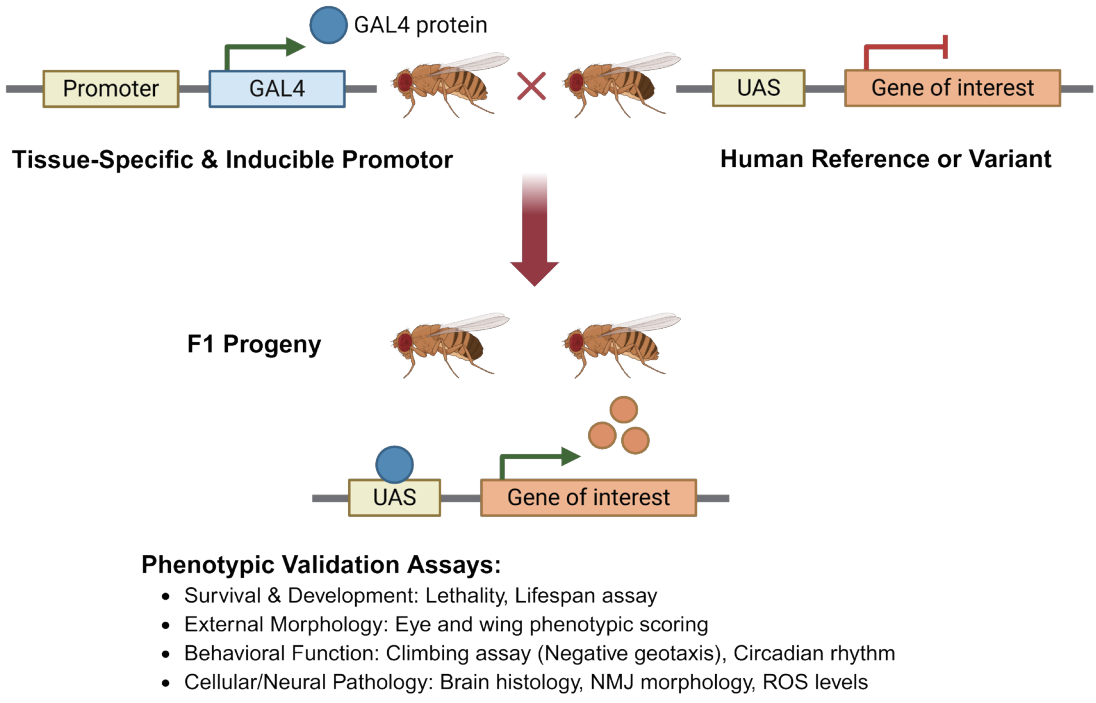


Fig. 1. Schematic of the GAL4/UAS system for enabling tissue-specific and inducible (temporal) gene expression in *Drosophila*.

transgenes (e.g., human cDNAs relevant to diseases) or RNA interference constructs for the silencing of targeted genes, within distinct cells or tissues and at defined developmental times, supported by extensive collections of driver lines [14]. Established transgenesis protocols further enhance functional genomics. These protocols permit the reliable introduction and expression of foreign DNA, including human gene orthologs, often by utilizing site-specific integration strategies, such as Minos-mediated integration cassettes (MiMIC) and the PhiC31 integrase system [15]. To investigate cell-autonomous gene functions, mosaic analysis methodologies, including FLP/FRT or mosaic analysis with a repressible cell marker (MARCM), are employed to generate and study mutant cell clones within an otherwise wild-type tissue background. Additionally, inducible systems, namely drug-activated GeneSwitch or temporal and regional gene expression targeting (TARGET), provide control over gene activity based on time and are activated by external signals, like drug exposure or temperature changes [13].

The advent of CRISPR/Cas9 technology has greatly improved our ability to accurately edit genomes [16]. This versatile tool enables scientists to introduce a variety of targeted genomic modifications, including precise insertions, deletions, and point mutations. Gene modifications are especially important for creating models of human genetic diseases, as they allow for the creation of fruit fly models genetically matched to patients. By introducing patient-specific mutations into corresponding *Drosophila* genes, researchers can test whether these gene modifications can cause disease. These tests are indispensable when the effects of these gene variants are unknown, as is often the case with rare diseases. These specially designed fly models also contribute to a better understanding of disease mechanisms.

Conservation of genetic pathways in *Drosophila melanogaster* and *Homo sapiens*

Comparative genomic analyses reveal that 60-85% of human genes have identifiable homologs in the *Drosophila* genome, with the proportion rising to approximately 75%-85% for disease-associated genes, demonstrating the fly's relevance as a model for human biology [17,18]. This conservation extends beyond individual genes, encompassing core biological pathways crucial for development and cellular function. Major signaling pathways (e.g., Wnt, Notch, Hippo, JAK/STAT, and RAS) and fundamental processes (e.g., apoptosis, the cell cycle, metabolism, neurogenesis, and innate immunity) are highly conserved between flies and humans [19]. This extensive conservation of genes, pathways, and cell functions provides a biological rationale for using fruit fly models to investigate the mechanisms of human health and disease.

Table 1. Summary of *Drosophila* models for human rare disease pathogenesis and therapeutic discovery

Disorder	Human Gene/ Protein Affected	Drosophila Model	Contribution to Pathogenesis	Therapeutic Insight
Rett Syndrome	*MECP2*	Overexpression of human *MECP2* and RTT patient-derived mutant alleles	Loss of *Sin3A* function exacerbated phenotypes, while loss of *Smr* and *crol* suppressed them	*MECP2*-interacting factors identified as potential therapeutic targets
MECP2 Duplication Syndrome	*MECP2* Duplication (Xq28)	Overexpression of human *MECP2*	Discovered pathways disrupted by *MECP2* duplication via genetic screens	Genetic screening elucidated candidate therapeutic targets
Fragile X Syndrome	*FMR1*	Knockout of fly homolog (*dfmr1*)	Synaptic dysfunction and dysregulated protein translation identified	Minocycline treatment restored synaptic structure, leading to subsequent clinical trials
Mitchell Syndrome	*ACOX1* (p.N237S)	*acox1* (p.N250S) mutant model	fly model with dACOX1 N250S mutation demonstrated neurodegenerative phenoypes, decreased axon number, and elevated ROS levels	Antioxidant treatment suppressed neurodegenerative phenotypes in fly models
Huntington's Disease	*HTT* protein	Mutant flies expressing human *HTT* in cardiomyocytes	Replicated neural pathology and clinical cardiac phenotypes (cardiac amyloidosis)	Combined rapamycin+lithium therapy provided enhanced neuroprotection
Spinal Muscular Atrophy	*SMN1*	Transgenic flies expressing patient-derived *SMN* missense mutations	Successfully replicated motor dysfunction observed in human patients	Provides a framework for disease modeling and therapeutic focus
Duchenne Muscular Dystrophy	*DMD* (*Dys*)	Ortholog mutant (*dys*) reproducing defective Dg-Dys complex	Demonstrated that DMD involves significant neurologic abnormalities	The multifactorial nature of DMD increases options for therapeutic targets
Myotonic Dystrophy	*CNBP*	Selective silencing of *cnbp* gene	Allowed for the study of dual mechanisms in MD pathogenesis	Future screening of therapeutic agents can be conducted using patient-specific models
Barth Syndrome	*TAFAZZIN*	*Tafazzin* (ortholog) mutant model	Allowed for the study of cardiolipin remodeling and mitochondrial dysfunction	Potential therapeutic avenues targeting mitochondrial membrane composition
Leigh Syndrome	*SURF1*	Post-transcriptional silencing of *surf1* ortholog	Replicated decreased COX activity and mitochondrial DNA haplotype-driven severity	Variable expressivity allows for patient-specific therapeutic studies
Niemann-Pick Disease type C	*NPC1/NPC2*	Null mutants of *npc1a* (ortholog)	Linked lipid dysregulation to altered neurotransmission and neurodegeneration	Findings led to clinical trials studying cyclodextrin for cholestoral homeostasis
Mucopolysaccharidosis	*HGSNAT/NAGLU*	Drosophila knockdown mutants	Replicated neurological defects, sleep disturbances, and clinical pathologies	Knockdown models help elucidate cellular pathways involved in MPS
Fabry Disease	*GLA* (alpha-Gal A)	Transgenic fly model expressing human *GLA* variants	Premature cell death in dopaminergic cells due to misfolded alpha-Gal A	Migalastat (chaperone) relieved ER stress response in certain fly mutants
Gaucher Disease	*GBA1* (Gcase)	Mutated *gba1a* and *gba1b* (orthologs)	Homozygous mutants replicated symptoms of neuronopathic GD	Rescued with Ambroxol, establishing GD mutants as a robust drug-screening models
Usher Syndrome	USH gene family (e.g., *USH1, USH2*)	Orthologs Usher network (e.g.,*sano*)	Useful in explaining why patients experience hearing and vision loss	Genetic interaction screens identified modifiers of Usher-associated degeneration
Retinitis Pigmentosa	*PRPF8*	Ortholog *prp8* mutant model	Enabled tracking of the clinical severity of patient-specific mutations	Better understanding of mutations helps understand the disease process
Charcot-Marie-Tooth	*ARS* family, *MFN2, SORD*	Flies expressing *YARS/TyrRS* or *SORD* deficiency	TyRS nuclear localization disrupts transcription; *SORD* deficiency identified polyol pathway	Highlighted axonal maintenance; i dentified polyol pathway as a target for intervention
Chronic Granulomatous Disease	NADPH Oxidase	Drosophila *dDuox* model	Important for studying conserved ROS-dependent antimicrobial mechanisms	Provide a foundation to study potential therapeutic targets for CGD
MyD88 & IRAK4 Deficiency	*MyD88* & *IRAK4*	dmyd88 (ortholog) and *tube* mutants	Increased susceptibility to fungal and gram-positive bacterial infections	Pinpointed protein functions and subsequent pathogen susceptibility
Crohn's Disease	*NOD2*, *JAK/STAT*	Conserved *JAK/STAT, MAPK*, and *Wnt* pathways	Studied effects of chronic intestinal inflammation on gut homeostasis	Studies on novel mechanisms in gut homeostasis provide future interventions
Gliomas	*EGFR*, *PI3K*	Co-activation of *EGFR* and *PI3K* in glial cells	Replicated high-grade gliomas characteristics; identified *cic* as a MAPK effector	Identified Artemisinin as a selective apoptosis inducer in tumor cells
Colorectal Cancer	*APC* (Loss of function)	*apc* knockout and multigenic *CRC* variants	Wnt/beta-catenin pathway dysregulation; enabled HTS for drug discovery	Led to the identification of combination therapies (e.g., trametinib and panitumumab)
Medullary Thyroid Carcinoma	*RET* proto-oncogene	Models expressing human *RET*	Allowed for rapid screening of potential anti-cancer compounds	Fly-based screens helped identify Vandetanib as a therapeutic target for MTC
Cancer Cachexia	*Upd3, ImpL2* (IL-6)	Tumor-bearing Drosophila models	Identify *ImpL2* as a protein driving tissue wasting and cachexia	Understanding the pathogenesis allows for refined candidate targets

Efficiency, scalability, and cost-effectiveness of *Drosophila* in research

A further significant advantage of the fruit fly is its rapid life cycle of approximately 10-14 days and high reproductive capacity, allowing for rapid generation of large experimental populations, multigenerational studies, and statistically robust analyses. Compared to vertebrate models, maintaining these insects in the laboratory is simpler and far more cost-effective, requiring only basic food and minimal space. These features, along with their small size and ease of handling, make this model organism an ideal platform for high-throughput screening (HTS). This enables large-scale genetic and pharmacological screens of compound libraries, facilitating the early assessment of bioavailability and potential toxicity [20,21].

APPLICATION OF *DROSOPHILA* MELANOGASTER IN RARE DISEASE MODELING

As a key model organism in biomedical research, *Drosophila* provides valuable insight into the mechanisms underlying a broad range of human diseases. Due to the fruit fly's evolutionarily conserved major signaling pathways, simplified biology, and genetic tractability, it serves as an ideal platform for studying human diseases. Through this model, researchers have identified disease-associated genes, uncovered pathogenic mechanisms, and investigated potential therapeutic strategies. Consequently, the fruit fly remains central to advancing our understanding of complex diseases. This section reviews the utilization of *Drosophila* in rare disease research, with a focus on neuronal diseases, muscular disorders, metabolic diseases, sensory disorders, immunological disorders, and rare cancers (Table 1). In addition, this section examines the experimental strategies utilized to dissect the genetic, molecular, and cellular mechanisms underlying these processes.

Research on neuronal disorders using *Drosophila* models

Rett syndrome

Rett Syndrome (RTT) is a severe neurodevelopmental disorder predominantly affecting females, primarily caused by mutations in the methyl-CpG binding protein 2 (MECP2) gene [22]. As the fruit fly lacks a direct homolog of MECP2, disease models were created by overexpressing the human MECP2 gene and RTT patient-derived mutant alleles (R106W, R294X, and D166) in the fly. This model displayed abnormal phenotypes in the eye, wing veins, and motor function [23]. A genetic screen using this overexpression model revealed that a loss of function in Sin homolog A (Sin3A), a component of the histone deacetylase complex, amplified the phenotypes, while a loss of function in the co-repressors Smr and the transcription factor crooked legs (crol) suppressed them. This finding suggests that potential RTT treatment strategies could target MeCP2-interacting factors [23].

Drosophila models of Rett syndrome have further demonstrated that MeCP2 dysfunction leads to alterations in synaptic plasticity and dendritic morphology, phenotypes that are amenable to genetic suppressor screening. These studies have identified chromatin-remodeling factors and signaling molecules that partially rescue MeCP2-associated neuronal defects, providing candidate targets for therapeutic development.

MECP2 duplication syndrome

MECP2 duplication syndrome is a rare X-linked genomic disorder caused by duplications encompassing the MECP2 gene at Xq28, primarily affecting males. Clinical features include severe intellectual disability, infantile hypotonia, progressive spasticity, recurrent respiratory infections, and epilepsy [22]. While Rett syndrome results from loss-of-function mutations in MECP2, duplication syndrome represents the reciprocal gain-of-function condition, underscoring the critical importance of MECP2 dosage balance for normal neurological development. In *Drosophila*, overexpression of human MeCP2 in neurons has been shown to cause locomotor dysfunction, neurodegeneration, and alterations in dendritic morphology, recapitulating key aspects of the human disease [23]. These fly models have been instrumental in dissecting the molecular pathways disrupted by MeCP2 overexpression, including alterations in chromatin remodeling and transcriptional regulation. Furthermore, *Drosophila*-based genetic screens have identified modifiers of MeCP2-induced toxicity, providing candidate therapeutic targets that warrant further investigation in mammalian systems [23].

Fragile X Syndrome

Fragile X Syndrome (FXS) is the most common heritable cause of intellectual disability, resulting from the loss of function of the FMR1 gene. Studies using the *Drosophila* model with a knockout of the FMR1 homolog (*dfmr1*) have provided a multifaceted understanding of pathogenic mechanisms [24]. In this model, GABAergic circuit dysfunction was observed in the mushroom body, the learning and memory center of the fly brain, accompanied by downregulation of the GABA syn-

thesis enzyme glutamic acid decarboxylase and reduced GABA receptor protein levels [23]. Beyond neuronal defects, *dfmr1* mutant flies exhibited impaired phagocytic activity by both innate immune cells and glial cells, resulting in delayed neuronal clearance following injury and postponed mushroom body development [25]. This dual phenotype highlights that FXS pathology is not confined to neuronal dysfunction but also involves complex interactions with brain immune responses. The fly model thus enables the dissection of these multi-systemic effects, offering valuable insight for identifying novel therapeutic targets, including strategies for immune modulation [25].

Mitchell Syndrome

Mitchell Syndrome, which results from *de novo* gain-of-function mutations in the Acyl-CoA oxidase 1 (ACOX1, human p.N237S; *Drosophila* p.N250S), has been modeled using the fruit fly [21,26]. Unlike ACOX1 loss-of-function mutations, this variant leads to elevated ACOX1 protein levels and enzymatic activity, resulting in excessive production of reactive oxygen species (ROS) in glial cells rather than accumulation of very long-chain fatty acids (VLCFAs). Flies expressing the equivalent dACOX1^{N250S} mutation exhibit severe neurodegenerative phenotypes, including marked lethality within two days, impaired locomotion, and extensive glial and axonal degeneration [26]. Transmission electron microscopy revealed a drastic reduction in axon number, along with abnormal multilamellar bodies and disrupted membrane structures. Notably, wrapping glia were vulnerable to elevated ROS levels induced by dACOX1^{N250S} expression [26]. These gain-of-function phenotypes driven by ROS were potently suppressed by antioxidant treatment, including N-acetyl cysteine amide (NACA) administration and catalase overexpression. Importantly, NACA treatment restored viability, locomotor activity, as well as mating and reproductive capacity [26].

Huntington's disease

Huntington's disease (HD) is a fatal neurodegenerative disorder caused by an abnormal polyglutamine (polyQ) repeat expansion in the Huntingtin (HTT) protein [27]. Although HD primarily affects the nervous system, more than 30% of patients die from heart failure, suggesting a strong association between neurodegeneration and cardiac dysfunction. The fly model has been instrumental in elucidating not only neural pathology but also cardiac amyloidosis associated with HD [27]. Flies expressing mutant HTT in cardiomyocytes recapitulate key clinical cardiac phenotypes, including severe cardiac dilation, contractile impairment, and arrhythmias, closely resembling those observed in patients. These abnormalities are linked to oxidative stress induced by amyloid aggregates, disorganization of myofibrils, and loss of actin-myosin-containing fibers. Notably, cardiac-specific expression of toxic polyQ alone was sufficient to induce both functional and structural cardiac defects [27].

Spinal Muscular Atrophy

Spinal Muscular Atrophy (SMA) is a neurodegenerative disorder caused by mutations in the SMN1 gene and is characterized by progressive muscle weakness and paralysis [28]. A loss-of-function *Drosophila* model for the Smn gene has been established, and notably, 14 transgenic fly lines expressing patient-derived *SMN* missense mutations have been generated [29]. These models faithfully recapitulate the progressive motor dysfunction observed in human patients. Consistent with the human condition, adult flies exhibited a progressive loss of motor function that initially manifested in the posterior limbs. The severity and onset of phenotypes varied depending on the specific mutation, and importantly, even late-onset SMA, which has been challenging to model in other animal systems, was effectively reproduced in *Drosophila* [28,29].

Research on muscular disorders using *Drosophila* models

Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is a progressive muscle-weakening disorder caused by mutations in the Dystrophin (Dys) gene [30]. A *Drosophila* model that reproduces defects in the Dystroglycan-Dystrophin (Dg-Dys) complex successfully mimics the muscle degeneration phenotypes observed in human patients. Mutant flies exhibit age-dependent muscle degeneration and locomotor impairment. Additionally, this model demonstrated that the Dg-Dys complex is essential for proper axon pathfinding in muscle cells, glial cells, and neurons. These findings indicate that DMD is not solely a myopathy but also involves neurological abnormalities, underscoring the utility of the fly model in dissecting the multi-systemic pathology of the disease [30].

Myotonic Dystrophy

Myotonic Dystrophy (DM) is a multisystem disorder caused by the expansion of CTG or CCTG repeats. Specifically, Myotonic Dystrophy type 2 (DM2) results from a CCTG repeat ex-

pansion in the CNBP gene and involves two main pathogenic mechanisms: loss of gene function and toxic RNA gain of function. The *Drosophila* model has been instrumental in investigating these two mechanisms independently [30]. Selective silencing of the CNBP gene in fruit flies leads to severe locomotor defects, which are attributed to the loss of CNBP-mediated regulation of polyamine metabolism. In contrast, the expression of the toxic CCTG repeat sequence alone reproduces key molecular phenotypes, such as the formation of ribonuclear foci, sequestration of the Muscleblind-like (MBNL) protein, and resulting defects in alternative splicing. Significantly, drug screening using this model has identified several candidate compounds that improve locomotor dysfunction [30].

Research on metabolic disorders using *Drosophila* models

Barth syndrome

Barth syndrome (BTHS) is an X-linked multi-systemic disorder caused by a mutation in the TAFAZZIN gene and characterized by dilated cardiomyopathy, skeletal muscle weakness, and abnormal metabolism of the mitochondrial phospholipid cardiolipin [31]. The *Drosophila* Tafazzin mutant model recapitulates the key pathological features of the disease, including an approximately 80% reduction in cardiolipin levels, abnormalities of mitochondrial morphology such as defects in the cristae membrane, and motor weakness as measured by flight and climbing assays [31].Importantly, the *Drosophila* Tafazzin model has facilitated the investigation of cardiolipin remodeling pathways and their relationship to mitochondrial dysfunction. Genetic interaction studies have identified suppressors of tafazzin-associated phenotypes, suggesting potential therapeutic avenues targeting mitochondrial membrane composition [31]. These findings complement mammalian studies and underscore the utility of the fly model for dissecting the metabolic and bioenergetic defects underlying Barth syndrome.

Leigh syndrome

Leigh syndrome is a mitochondrial disease caused by loss-of-function mutations in mitochondrial proteins involved in the mitochondrial respiratory chain, particularly the Surfeit locus protein 1 (SURF1) protein, which is required for the proper assembly of cytochrome c oxidase (COX). It is phenotypically varied but is generally characterized by early-onset neurological decline, altered energy metabolism, intellectual and motor impairment, and early death [32]. *Drosophila* models of Leigh syndrome, generated through post-transcriptional silencing of the Surf1 ortholog, accurately recapitulate the clinical hallmark of decreased COX activity *in vivo*. These flies exhibit reduced lifespan, locomotor impairment, and mitochondrial morphological abnormalities, including disorganized cristae. Additionally, mito-nuclear interaction studies in the fly have revealed that the severity of Leigh syndrome phenotypes is modulated by mitochondrial DNA haplotype, providing insight into the variable expressivity observed in patients [32].

Lysosomal Storage Disorders

Lysosomal Storage Disorders (LSDs) are a heterogeneous group of rare inherited metabolic diseases caused by mutations in genes encoding lysosomal acid hydrolases, membrane proteins, or transporters, leading to the accumulation of undegraded substrates within the endolysosomal system [33]. This impaired degradative capacity disrupts key cellular processes, including endolysosomal trafficking and autophagy, and results in a wide spectrum of clinical manifestations, frequently involving the central nervous system as well as multiple peripheral organs [33]. Although significant progress has been made in understanding LSD pathogenesis, effective therapies remain limited, particularly for neurological symptoms. In this context, the fruit fly has emerged as a useful *in vivo* model for studying lysosomal function and related cellular pathways. Among the various LSDs, several representative disorders have been extensively modeled in *Drosophila*, including Niemann-Pick Disease (NPD), Mucopolysaccharidoses (MPS), Fabry Disease, and Gaucher Disease (GD). NPD is a rare lysosomal storage disorder caused by mutations that result in loss of function in the Sphingomyelin Phosphodiesterase 1 (SMPD1) gene (type A and B) or the Niemann-Pick type C1 (NPC1)/NPC2 genes (type C). These mutations impair intracellular lipid trafficking, leading to the accumulation of cholesterol, glycosphingolipids, and other lipid species within late endosomes and lysosomes. This accumulation disrupts cellular lipid homeostasis, contributing to progressive neurodegeneration and systemic complications. [34]. In *Drosophila*, null mutants of the NPC1 homolog Npc1a, which is ubiquitously expressed, show cholesterol accumulation and disrupted lipid trafficking, recapitulating key features of NPD [35]. These mutant flies exhibit increased glutamatergic neurotransmission, a trait also found in flies with mutations in the brainiac gene, which inhibits glycosphingolipid synthesis. This suggests a connection between lipid dysregulation, altered neurotransmission, and neurodegeneration [35]. In parallel, flies lacking the acid sphin-

gomyelinase orthologue dASM, which corresponds to the human SMPD1 gene, display developmental and respiratory defects associated with sphingolipid storage dysfunction, and their phenotypes can be partially rescued by genetic or pharmacological interventions [34]. These findings in the *Drosophila* NPD have provided a framework for understanding the neurodegenerative cascade triggered by lipid accumulation, and have facilitated the testing of pharmacological agents, including cyclodextrin derivatives, aimed at restoring cholesterol homeostasis. MPS are LSDs that result from an inability to properly break down cellular glycosaminoglycans (GAGs) and their subsequent accumulation in lysosomes. Enzymes responsible for GAG breakdown include heparan-α-glucosaminide N-acetyltransferase and alpha-*N*-acetylglucosaminidase. These are implicated in MPS types IIIC and IIIB, respectively [36,37]. To simulate enzymatic dysfunction, researchers have used *Drosophila* knockdown mutants and have observed neurological defects, sleep and activity disturbances, and clinically equivalent disease pathologies [36,37]. Further use of *Drosophila* as an MPS model may provide further insight into the cellular pathways involved in MPS-mediated neurodegeneration and a platform to develop therapeutics targeting implicated genes.

Fabry disease is an X-linked recessive LSD caused by mutations in the galactosidase alpha (GLA) gene, which encodes lysosomal α-galactosidase A (α-Gal A). Mutant α-Gal A is retained in the endoplasmic reticulum (ER), leading to organelle stress, ER-associated degradation (ERAD), and unfolded protein response (UPR), which were tracked by Braunstein et al. *in vivo* with transgenic *Drosophila* expressing α-Gal A variants [38]. In dopaminergic cells, misfolded α-Gal A was shown to have led to premature cell death due to the associated consequences of ER retention, but an α-Gal A chaperone, migalastat, was shown to relieve the ER stress response with certain α-Gal A mutants [38]. GD is an LSD caused by mutations in the GBA1 gene, which codes for acid-β-glucocerebrosidase (GCase). Similarly to Fabry disease, mutant GCase is retained in the ER, leading to ERAD and UPR [38]. Cabasso et al. mutated GBA1 orthologs in *Drosophila*, GBA1a and GBA1b and examined the effects of sequence truncations. The GBA1b homozygous mutant exhibited ERAD, UPR activation, reduced GCase activity, inflammation, and neuroinflammation that is seen in neuronopathic GD [39]. This mutant was partially rescued with Ambroxol, relieving all inflammatory effects and UPR, but did not increase GCase activity. Altogether, researchers have concluded that GBA1b homozygous mutant flies can serve as a robust model for neuronopathic GD and can be used to test therapies and for further study of disease mechanisms [39]. Overall, *Drosophila* models of LSD effectively replicate the common pathogenic cascade of protein misfolding, ER stress, and subsequent cellular dysfunction. This provides a robust *in vivo* platform for validating pharmacological chaperones as a universal therapeutic strategy.

Research on sensory disorders using *Drosophila* models

Usher Syndrome

Usher Syndrome (USH) is a genetic disorder that leads to both hearing and vision loss, and is characterized by defects in proteins involved in sensory cell function. Several USH-associated proteins have conserved functional counterparts in flies, enabling genetic analysis of sensory protein networks *in vivo* [40], For example, components of the Usher protein network, including scaffold proteins such as harmonin (USH1C) and SANS (USH1G), have conserved functional roles in sensory systems, and *Drosophila* orthologs or functional homologs of these proteins are used to study their roles in cellular organization and trafficking in sensory cells [40]. Additionally, fruit fly sensory organs such as Johnston's organ are used to study mechanosensory transduction mechanisms that are evolutionarily conserved and relevant to understanding hereditary hearing loss [40].

The *Drosophila* Usher syndrome model has been valuable for understanding the mechanistic basis of sensory cell degeneration. Studies have shown that disruption of the Usher protein complex leads to defects in mechanotransduction and photoreceptor maintenance, providing insight into why patients experience both hearing and vision loss. Furthermore, genetic interaction screens in the fly have identified modifiers of Usher-associated photoreceptor degeneration, offering candidate targets for therapeutic intervention aimed at slowing retinal deterioration.

Retinitis pigmentosa

Retinitis pigmentosa (RP) is a group of hereditary retinal disorders characterized by the progressive degeneration of retinal photoreceptor cells [41]. RP type 13 is linked to pre-mRNA processing factor 8 (PRPF8), an important spliceosome component. Over 20 different mutations of PRPF8 have been clinically identified, and Stanković et al. have used the *Drosophila* model to elucidate the mechanisms of its expression *in vivo* using prp8, a fly ortholog. They used the GAL4/UAS system to induce tissue-specific expression of mutant prp8 transgenes and tracked the development of prothoracic gland cells and

eye/antennal imaginal discs. The two most pathogenic prp8 mutants contributed to adult eye defects, suggesting induction of cell death, and the clinical severity of each respective mutation matched the pathogenicity of the same mutants overexpressed in *Drosophila* [41].

Charcot-Marie-Tooth disease

Charcot-Marie-Tooth disease (CMT) is a group of inherited motor and sensory neuropathies characterized by progressive distal muscle weakness and sensory loss. More than 100 genes containing disease-causing mutations have been identified to date, reflecting the high genetic heterogeneity of the condition [42]. One of the most common implicated groups of genes is the aminoacyl-tRNA synthetase (ARS) family, which is the largest class of proteins associated with axonal CMT and is crucial for protein translation and neuronal maintenance [43].

In *Drosophila* models, several CMT-associated ARS genes have been functionally analyzed. For instance, expressing mutant tyrosyl-tRNA synthetase (YARS/TyrRS) in flies leads to progressive motor dysfunction and neurodegeneration, reproducing key features of human CMT [44]. These models demonstrate that CMT-linked ARS mutations act mainly through toxic gain-of-function mechanisms rather than a simple loss of enzymatic activity [44]. Notably, the nuclear localization of mutant TyrRS has been shown to contribute to the disease by disrupting transcriptional regulation in neuronal tissues. Preventing the entry of mutant TyrRS into the nucleus in *Drosophila* can significantly rescue neurodegenerative symptoms, emphasizing nuclear dysfunction as a potential therapeutic target [44].

Beyond ARS genes, fly models have also provided crucial insights into mitochondrial dynamics, particularly regarding mutations in Mitofusin 2 (MFN2), which is the most common cause of CMT2A. The *Drosophila* ortholog Mitochondrial assembly regulatory factor (Marf) is essential for mitochondrial fusion and axonal transport. Research using Marf-deficient models has shown that mitochondrial depletion in distal axons is accompanied by increased endoplasmic reticulum stress, evidenced by the upregulation of markers such as Xbp1 and Bip [42]. Furthermore, these models suggest that neuronal survival is more dependent on the mitochondrial capacity for ATP production than on their specific physical distribution, providing a deeper understanding of axonal maintenance [42]. Recent studies in flies have also expanded the focus to metabolic and membrane trafficking pathways. The loss of sorbitol dehydrogenase (SORD) was recently identified as a common cause of recessive CMT2. *Drosophila* models lacking SORD show elevated intracellular sorbitol levels and progressive motor impairment, identifying the polyol pathway as a novel target for intervention [42]. Similarly, models of FIG4 (associated with CMT4J) have revealed that defects in phosphoinositide metabolism led to enlarged endolysosomal vesicles and impaired membrane trafficking [42]. Collectively, these fly models have provided vital insights into conserved disease mechanisms, including axonal degeneration, transcriptional dysregulation, and metabolic imbalances, serving as powerful platforms for evaluating new therapeutic strategies.

Research on immunological disorders using *Drosophila* models

Although *Drosophila melanogaster* lacks an adaptive immune system, it has emerged as a pivotal model for studying innate immunity due to its evolutionary conservation of key signaling pathways with humans [45]. The immune response in flies can be divided into two main systems, humoral and cellular. The humoral response involves the production of antimicrobial peptides (AMPs) through the Toll and signaling network. In contrast, the cellular response is mediated by specialized cells called hemocytes, including Plasmatocytes and lamellocytes, which function similarly to human macrophages [45]. Interestingly, the discovery of mammalian Toll-like receptors (TLRs) originated from research on the *Drosophila* Toll pathway. This highlights how studying this simplified model can provide valuable insight into the complex cell-signaling mechanisms that are involved in human immunological disorders [45].

Chronic Granulomatous Disease

Chronic Granulomatous Disease (CGD) is caused by a defect in Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase, an enzyme used by mammalian phagocytes to eliminate invading pathogens through reactive oxygen species (ROS) [46]. Impaired ROS generation leads to recurrent infections and dysregulated inflammatory responses [46]. In *Drosophila*, the NADPH oxidase homolog dual oxidase (dDuox) plays a central role in mucosal immunity by generating ROS in the gut epithelium, thereby restricting microbial growth [47]. In addition to dDuox, ROS-mediated immune responses are tightly regulated to maintain intestinal homeostasis, preventing excessive damage to host tissues [48]. Moreover, both ROS production and its regulation are essential for sustaining intestinal immune homeostasis in *Drosophila* [48]. Although the fruit fly does not have professional phagocytes equivalent to

those found in mammals, it serves as a valuable model for investigating conserved ROS-dependent antimicrobial mechanisms and their regulation. These features make the fly a useful complementary model for understanding key aspects of CGD-related pathophysiology.

MyD88 and IRAK4 deficiency

MyD88 deficiency is an autosomal recessive mutation in myeloid differentiation primary response 88 (MyD88) adaptor protein that impairs toll-like receptor signaling [49]. When a PAMP binds to a TLR in the innate immune response, MyD88 is recruited and subsequently activates interleukin-1 receptor-associated kinase-4 (IRAK-4) [49]. IRAK-4 later activates NF-κB, which calls for transcription of pro-inflammatory cytokines, such as IL-1, IL-6, and TNF- α. Impaired MyD88 and IRAK4 increase susceptibility to bacterial infections, especially *Streptococcus pneumonia*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* [49]. Additionally, MyD88 deficiency disrupts NF-κB activation, thereby impairing IL-1 receptor and TLR ligand responses [49]. The conservation and homology of the TLR pathway in flies and humans have allowed researchers to study these primary immunodeficiencies [50]. The *Drosophila* protein Tube acts downstream of TLR, like IRAK-4 in mammals, to promote signal transduction [50]. DmMyD88 in *Drosophila* is the homolog of mammalian MyD88, and a deficiency in this protein was shown to increase susceptibility to fungal and gram-positive bacterial infections in flies [51].

Crohn's disease

Drosophila melanogaster has been used to study inflammatory bowel disease, like Crohn's disease, for many years [52]. Crohn's disease is characterized by chronic intestinal inflammation, leading to repeated epithelial injury. The fly models helped elucidate that EGFR/MAPK/Ras activation promotes cell growth and proliferation in order to repair the intestinal epithelium [53]. Additionally, epithelial immune responses in mammals are similar to those seen in *Drosophila*. JAK/STAT, EGFR/MAPK, JNK, and Wnt/β-catenin regulate inflammation and epithelial regeneration in flies and mammals [54]. Through the study of intestinal inflammation using conserved pathways, novel insights into the molecular regulation of gut homeostasis and epithelial repair have been identified [52].

Research on Cancer Using *Drosophila* Models

The fruit fly has emerged as a valuable model for cancer research, offering unique advantages for studying the fundamental mechanisms of tumorigenesis, metastasis, and therapeutic responses. Approximately 70% of known human cancer genes have conserved orthologs in the fly, including key tumor suppressors and oncogenes such as Rb, p53, PTEN, APC, Ras, and Notch [20]. The genetic tractability of *Drosophila* allows researchers to model specific oncogenic mutations and study their effects on cell growth, invasion, and tissue homeostasis *in vivo*.

Glioma and neurological malignancies

Malignant gliomas are characterized by the co-activation of EGFR and PI3K signaling pathways, leading to aggressive brain tumor progression. The co-activation of EGFR and PI3K in *Drosophila* glial cells successfully replicates the characteristics of human high-grade gliomas. Additionally, studies on the capicua (cic) gene in flies have elucidated its role as a MAPK effector and its involvement in oligodendrogliomas [55]. Pharmacological interventions in these models have shown that compounds like artemisinin can selectively induce apoptosis in tumor cells and improve locomotor activity in affected larvae [55].

Colorectal cancer

Colorectal cancer (CRC) is primarily caused by the abnormal activation of the Wnt signaling pathway, with loss-of-function mutations in the Adenomatous Polyposis Coli (APC) gene occurring in approximately 80% of human cases. In *Drosophila* midgut models, the loss of APC induces epithelial hyperplasia, resembling human adenomas. This process has been found to require the activation of the non-receptor tyrosine kinase c-Src [55]. Furthermore, personalized fly models incorporating complex multigenic CRC patients have enabled HTS. This approach has led to the identification of effective combinations such as trametinib and panitumumab that were subsequently validated in clinical settings [20].

Thyroid and lung cancer

Medullary thyroid carcinoma (MTC) frequently arises from oncogenic mutations in the RET proto-oncogene. *Drosophila* models expressing human RET mutants have served as robust platforms for identifying therapeutic inhibitors. Notably, the multi-kinase inhibitor Vandetanib (ZD6474) was identified through fly-based screens and later approved for clinical use in MTC patients [20,55]. Similarly, models of EGFR-mutant lung cancer in flies have been used to evaluate the *in vivo* efficacy of various tyrosine kinase inhibitors against specific oncogenic

variants, enabling rapid screening of potential anti-cancer compounds [20,55].

Cancer cachexia and systemic paraneoplastic syndromes

Advanced cancer often triggers cachexia, a systemic wasting syndrome responsible for up to 30% of cancer-related deaths, characterized by progressive loss of muscle and adipose tissue. Research using tumor-bearing *Drosophila* has identified ImpL2, an insulin-binding protein, as a critical tumor-derived factor that antagonizes systemic insulin signaling to drive wasting [56]. Beyond localized energy loss, these models have revealed that inflammatory mediators like TNF-α (Eiger) and IL-6 (Upd) induce multifactorial pathologies, including renal dysfunction, hepatic gluconeogenesis, and the breakdown of the blood-brain barrier (BBB), highlighting the complexity of tumor-host interactions [56].

LIMITATIONS AND ADVANCES IN MODELING RARE DISEASE USING *DROSOPHILA*

Despite its widespread use as a genetic model organism, *Drosophila melanogaster* has several intrinsic limitations when applied to human rare disease research. Anatomically, it lacks key organs involved in many human pathologies, including the lungs, kidneys, and components of the adaptive immune system. These structural differences constrain its ability to recapitulate certain tissue-specific conditions [12]. Moreover, while many core signaling pathways are evolutionarily conserved, approximately 25 to 30 percent of human genes lack direct orthologs in the fly genome, posing challenges in modeling gene-specific disorders [57]. To overcome these limitations, researchers have developed innovative strategies that extend the utility of the fly model (Fig. 2). One such approach is the use of "humanized flies", where human disease-causing genes or their variants are expressed in flies to assess function, pathogenicity, and interaction with conserved pathways [12,58]. Moreover, genome editing tools such as CRISPR/Cas9 allow precise substitution of endogenous sequences with human counterparts, facilitating the creation of phenotypes with higher degrees of clinical accuracy [57]. In parallel, behavioral tests such as climbing assays or circadian rhythm measurements are used as functional indicators of disease-related phenotypes, including motor impairment and muscle degeneration [58]. Importantly, insights gained from the fruit fly experiments are often complemented with data from other model systems, including zebrafish, murine models, and patient-derived cells (e.g., human induced pluripotent stem cells). This integrative approach helps validate findings across species and adds translational depth to genetic and molecular discoveries [12,57]. While the fruit fly, as a model organism, cannot fully replicate human complexity, its genetic tractability and efficiency make it an indispensable first-line tool for functional genomics and

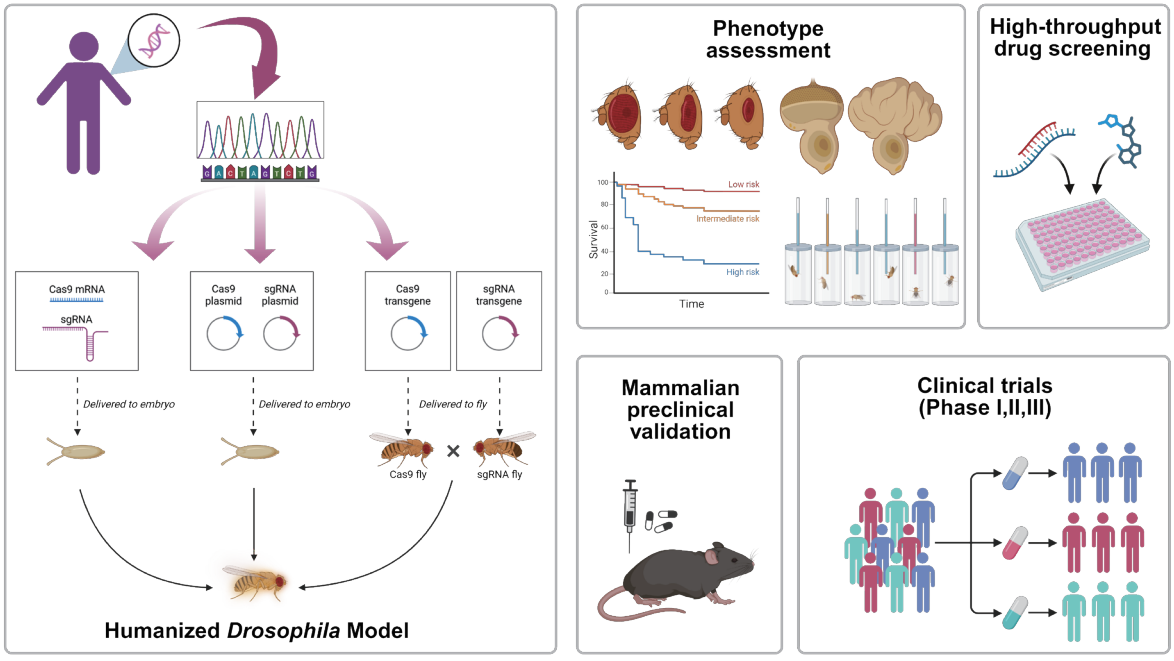


Fig. 2. From Gene to Clinic: A Translational Framework for Human Disease Modeling in *Drosophila*.

rare disease research [58,59]. Notably, collaborative initiatives such as the Undiagnosed Diseases Network (UDN) have demonstrated the power of integrating *Drosophila* functional studies with clinical genomics to resolve undiagnosed cases. In the UDN pipeline, candidate variants of uncertain significance identified through exome or genome sequencing are modeled in *Drosophila* to assess pathogenicity, leveraging the rapid generation of transgenic and knockout flies to provide functional evidence within clinically relevant timescales. Complementary bioinformatic resources, including the Model Organism Aggregated Resources for Rare Variant ExpLoration (MARRVEL) database, facilitate the cross-referencing of human genetic data with model organism phenotypes, streamlining variant interpretation and accelerating diagnosis.

CONCLUSION

The wide variety of animal models available in biomedical research has greatly enhanced our understanding of human physiology and disease. Among these, *Drosophila melanogaster* has played an essential role in scientific advancements for over 120 years, making it an attractive model for studying both established and emerging diseases. Although fly models have inherent limitations, these can be mitigated by focusing on conserved biological pathways, integrating complementary model systems, and employing humanization strategies. Moreover, combining *Drosophila* with other animal models provides a more comprehensive framework for understanding complex disease mechanisms. This approach is particularly valuable in preclinical drug evaluation and translational research.

Rare diseases continue to pose significant medical and social challenges, and research in this area faces numerous obstacles. Over more than a century, research using the fruit fly has generated extensive genetic knowledge and yielded robust experimental tools, establishing it as an indispensable platform for investigating complex biological processes. Its applications span elucidating fundamental biological principles, dissecting mechanisms of human disease, validating patient-derived genetic variants, and identifying novel therapeutic candidates.

Despite evolutionary and physiological differences between fruit flies and humans, these challenges can be mitigated through the study of conserved pathways, the use of complementary models, and humanization approaches. In addition, recent advances in multi-omics approaches, integrating transcriptomics, proteomics, and metabolomics, have enabled a more comprehensive understanding of disease mechanisms at the systems level in *Drosophila*. By combining fly models with patient-derived organoids and induced pluripotent stem cell-based systems, researchers can enhance translational relevance and support precision medicine efforts. Together, these strategies will ensure that *Drosophila* models continue to play an important role in diagnosing and developing therapies for rare diseases.

NOTES

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Conflicts of interest

The authors declare no conflicts of interest.

Ethical statement

This article is a review and does not involve any studies with human participants or animals performed by the authors. No ethical approval was required.

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Author contributions

S.K. drafted the initial and revised manuscript. A.K. contributed to writing the sections on retinitis pigmentosa and Charcot-Marie-Tooth disease, as well as Fig. preparation. S.F. contributed to writing the sections on lysosomal storage disorders and table preparation. H.-L.C. conceived the review, supervised all aspects of the work, and critically revised the manuscript. All authors read and approved the final manuscript.

Use of artificial intelligence

During the preparation of this manuscript, the authors used PubMed, Google Scholar, ChatGPT, and Gemini to assist with literature searches. All references were independently reviewed and verified by the authors before being selected for inclusion. AI tools were also used for English-language editing, including grammar correction and tone refinement. These tools were not used to generate scientific content or figures. The authors carefully reviewed all content, including the references, and assumed full responsibility for the published work.

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Preimplantation Genetic Testing for Rare Monogenic Disease: A Female Reproductive Medicine Perspective on the Clinical Pathway

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Preimplantation genetic testing for monogenic disorders (PGT-M) allows couples at high risk of transmitting a rare genetic disease to achieve an unaffected pregnancy while substantially reducing the likelihood of pregnancy termination after prenatal diagnosis. Although PGT-M is often presented as a molecular genetic technique, its clinical success also depends on reproductive variables: ovarian reserve, ovarian response to controlled stimulation, blastocyst yield, and obstetric management of the resulting pregnancy. This review follows the PGT-M pathway chronologically, from genetic diagnosis and pre-cycle workup through ovarian stimulation, embryo biopsy and genetic analysis, embryo transfer, and confirmatory prenatal diagnosis, and considers each step from the perspective of female reproductive medicine. Particular attention is given to the embryo attrition cascade that determines whether a transferable embryo will be available; the interpretation of outcomes per couple rather than per transfer; carrier states in which the pathogenic variant itself compromises ovarian function; and the counseling and regulatory framework applicable in Korea. PGT-M is best regarded not as a laboratory service but as a multidisciplinary program in which clinical genetics, the PGT laboratory, embryology, reproductive medicine, and obstetric care each contribute to the goal of a healthy live birth.

Key words: Preimplantation diagnosis, Genetic testing, Rare diseases, Genetic counseling, Fertilization in vitro

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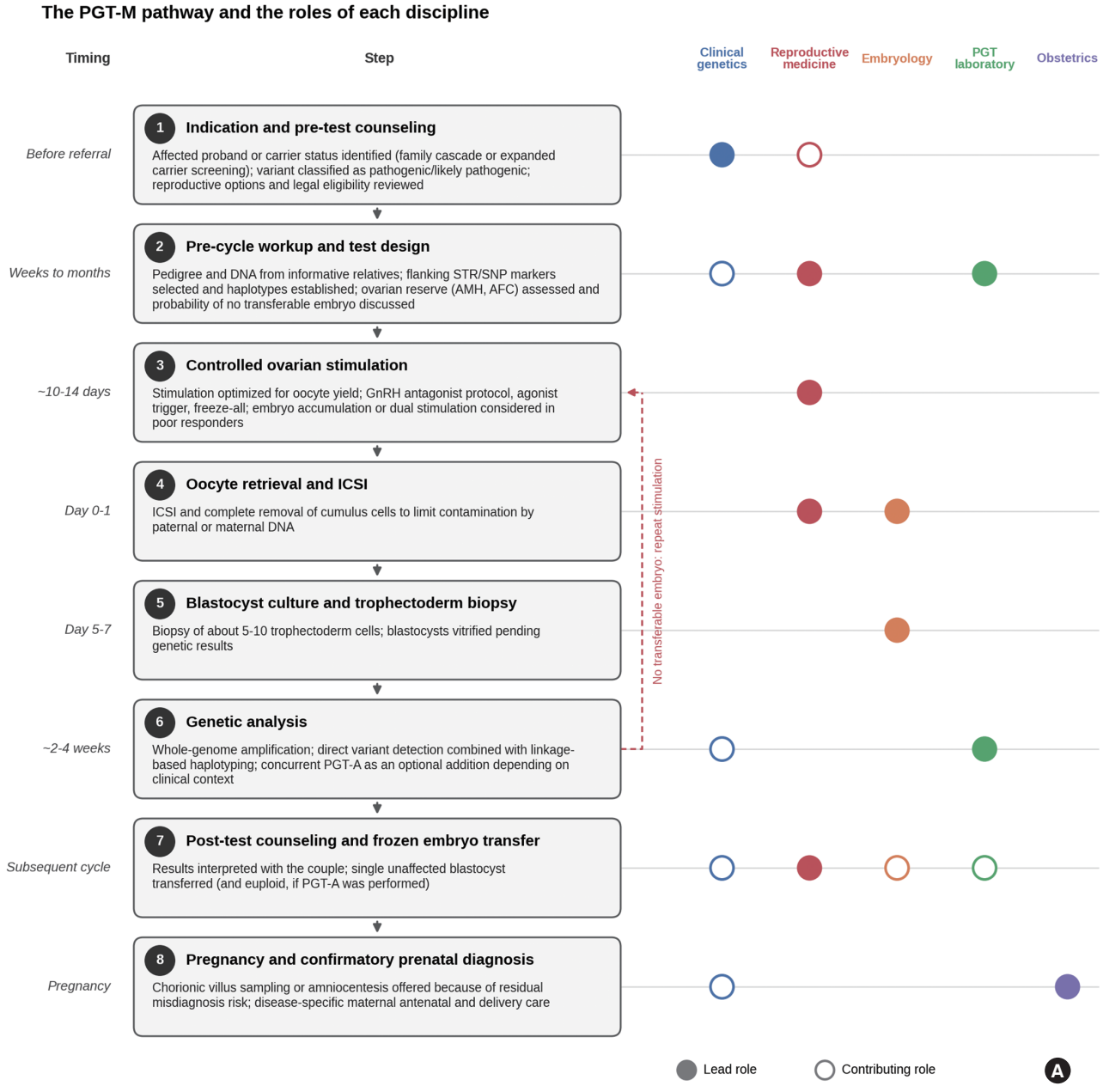
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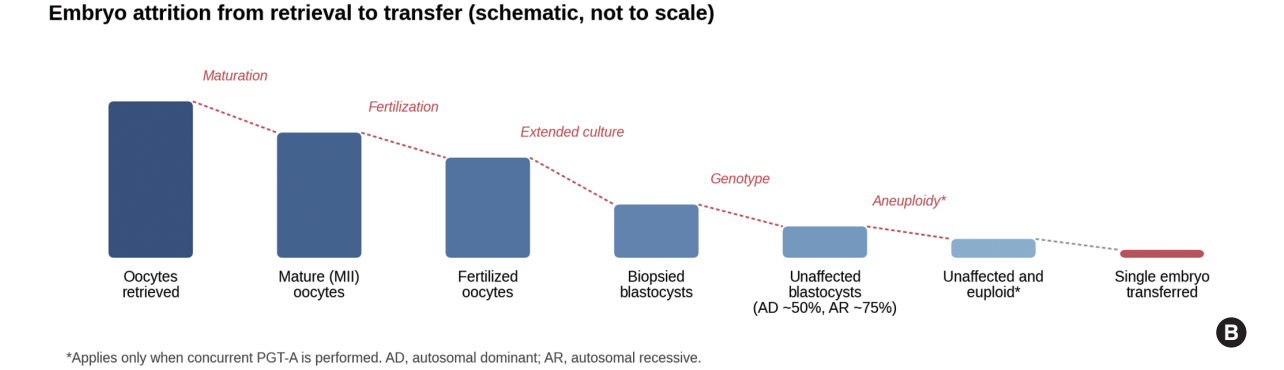
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INTRODUCTION

Rare genetic diseases are individually uncommon but collectively affect a substantial proportion of the population, and most are monogenic. For couples who have already had an affected child or who have been identified as carriers through expanded carrier screening or family cascade testing, the reproductive options are limited: accepting the recurrence risk, gamete donation, adoption, prenatal diagnosis with the possibility of termination, or preimplantation genetic testing for monogenic disorders (PGT-M) [1]. Among these options, PGT-M is the only approach that offers the possibility of a genetically related, unaffected child while largely avoiding the physical and psychological burden of pregnancy termination after an affected prenatal diagnosis.

Following the 2017 terminology revision, preimplantation genetic diagnosis was subdivided into PGT-M for monogenic conditions, PGT for structural rearrangements (PGT-SR), and PGT for aneuploidy (PGT-A) [2]. The PGT-M literature has been dominated by molecular genetics. From a reproductive endocrinology perspective, however, the number of blastocysts available for testing can be as decisive as the assay itself, although assay development and the establishment of informative haplotypes may also be limiting in individual families [3]. Accordingly, this review follows the PGT-M pathway chronologically (Fig. 1A) and examines each step through an explicit female reproductive medicine lens rather than attempting a comprehensive review of the molecular methods.





*Applies only when concurrent PGT-A is performed. AD, autosomal dominant; AR, autosomal recessive.

Fig. 1. Continued to the next page.

Fig. 1. Continued. Schematic overview of preimplantation genetic testing for monogenic disorders (PGT-M). (A) The PGT-M pathway from indication to confirmatory prenatal diagnosis, with approximate timing and the lead (filled circles) and contributing (open circles) roles of clinical genetics, reproductive medicine, embryology, the PGT laboratory, and obstetrics at each step. The dashed arrow indicates return to ovarian stimulation when no transferable embryo is obtained. (B) Schematic representation of embryo attrition from oocyte retrieval to transfer (not to scale). The expected unaffected fraction is approximately 50% for autosomal dominant (AD) and 75% for autosomal recessive (AR) conditions; the aneuploidy step applies only when concurrent preimplantation genetic testing for aneuploidy (PGT-A) is performed. ICSI, intracytoplasmic sperm injection; STR, short tandem repeat; SNP, single-nucleotide polymorphism; AMH, anti-Müllerian hormone; AFC, antral follicle count.

THE PGT-M PATHWAY

Genetic diagnosis and pre-test counseling

PGT-M cannot begin until the causative variant has been confirmed and classified as pathogenic or likely pathogenic in the affected proband [3]. Pre-test counseling, ideally provided jointly by a clinical geneticist or genetic counselor and the reproductive physician, reviews the reproductive options, the inheritance pattern and expected proportion of unaffected embryos, the legal eligibility of the condition in Korea (Section 4), and the probability that treatment will not yield a transferable embryo. Indications have broadened beyond classic severe childhood-onset recessive diseases to include autosomal dominant late-onset conditions, inherited cancer predisposition, X-linked disorders, *de novo* variants, mitochondrial DNA disease assessed using heteroplasmy thresholds, and human leukocyte antigen (HLA) matching for an affected sibling [1].

Pre-cycle workup and test design

A detailed pedigree and DNA samples from as many informative relatives as possible are required because contemporary protocols do not rely on direct mutation detection alone. Informative polymorphic markers, either short tandem repeats (STRs) or single-nucleotide polymorphisms (SNPs), that flank the locus are selected so that the high- and low-risk haplotypes can be traced [3]. This step can itself be demanding: *de novo* variants, the absence of informative relatives, or limited marker informativeness may require additional family samples, alternative phasing strategies, or custom assay development and may delay or occasionally preclude testing [3]. This preparatory phase takes several weeks to a few months and should be initiated before, rather than during, ovarian stimulation. In parallel, the reproductive assessment should include ovarian reserve evaluation using anti-Müllerian hormone (AMH) and antral follicle count, semen analysis, and uterine evaluation so that the likely number of stimulation cycles can be estimated at the outset.

Controlled ovarian stimulation

Because the number of blastocysts is a principal determinant of whether a transferable embryo will be obtained (Section 3), ovarian stimulation for PGT-M should be optimized for oocyte yield rather than a conventional fresh-transfer endpoint. AMH and antral follicle count guide gonadotropin dosing; antagonist protocols with a dual trigger and a freeze-all strategy are standard because all embryos are cryopreserved pending genetic results. In poor responders, embryo accumulation over consecutive cycles and, in selected cases, dual stimulation within a single ovarian cycle have been proposed to increase the cumulative probability of obtaining at least one transferable blastocyst [4]. Evidence for the latter strategy is mixed: a randomized comparison of dual stimulation with two conventional stimulations found no significant difference in the number of euploid blastocysts obtained. Thus, the strategy is best presented as a means of shortening the time to a transferable embryo rather than increasing yield [5].

Carrier states that compromise the ovary

Several monogenic conditions relevant to PGT-M directly affect female reproductive potential, making reproductive medicine input into the pathway particularly important. *FMR1* premutation carriers are at risk of fragile X-associated primary ovarian insufficiency [6]. Women with pathogenic *BRCA1* variants have been reported to have reduced ovarian reserve; however, a systematic review found no consistent difference in AMH or response to stimulation, so this possibility should be discussed during counseling without being overstated [7]. Myotonic dystrophy type 1 is characterized by both anticipation, which makes the severity of recurrence unpredictable, and well-described obstetric risks, including polyhydramnios, preterm labor, disordered labor, and an increased operative delivery rate [8]. In these families, PGT-M must be planned early and considered alongside fertility preservation because delay may render treatment impossible.

Oocyte retrieval, fertilization, and embryo biopsy

Intracytoplasmic sperm injection (ICSI) is used to avoid contamination by extraneous sperm, and cumulus cells are carefully removed [3]. Trophectoderm biopsy of five to ten cells at the blastocyst stage has replaced cleavage-stage blastomere biopsy, providing more template DNA, a lower relative impact on the embryo, and better implantation outcomes [9]. Biopsied blastocysts are vitrified while genetic analysis is completed.

Genetic analysis

Following biopsy, DNA is amplified by whole-genome amplification or, in targeted protocols, direct multiplex polymerase chain reaction (PCR). These amplification procedures introduce the principal analytical risks: allele dropout, preferential amplification, and contamination by cumulus or sperm DNA [3]. Robust PGT-M combines direct detection of the variant with indirect linkage-based haplotyping so that each result is internally corroborated and allele dropout is recognized rather than misread [3]. Several analytical strategies are in use. Targeted approaches based on multiplex PCR of flanking STR or SNP markers, with or without direct variant detection, remain widely used and do not assess chromosomal copy number. In the most recent ESHRE PGT Consortium data, targeted PCR-based testing still predominated for PGT-M, whereas comprehensive whole-genome methods were applied mainly to PGT-A and concurrent testing [10]. Genome-wide SNP array approaches such as karyomapping [11] and, more recently, next-generation sequencing with haplotype reconstruction allow the disease locus to be analyzed without the need for fully family-specific assay design and can also report chromosomal copy number from the same biopsy. Concurrent PGT-A is therefore an optional addition rather than an inherent component of PGT-M. It may be considered depending on maternal age, reproductive history, and local practice, but its benefit is not universal. In a randomized trial of 1,212 women aged 20–37 years with a good prognosis and at least three good-quality blastocysts, the cumulative live birth rate within 1 year was 77.2% (468/606) with PGT-A and 81.8% (496/606) with conventional IVF (absolute difference, −4.6 percentage points; 95% confidence interval [CI], −9.2 to −0.0), and conventional IVF was noninferior to PGT-A [12]. When concurrent PGT-A is performed, the requirement that an embryo be both unaffected and euploid further reduces the transferable fraction, particularly in women of advanced age.

Post-test counseling and embryo transfer

The genetic and reproductive teams jointly review the results with the couple, including the handling of carrier embryos in recessive conditions, inconclusive results, and, when PGT-A has been performed, mosaic findings. Transfer of a single unaffected blastocyst, which is also euploid when concurrent PGT-A has been performed, should be the default to avoid multiple pregnancy in couples who have already accepted substantial reproductive risk.

Pregnancy and confirmatory prenatal diagnosis

Because PGT-M carries a small residual risk of misdiagnosis arising from allele dropout, undetected recombination, contamination, or embryo mosaicism, it does not replace confirmatory prenatal diagnosis. Chorionic villus sampling or amniocentesis should therefore be offered and discussed at the outset [3]. Disease-specific antenatal and delivery planning, such as cardiovascular surveillance in Marfan syndrome, renal and blood pressure monitoring in autosomal dominant polycystic kidney disease, and anesthetic precautions in neuromuscular disease, follows from the same maternal diagnosis that prompted PGT-M and requires early communication between the reproductive and obstetric teams.

EMBRYO ATTRITION AND CLINICAL UTILITY

The attrition cascade

The number of oocytes retrieved is not equivalent to the number of embryos ultimately tested. Attrition occurs during maturation, fertilization, and extended culture to the biopsiable blastocyst stage, and only a proportion of the blastocysts that are eventually tested are suitable for transfer (Fig. 1B). In a review of PGT-M cycles, the probability that a given embryo would be classified as appropriate for transfer differed by inheritance pattern and decreased further when aneuploidy testing was performed concurrently [13]. Before any aneuploidy testing is considered, the expected unaffected fraction is 50% for an autosomal dominant condition and 75% for an autosomal recessive condition. A woman with diminished ovarian reserve who carries an autosomal dominant variant may therefore complete a full cycle without a transferable embryo. Counseling before the first cycle should explicitly address the probability of a “no-transfer” outcome, and treatment planning should anticipate the possibility of more than one retrieval.

Diagnostic performance

Diagnostic efficiency in experienced centers is high, with the large majority of biopsied embryos yielding a conclusive result. Trophectoderm biopsy combined with comprehensive testing strategies is associated with higher diagnostic efficiency and better clinical outcomes per transfer than earlier approaches, and misdiagnosis is rare when direct and indirect analyses are used together [10,14].

Live birth outcomes and the choice of denominator

Reported outcomes depend strongly on the denominator used. A single-center retrospective cohort from Australia analyzed 572 PGT-M cycles performed in 299 patients between 2015 and 2022; only cycles in which at least one embryo was biopsied were included, and all embryos underwent karyomapping with concurrent aneuploidy screening [15]. With these 572 biopsy cycles as the denominator, 392 (68.5%) yielded at least one embryo suitable for transfer and 355 (62.1%) resulted in at least one embryo transfer. With embryos transferred as the denominator, the 513 embryos transferred in 490 transfer procedures resulted in 263 clinical pregnancies and 230 live births, corresponding to a clinical pregnancy rate of 51.3% (95% CI, 47.0%–55.6%) and a live birth rate of 44.8% (95% CI, 40.6%–49.2%) per embryo transferred [15]. Within the cohort, a recorded subfertility factor was associated with a lower likelihood of clinical pregnancy per embryo transferred ($\beta = -0.48$, $p = 0.026$), whereas its association with live birth did not reach statistical significance ($\beta = -0.42$, $P = 0.052$) [15]. Cycles that were canceled before oocyte retrieval or that produced no biopsiable blastocyst were not included in any of these denominators; the proportion of all initiated cycles reaching transfer would therefore be lower than 62.1% [15]. Per-transfer figures therefore describe the prognosis of couples who reach transfer rather than that of all couples who begin treatment. Cumulative live birth rates per couple or per intention to treat, which would account for canceled cycles, cycles without a transferable embryo, and repeated stimulation, are rarely reported for PGT-M and represent an important gap in the evidence. Until such data are available, counseling should present per-transfer outcomes together with the realistic probability of reaching transfer in a given cycle, which depends on maternal age, ovarian reserve, and inheritance pattern [13,15]. Clinical utility is best expressed as a cumulative outcome per couple, specifically the probability of an unaffected live birth per intention to treat, and as the avoidance of successive terminations after prenatal diagnosis.

LIMITATIONS, COUNSELING, AND REGULATION

The principal limitations are often biological: insufficient oocytes, absence of transferable embryos, mosaic results of uncertain significance, and variants of uncertain significance that cannot be used for embryo selection. Technical limitations, including failure to establish informative haplotypes and inconclusive embryo results, can also occur. Cost and incomplete reimbursement remain barriers. Non-directive genetic counseling, ideally provided jointly by the reproductive physician and a clinical geneticist, must address the possibility of cycle failure, the disposition of affected embryos, and the ethical questions raised by selection for variable-penetrance or adult-onset conditions.

In Korea, genetic testing of embryos is governed by the Bioethics and Safety Act, which restricts PGT-M to designated conditions [16]. The list has expanded well beyond the original small set, and a 2024 amendment to the Enforcement Decree changed the designation procedure from ministerial notification to public announcement, with the aim of allowing new conditions to be added more rapidly at the request of treating institutions or academic societies. Clinicians managing families affected by rare diseases should confirm the current status of a given condition before counseling and should be aware that an unlisted condition may be submitted for addition rather than simply declined.

CONCLUSION

PGT-M has become a reliable means of preventing the transmission of rare monogenic disease. Its outcomes, however, depend on ovarian reserve, stimulation strategy, blastocyst yield, and obstetric management as much as on molecular technique. Framing PGT-M as a multidisciplinary reproductive program, in which clinical genetics, the PGT laboratory, embryology, reproductive medicine, and obstetric care each contribute across a single coordinated pathway and outcomes are counseled per couple rather than per transfer, offers the most realistic path to the outcome that matters to families: an unaffected child delivered safely.

NOTES

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Conflicts of interest

No potential conflict of interest relevant to this article was reported.

Ethical statement

This article is a narrative review based entirely on previously published literature and publicly available regulatory documents. It does not report any study involving human participants, human-derived material, or animals; therefore, institutional review board approval and informed consent were not required.

Author contributions

Conceptualization: Park D. Literature review and acquisition of data: Choi H. Drafting of the manuscript: Park D., Choi H. Critical revision of the manuscript for important intellectual content: Park D. Final approval of the version to be published: Park D., Choi H. Both authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Use of artificial intelligence

The authors used Claude (Anthropic, PBC; Claude Opus 5) in the preparation of this manuscript. The tool was used for three purposes: (1) drafting of the manuscript text based on an outline and content framework specified by the authors, (2) assistance in searching for and identifying candidate published references, and (3) drafting of the layout of Fig. 1 based on content specified by the authors. In accordance with the JIG Artificial Intelligence Use Policy, the authors verified the accuracy, validity, and appropriateness of all artificial intelligence–generated content, and independently confirmed every cited reference against the original publication. The content of Fig. 1 was defined, reviewed, and approved by the authors; no photographic or realistic image-generation tool was used. No identifiable patient information, genomic data, or confidential research data were uploaded to any artificial intelligence platform. Artificial intelligence does not qualify for authorship, and the authors take full responsibility for all content of this manuscript.

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Genes Associated with Short Stature: An Update

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Short stature is one of the most common reasons for referral to pediatric endocrinology clinics. In many children, no cause is identified, and they are classified as having idiopathic short stature (ISS). With recent advances in genetic testing, however, a growing number of these children are found to have monogenic defects. Linear growth is driven by chondrocyte proliferation, hypertrophy, and extracellular matrix production in the growth plate, and genetic variants that disrupt these processes impair growth. The affected mechanisms include endocrine signaling through the growth hormone (GH)–insulin-like growth factor I (IGF-I) axis; paracrine signaling and transcriptional regulation within the growth plate (*SHOX, FGFR3*, and *NPR2*); the cartilage extracellular matrix (*ACAN*); intracellular signaling (the RAS/MAPK pathway); fundamental cellular processes such as DNA replication and centrosome function; and genomic imprinting. Each mechanism is associated with a characteristic clinical pattern of birth size, body proportions, bone age, and biochemical findings. An accurate genetic diagnosis establishes the cause of growth failure, provides information on prognosis and associated risks, predicts the response to GH therapy, and enables genetic counseling. Mechanism-based therapies, such as C-type natriuretic peptide analogs for achondroplasia, are now entering clinical practice. In conclusion, the integration of clinical, biochemical, and genetic information according to the underlying cellular mechanisms is essential for the accurate diagnosis and management of children with short stature.

Key words: Body height, Growth plate, Growth disorders, Genetic testing

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INTRODUCTION

Short stature is defined as a height more than 2 standard deviations (SD) below the mean for age and sex. It is one of the most common reasons for referral to pediatric endocrinologists [1]. Short stature has traditionally been classified according to its underlying etiology or clinical presentation, including growth hormone (GH) deficiency, chromosomal abnormalities, skeletal dysplasias, syndromic conditions, small for gestational age (SGA) without catch-up growth, and idiopathic short stature (ISS). However, the cause of growth failure remains unidentified in most children evaluated for short stature [2]. In this review, SGA refers to a birth weight and/or length below −2 SD for gestational age, whereas intrauterine growth restriction (IUGR) refers to a pathological process of restricted fetal growth; the two terms are therefore not used interchangeably [3].

Human height is a highly heritable trait. Common variants at hundreds of loci each exert a small effect, whereas rare variants in single genes can cause marked growth failure [1,4]. Next-generation sequencing, including gene panels and exome sequencing, has identified monogenic causes in a considerable proportion of children previously classified as having ISS or familial short stature [3,4]. However, the diagnostic yield depends strongly on the clinical phenotype. In a 2026 systematic review, the diagnostic yield of exome sequencing was 15.1% in isolated short stature and 50.8% in syndromic short stature [5]. These figures therefore cannot be applied uniformly to all children with ISS, and the yield is highest in

children with severe short stature, dysmorphic features, or skeletal abnormalities [5]. In the same year, the first international guideline on genetic testing in children with short stature was published [3].

A mechanism-based classification is now widely used. This approach places the growth plate chondrocyte at the center and classifies genetic defects according to where they disrupt chondrocyte function [1]. In this review, we summarize established genes and syndromes associated with short stature according to their underlying cellular mechanisms, together with recently implicated genes and the strengths and limitations of current genetic testing. Gene and variant nomenclature follows the Human Genome Variation Society (HGVS) recommendations, and variant terminology follows the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) guidelines.

GROWTH PLATE BIOLOGY

Linear growth occurs at the growth plate, a thin layer of cartilage located near the ends of long bones. The growth plate consists of three zones: the resting zone, the proliferative zone, and the hypertrophic zone [6]. Resting chondrocytes give rise to proliferative chondrocytes, which divide rapidly and form columns parallel to the long axis of the bone. These cells then enlarge into hypertrophic chondrocytes and secrete extracellular matrix. Finally, blood vessels and bone cells invade the hypertrophic zone, and the cartilage is remodeled into bone tissue [6].

Chondrocyte function is regulated at multiple levels. Endocrine factors include GH, insulin-like growth factor I (IGF-I), thyroid hormone, glucocorticoids, and sex steroids. Paracrine factors include C-type natriuretic peptide (CNP), fibroblast growth factors (FGFs), Indian hedgehog (IHH), parathyroid hormone-related protein (PTHrP), and bone morphogenetic proteins [1,6]. The cartilage extracellular matrix provides a structural and signaling environment for chondrocytes. In addition, intracellular processes common to all cells, such as DNA replication, cell cycle regulation, and centrosome function, are essential for chondrocyte proliferation. A defect at any of these levels can reduce growth plate output and cause short stature [1]. Clinical characteristics of short stature according to genetic defects are described in Table 1.

DEFECTS IN THE GH–IGF-I AXIS

GH secreted from the pituitary gland binds to the GH receptor (GHR) and activates the JAK2–STAT5B pathway. This induces hepatic and local IGF-I production. IGF-I circulates in a ternary complex with IGF-binding protein 3 (IGFBP-3) and the acid-labile subunit (ALS). IGF-I is released from this complex by proteases such as pregnancy-associated plasma protein A2 (PAPP-A2) and acts on chondrocytes through the IGF-I receptor (IGF1R) [1,2]. Defects at each step produce distinct biochemical profiles.

GH SYNTHESIS DEFECT: *GH1* VARIANTS (OMIM #262400, ISOLATED GH DEFICIENCY TYPE IA; OMIM #173100, ISOLATED GH DEFICIENCY TYPE II)

The *GH1* gene is located on chromosome 17q23. Isolated GH deficiency (IGHD) type IA is an autosomal recessive disorder caused by homozygous deletion of *GH1*. Affected children show severe growth failure from early infancy and may develop anti-GH antibodies during GH treatment. IGHD type II is inherited in an autosomal dominant manner and is mainly caused by splice-site variants that lead to skipping of exon 3. The resulting 17.5-kDa GH isoform exerts a dominant-negative effect on normal GH secretion [2]. Defects in pituitary transcription factors, such as *POU1F1* and *PROP1*, cause combined pituitary hormone deficiency, including GH deficiency [2].

Phillips et al. [7] first identified *GH1* gene deletions in patients with familial IGHD in 1981.

GH RECEPTOR DEFECT: *GHR* VARIANTS (OMIM #262500, LARON SYNDROME)

Laron syndrome is caused by homozygous or compound heterozygous variants in the *GHR* gene on chromosome 5p13. It is characterized by severe postnatal growth failure, midface hypoplasia, and truncal obesity. Serum GH levels are normal or elevated, whereas IGF-I, IGFBP-3, and ALS levels are markedly low. GH-binding protein is usually low or absent but may be normal when variants affect the transmembrane or intracellular domain [2,4]. Patients do not respond to GH therapy, and recombinant human IGF-I (rhIGF-I) is the treatment of choice.

Godowski et al. [8] first demonstrated a partial deletion of the *GHR* gene in patients with Laron syndrome in 1989.

Table 1. Clinical characteristics of short stature according to genetic defects

Gene	Cellular mechanism	Disorder	Inheritance	Birth size	Key clinical and biochemical clues	Growth-promoting therapy
GH1	GH synthesis	IGHD type IA, type II	AR (type IA); AD (type II)	Normal	Low stimulated GH, low IGF-I	GH effective
GHR	GH receptor	Laron syndrome	AR	Normal	Elevated GH, very low IGF-I and IGFBP-3	rhIGF-I
STAT5B	GH signal transduction	GH insensitivity with immune dysregulation	AR	Normal	Normal or elevated GH, low IGF-I, immune dysregulation, elevated prolactin	rhIGF-I
IGFALS	IGF-I complex stabilization	ALS deficiency	AR	Normal	Very low IGF-I and IGFBP-3, mild short stature	Limited GH response
PAPPA2	Release of IGF-I from IGFBPs	Short stature, Dauber-Argente type	AR	Normal	Elevated total IGF-I, IGFBP-3, and ALS; low free IGF-I	rhIGF-I (limited data)
IGF1	IGF-I production	IGF-I deficiency	AR	SGA	Microcephaly, hearing loss, intellectual disability	rhIGF-I
IGF1R	IGF-I receptor	IGF-I resistance	AD; rarely AR	SGA	Microcephaly, normal or elevated IGF-I	Partial GH response
SHOX	Chondrocyte transcriptional regulation	Léri-Weill dyschondrosteosis, ISS; Langer mesomelic dysplasia	Pseudoautosomal dominant (LWD, ISS); pseudoautosomal recessive (Langer)	Normal	Mesomelia, Madelung deformity	GH effective
FGFR3	Paracrine inhibition of chondrocytes	Achondroplasia, hypochondroplasia	AD (mostly *de novo*)	Normal	Rhizomelia, macrocephaly	Vosoritide (achondroplasia)
NPR2	CNP signaling	Acromesomelic dysplasia; SNSK	AR (acromesomelic dysplasia); AD (SNSK)	Normal	Short metacarpals, mild disproportion	Variable GH response
NPPC	CNP production	Autosomal dominant short stature	AD	Normal	Mild short stature, small hands	CNP analogs under investigation
IHH	IHH–PTHrP feedback loop	SNSK, brachydactyly type A1; acrocapitofemoral dysplasia	AD (SNSK, BDA1); AR (acrocapitofemoral dysplasia)	Normal	Mild disproportion, short middle phalanx of the fifth finger	GH (observational data)
ACAN	Cartilage extracellular matrix	Short stature with advanced bone age	AD	Normal or mildly reduced	Advanced bone age, early growth cessation, early-onset osteoarthritis	Some GH response
PTPN11	RAS/MAPK signaling	Noonan syndrome 1	AD	Normal	Characteristic facies, pulmonary valve stenosis, cardiomyopathy	GH effective
CUL7	Ubiquitin ligase, cell cycle	3-M syndrome 1	AR	SGA	Relative macrocephaly, normal intelligence, slender long bones	Poor GH response
ATR	DNA damage response	Seckel syndrome 1	AR	SGA	Severe microcephaly, intellectual disability	Poor GH response
PCNT	Centrosome function	MOPD II	AR	SGA	Microcephaly, cerebrovascular disease, insulin resistance	Poor GH response
ORC1 and others	DNA replication licensing	Meier-Gorlin syndrome	AR	SGA	Microtia, absent or hypoplastic patellae	Variable GH response
11p15 ICR1, UPD7	Genomic imprinting	Silver-Russell syndrome	Mostly sporadic	SGA	Relative macrocephaly at birth, body asymmetry, feeding difficulties	GH effective

AD, autosomal dominant; ALS, acid-labile subunit; AR, autosomal recessive; BDA1, brachydactyly type A1; CNP, C-type natriuretic peptide; GH, growth hormone; ICR1, imprinting control region 1; IGF-I, insulin-like growth factor I; IGFBP, IGF-binding protein; IGHD, isolated GH deficiency; IHH, Indian hedgehog; ISS, idiopathic short stature; LWD, Léri-Weill dyschondrosteosis; MAPK, mitogen-activated protein kinase; MOPD II, microcephalic osteodysplastic primordial dwarfism type II; PTHrP, parathyroid hormone-related protein; rhIGF-I, recombinant human IGF-I; SGA, small for gestational age; SNSK, short stature with nonspecific skeletal abnormalities; UPD7, maternal uniparental disomy of chromosome 7.

GH SIGNAL TRANSDUCTION DEFECT: *STAT5B* VARIANTS (OMIM #245590, GROWTH HORMONE INSENSITIVITY WITH IMMUNE DYSREGULATION)

STAT5B is the key intracellular mediator of GH-induced IGF-I transcription. Biallelic *STAT5B* variants on chromosome 17q21 cause severe postnatal growth failure with normal or elevated GH levels but low IGF-I, IGFBP-3, and ALS levels. Because STAT5B also mediates cytokine signaling, the condition is accompanied by immune dysregulation, including eczema, recurrent infections, lymphocytic interstitial pneumonia, and autoimmune disease. Elevated prolactin is another characteristic finding [9].

Kofoed et al. [9] first described a homozygous *STAT5B* variant in a patient with GH insensitivity in 2003.

IGF-I COMPLEX STABILIZATION DEFECT: *IGFALS* VARIANTS (OMIM #615961, ACID-LABILE SUBUNIT DEFICIENCY)

ALS stabilizes the IGF-I–IGFBP-3 ternary complex and prolongs the half-life of circulating IGF-I. Biallelic variants in the *IGFALS* gene on chromosome 16p13.3 cause marked reductions in serum IGF-I and IGFBP-3 that are disproportionately low relative to the degree of growth failure. Height is usually only mildly reduced, at approximately −2 to −3 SD, and delayed puberty and insulin resistance are common [10].

Domené et al. [10] first reported pathogenic *IGFALS* variants in 2004.

IGF-I BIOAVAILABILITY DEFECT: *PAPPA2* VARIANTS (OMIM #619489, SHORT STATURE, DAUBER-ARGENTE TYPE)

PAPP-A2 is a metalloproteinase that cleaves IGFBP-3 and IGFBP-5, releasing free IGF-I from the ternary complex. Homozygous loss-of-function variants in *PAPPA2* on chromosome 1q25 cause progressive growth failure, mild microcephaly, and thin long bones. The biochemical profile is distinctive: total IGF-I, IGFBP-3, IGFBP-5, and ALS levels are elevated, whereas free IGF-I is reduced [11].

Dauber et al. [11] first described this syndrome in two unrelated families in 2016.

IGF-I PRODUCTION DEFECT: *IGF1* VARIANTS (OMIM #608747, INSULIN-LIKE GROWTH FACTOR I DEFICIENCY)

Homozygous variants or deletions of the *IGF1* gene on chromosome 12q23 cause severe prenatal growth failure, resulting in SGA birth, and postnatal growth failure. Other features include microcephaly, sensorineural hearing loss, and intellectual disability. Serum IGF-I is undetectable or very low, but it may be elevated when the variant protein is biologically inactive [4].

Woods et al. [12] first reported a partial deletion of *IGF1* in a patient with severe prenatal and postnatal growth failure in 1996.

IGF-I RECEPTOR DEFECT: *IGF1R* VARIANTS (OMIM #270450, INSULIN-LIKE GROWTH FACTOR I RESISTANCE)

Most patients carry heterozygous variants in the *IGF1R* gene on chromosome 15q26, inherited in an autosomal dominant manner; biallelic variants cause a more severe phenotype. Patients are typically born SGA and present with postnatal growth failure and microcephaly. Serum IGF-I levels are normal or elevated, reflecting resistance at the receptor level [4,13].

Abuzzahab et al. [13] first identified pathogenic *IGF1R* variants in children with prenatal and postnatal growth failure in 2003.

DEFECTS IN PARACRINE SIGNALING AND TRANSCRIPTIONAL REGULATION IN THE GROWTH PLATE

Within the growth plate, chondrocyte proliferation and differentiation are regulated by local signaling networks. The FGFR3 pathway and the CNP–NPR2 pathway act in opposite directions on the mitogen-activated protein kinase (MAPK) cascade: FGFR3 signaling inhibits chondrocyte proliferation, whereas CNP–NPR2 signaling promotes it [1,6]. The transcription factor SHOX also regulates chondrocyte differentiation. These genes are now recognized as common monogenic causes of short stature [4].

TRANSCRIPTIONAL REGULATION DEFECT: *SHOX* DEFICIENCY (OMIM #127300, LÉRI-WEILL DYSCHONDROSTEOSIS; OMIM #300582, IDIOPATHIC FAMILIAL SHORT STATURE)

The *SHOX* gene is located in pseudoautosomal region 1

(PAR1) of the X and Y chromosomes (Xp22.33/Yp11.2). It encodes a homeobox transcription factor expressed in the limb growth plates. *SHOX* haploinsufficiency causes a spectrum of phenotypes, ranging from nonspecific short stature to Léri-Weill dyschondrosteosis, which is characterized by mesomelic limb shortening and Madelung deformity. Biallelic defects cause the more severe Langer mesomelic dysplasia. *SHOX* haploinsufficiency also accounts for a large part of the short stature in Turner syndrome [4]. Deletions of the gene or its downstream enhancers are more common than sequence variants. GH therapy is approved for *SHOX* deficiency.

Rao et al. [14] identified *SHOX* as the gene responsible for growth failure in ISS and Turner syndrome in 1997.

PARACRINE INHIBITION: *FGFR3* VARIANTS (OMIM #100800, ACHONDROPLASIA; OMIM #146000, HYPOCHONDROPLASIA)

The *FGFR3* gene on chromosome 4p16.3 encodes a negative regulator of chondrocyte proliferation and differentiation. Heterozygous gain-of-function variants constitutively activate FGFR3 and its downstream STAT1 and MAPK signaling. Achondroplasia, the most common skeletal dysplasia, is caused by the recurrent p.(Gly380Arg) variant (c.1138G>A or c.1138G>C) in nearly all patients, and most cases arise *de novo* [1]. It is characterized by rhizomelic short stature, macrocephaly, and midface hypoplasia. Hypochondroplasia has a milder phenotype and is frequently caused by the p.(Asn540Lys) variant. Vosoritide, a CNP analog that antagonizes FGFR3-mediated MAPK signaling, increased the annualized growth velocity by approximately 1.6 cm/year compared with placebo in a phase 3 trial [15].

Shiang et al. [16] identified pathogenic *FGFR3* variants as the cause of achondroplasia in 1994.

CNP SIGNALING DEFECT: *NPR2* VARIANTS (OMIM #602875, ACROMESOMELIC DYSPLASIA, MAROTEAUX TYPE; OMIM #616255, SHORT STATURE WITH NONSPECIFIC SKELETAL ABNORMALITIES)

The *NPR2* gene on chromosome 9p13.3 encodes natriuretic peptide receptor B, the receptor for CNP. Binding of CNP generates cyclic GMP, which promotes chondrocyte proliferation and hypertrophy. Biallelic loss-of-function variants cause acromesomelic dysplasia, Maroteaux type, a severe skeletal dysplasia with disproportionate short stature [17]. Heterozygous variants cause mild autosomal dominant short stature, sometimes accompanied by subtle skeletal findings such as short metacarpals.

Bartels et al. [17] first identified pathogenic *NPR2* variants in acromesomelic dysplasia in 2004. Subsequently, Vasques et al. [18] reported heterozygous *NPR2* variants in 6% of patients initially classified as having ISS.

NEWLY IMPLICATED GENES AND EXPANDING PHENOTYPES: *NPPC* AND *IHH*

The list of genes in the growth plate paracrine network continues to grow, and the phenotypes of known genes are expanding as genetic testing is applied to children with milder, nonsyndromic short stature. *NPPC*, located on chromosome 2q37.1, encodes CNP, the ligand of natriuretic peptide receptor B. Heterozygous loss-of-function *NPPC* variants cause autosomal dominant short stature, often with small hands, and thus represent the ligand-side counterpart of heterozygous *NPR2* defects. Because these children have reduced CNP signaling, CNP analogs originally developed for achondroplasia are now being evaluated for a broader range of growth disorders, including those involving the CNP–NPR2 pathway [19].

IHH encodes Indian hedgehog, which forms a negative feedback loop with PTHrP to regulate chondrocyte proliferation and hypertrophy [6]. Biallelic *IHH* variants cause acrocapitofemoral dysplasia, and heterozygous variants were classically associated with brachydactyly type A1. However, heterozygous *IHH* variants are now recognized as a relatively frequent cause of autosomal dominant short stature with nonspecific skeletal abnormalities, typically presenting as mildly disproportionate short stature with shortening of the middle phalanx of the fifth finger [3]. *IHH* is included among the genes with the strongest evidence of association with isolated short stature in the 2026 international guideline [3]. In a retrospective cohort of 19 children with heterozygous deleterious *IHH* variants treated with GH, 10 of whom reached adult height, GH therapy was associated with clinically meaningful height gains, and the response may differ according to variant class [20].

DEFECTS IN THE CARTILAGE EXTRACELLULAR MATRIX

The cartilage extracellular matrix is composed mainly of type II collagen and proteoglycans. It provides mechanical support and regulates the availability of growth factors to

chondrocytes. Severe matrix defects cause classic skeletal dysplasias, such as spondyloepiphyseal dysplasia (*COL2A1*), pseudoachondroplasia (*COMP*), and metaphyseal chondrodysplasia, Schmid type (*COL10A1*) [4]. Milder defects may present as apparently nonsyndromic short stature.

EXTRACELLULAR MATRIX DEFECT: *ACAN* VARIANTS (OMIM #165800, SHORT STATURE AND ADVANCED BONE AGE, WITH OR WITHOUT EARLY-ONSET OSTEOARTHRITIS AND/OR OSTEOCHONDRITIS DISSECANS)

The *ACAN* gene on chromosome 15q26.1 encodes aggrecan, the major proteoglycan of the growth plate and articular cartilage. Heterozygous variants cause autosomal dominant short stature. Unlike most children with ISS, affected children often have advanced bone age and early growth cessation, resulting in markedly reduced adult height [21]. Other features include mild midface hypoplasia, brachydactyly, early-onset osteoarthritis, and intervertebral disc disease. In a cohort of 103 individuals from 20 families, the median adult height was −2.8 SD, and bone age was advanced in most children [22]. Increased growth velocity was observed in some patients treated with GH [22].

Nilsson et al. [21] first identified heterozygous *ACAN* variants as a cause of short stature with advanced bone age in 2014.

DEFECTS IN INTRACELLULAR SIGNALING: RASOPATHIES RAS/MAPK SIGNALING DEFECT: *PTPN11* VARIANTS (OMIM #163950, NOONAN SYNDROME 1)

The RAS/MAPK pathway transmits signals from growth factor receptors and regulates cell proliferation and differentiation. Germline variants in genes of this pathway cause a group of disorders called RASopathies. Noonan syndrome is the most common of these, and short stature is one of its main features [4]. Noonan syndrome 1 is caused by heterozygous gain-of-function variants in the *PTPN11* gene on chromosome 12q24.13, which encodes the protein tyrosine phosphatase SHP-2. *PTPN11* variants account for approximately half of all patients [23]; other causative genes include *SOS1*, *RAF1*, *RIT1*, and *KRAS* [4]. Clinical features include characteristic facial features, pulmonary valve stenosis, hypertrophic cardiomyopathy, cryptorchidism, and bleeding tendency. Excessive RAS/MAPK activity impairs GH receptor signaling, resulting in a mild GH-resistant state with low IGF-I, and also directly inhibits growth plate chondrogenesis [4]. GH therapy is approved for Noonan syndrome.

Tartaglia et al. [23] identified pathogenic *PTPN11* variants as the cause of Noonan syndrome in 2001.

DEFECTS IN FUNDAMENTAL CELLULAR PROCESSES: PRIMORDIAL DWARFISM

Primordial dwarfism is a group of disorders characterized by severe prenatal and postnatal growth failure. These disorders are caused by defects in processes essential for all proliferating cells, such as protein degradation, the DNA damage response, centrosome function, and DNA replication licensing. Because the total number of cells is reduced, growth failure is usually proportionate and often accompanied by microcephaly. The response to GH therapy is generally poor [4].

UBIQUITIN LIGASE DEFECT: *CUL7* VARIANTS (OMIM #273750, 3-M SYNDROME 1)

3-M syndrome is an autosomal recessive disorder characterized by severe prenatal and postnatal growth failure, relative macrocephaly, a triangular face, and normal intelligence. Radiographs show slender long bones and tall vertebral bodies. Biallelic variants in *CUL7* on chromosome 6p21.1 account for most cases, and the remainder are caused by variants in *OBSL1* and *CCDC8*. CUL7 is a component of an E3 ubiquitin ligase complex, and the three proteins interact to regulate cell cycle progression and microtubule dynamics [4].

Huber et al. [24] identified pathogenic *CUL7* variants as the cause of 3-M syndrome in 2005.

DNA DAMAGE RESPONSE DEFECT: *ATR* VARIANTS (OMIM #210600, SECKEL SYNDROME 1)

Seckel syndrome is an autosomal recessive disorder characterized by severe prenatal growth failure resulting in SGA birth, postnatal growth failure, marked microcephaly, intellectual disability, and a characteristic beak-like nose. *ATR*, located on chromosome 3q23, encodes a key kinase of the DNA damage response that is activated by replication stress. Impaired ATR signaling disrupts cell cycle checkpoints and reduces cell proliferation during development.

O'Driscoll et al. [25] identified a splice-site variant in *ATR* in families with Seckel syndrome in 2003.

CENTROSOME DEFECT: *PCNT* VARIANTS (OMIM #210720, MICROCEPHALIC OSTEODYSPLASTIC PRIMORDIAL DWARFISM TYPE II)

Microcephalic osteodysplastic primordial dwarfism type II (MOPD II) is one of the most severe forms of human growth failure, with an adult height of approximately 100 cm. It is caused by biallelic loss-of-function variants in *PCNT* on chromosome 21q22.3, which encodes pericentrin, a centrosomal protein required for mitotic spindle formation. Other features include microcephaly, skeletal dysplasia, dental anomalies, insulin resistance, and cerebrovascular disease, including moyamoya disease and intracranial aneurysms, which require regular screening [26].

Rauch et al. [26] identified pathogenic *PCNT* variants as the cause of MOPD II in 2008.

DNA REPLICATION LICENSING DEFECT: *ORC1* AND OTHER PRE-REPLICATION COMPLEX GENE VARIANTS (OMIM #224690, MEIER-GORLIN SYNDROME 1)

Meier-Gorlin syndrome, also known as ear-patella-short stature syndrome, is an autosomal recessive disorder characterized by microtia, absent or hypoplastic patellae, and prenatal and postnatal growth failure. Intelligence is usually normal. The causative genes, *ORC1* (chromosome 1p32.3), *ORC4*, *ORC6*, *CDT1*, and *CDC6*, encode components of the pre-replication complex, which licenses DNA replication origins before S phase [27]. Impaired replication licensing delays cell cycle progression during periods of rapid growth.

Bicknell et al. [27] identified pathogenic variants in these five genes in patients with Meier-Gorlin syndrome in 2011.

EPIGENETIC DEFECTS: IMPRINTING DISORDERS IMPRINTING DEFECT: LOSS OF METHYLATION AT 11P15 AND MATERNAL UPD7 (OMIM #180860, SILVER-RUSSELL SYNDROME)

Genomic imprinting results in gene expression that depends on the parent of origin, and several imprinted genes regulate fetal growth. *IGF2*, a paternally expressed gene at 11p15.5, encodes a major fetal growth factor. Silver-Russell syndrome (SRS) is characterized by SGA birth, postnatal growth failure, relative macrocephaly at birth, a prominent forehead, body asymmetry, and feeding difficulties [28]. Loss of methylation at imprinting control region 1 (ICR1) on the paternal 11p15 allele is the most common cause and is found in 30%–60% of patients. This leads to biallelic expression of *H19* and reduced expression of *IGF2* [29]. Maternal uniparental disomy of chromosome 7 (UPD7) accounts for 5%–10% of cases. Rare monogenic causes include loss-of-function variants in *IGF2*, *HMGA2*, and *PLAG1* and gain-of-function variants in *CDKN1C* [29]. Temple syndrome, caused by 14q32 imprinting defects such as maternal UPD14, shows substantial phenotypic overlap with SRS [29]. The international consensus statement recommends clinical diagnosis using the Netchine-Harbison clinical scoring system, early nutritional support, and GH therapy [29].

Gicquel et al. [28] first identified 11p15 ICR1 hypomethylation in patients with SRS in 2005.

CLINICAL IMPLICATIONS

The cellular mechanism of a genetic defect manifests as a recognizable clinical pattern (Table 1). SGA birth with postnatal growth failure suggests a defect in IGF-I action, primordial dwarfism, or SRS. Disproportionate short stature suggests a growth plate defect, such as *SHOX*, *FGFR3*, *NPR2*, *IHH*, or *ACAN* variants, and a skeletal survey may be helpful [2]. Advanced bone age in a child with familial short stature suggests an *ACAN* variant [21]. The pattern of GH, IGF-I, IGFBP-3, and ALS levels helps distinguish defects along the GH–IGF-I axis [2].

The 2026 international guideline recommends genetic testing in a short child with any of the following: major malformations and/or neurodevelopmental disorders; a family history suggesting autosomal dominant, autosomal recessive, X-linked, or mitochondrial inheritance, or a height SDS much lower than that of both parents; severe short stature (height <-3 SDS); microcephaly; absolute or relative macrocephaly; or body disproportion (sitting height/height or arm span/height outside ± 2.5 SDS) [3]. Genetic testing is also recommended for children with syndromic features or clinical or radiographic skeletal abnormalities [3].

When a specific diagnosis is suspected, targeted testing, such as 11p15 methylation analysis for SRS or *SHOX* deletion analysis, is efficient. When the phenotype is nonspecific, gene pan-

el or exome analysis combined with copy number analysis is recommended [3]. The diagnostic yield is higher in children with severe short stature or additional features [5]. A genetic diagnosis provides prognostic information, predicts the response to GH or other therapies, guides surveillance for associated complications, such as cardiac disease in Noonan syndrome or cerebrovascular disease in MOPD II, and enables genetic counseling for the patient's family [2,3].

The limitations of genetic testing should also be recognized. Exome sequencing analyzes only protein-coding regions, which constitute approximately 2% of the genome, and its sensitivity for copy number variants (CNVs), particularly small CNVs involving one or a few exons, is limited [3]. It cannot detect imprinting or methylation defects and may miss variants in deep intronic or regulatory regions, such as deletions of the *SHOX* downstream enhancers, as well as low-level mosaicism [3]. Therefore, chromosomal microarray or multiplex ligation-dependent probe amplification for CNVs and methylation-specific testing for imprinting disorders should be added when clinically indicated. A negative result does not exclude a genetic cause when clinical suspicion is strong, and periodic reanalysis of sequencing data is recommended [3]. In addition, variants should be classified according to the ACMG/AMP guidelines, and a variant of uncertain significance alone should not be used to establish a genetic diagnosis; segregation analysis and functional studies may help clarify its significance [3].

CONCLUSIONS

Short stature is a genetically heterogeneous condition, and many children previously classified as having ISS carry monogenic defects. These defects disrupt growth plate function at different levels, including the GH–IGF-I axis, paracrine signaling and transcriptional regulation, the cartilage extracellular matrix, intracellular signaling, fundamental cellular processes, and genomic imprinting. Each mechanism is associated with characteristic findings in birth size, body proportions, bone age, and biochemical profile. A mechanism-based approach helps clinicians select appropriate genetic tests and interpret their results, while recognizing the limitations of each testing method. An accurate genetic diagnosis guides individualized treatment, predicts the response to GH therapy, and enables surveillance for associated complications and genetic counseling. Mechanism-based therapies, such as CNP analogs, demonstrate how an understanding of growth plate biology can be translated into precision medicine for children with short stature.

NOTES

Conflicts of interest

No potential conflict of interest relevant to this article was reported.

Ethical statement

Not applicable. This review article did not involve human participants or animals.

Author contributions

Conceptualization, literature review, writing–original draft, and writing–review & editing: Moon JE.

Use of artificial intelligence

During the preparation of this manuscript, the author used Claude Opus 5.5 (Anthropic) to translate the author's Korean draft into English, to edit the language, and to format the manuscript according to the journal's instructions. The tool was not used to generate scientific content, create figures, or select references. The author reviewed and verified all content, including the references, and takes full responsibility for the content of the publication.

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Beyond Copy-Number and Coding-Region Analysis: Resolving the Molecularly Unresolved Dystrophinopathy

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Exon-level copy-number analysis followed by sequencing of all 79 *DMD* exons and their flanking intronic sequence achieves a cumulative sensitivity approaching 99%, and no current protocol reaches 100% sensitivity. In clinical series, however, approximately 2–5% of patients with convincing dystrophinopathy remain molecularly unresolved after such testing. This review addresses that residual group. The reasons for failure lie in the architecture of the locus: more than 99% of the 2.22 Mb locus is intronic, dosage-based assays cannot detect balanced rearrangements, and short reads resolve repeat-embedded breakpoints poorly. Published studies identify two main categories — deep intronic splice-altering variants and complex structural variants — but their relative proportions across populations remain uncertain. The evidence required to classify such a variant as causative depends on its class: transcript analysis is effectively indispensable for a novel deep intronic variant, whereas a rearrangement that directly disrupts coding exons may be resolved from breakpoint, phenotype and dystrophin findings. I set out how splicing evidence enters classification under the ClinGen Sequence Variant Interpretation Splicing Subgroup recommendations, a stage at which the same RNA result can inadvertently be counted twice. I also set out how the evidence for a balanced structural variant must be assembled where neither the copy-number nor the splicing scoring framework covers it, and propose a staged approach to the unresolved patient. Resolving the variant is a precondition for assessing genotype-dependent therapeutic eligibility and for variant-specific carrier testing in the family.

Key words: Duchenne muscular dystrophy, Deep intronic variants, RNA splicing, Long-read sequencing, Variant classification

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INTRODUCTION

The European Molecular Genetics Quality Network (EMQN) best-practice guidelines report a cumulative sensitivity approaching 99% for exon-level copy-number analysis followed by sequencing of all 79 *DMD* exons and their flanking intronic sequence [1]. The same guidelines add that no molecular testing protocol currently reaches 100% sensitivity [1]. Most laboratories proceed stepwise, screening for exon-level copy-number change and moving to small-variant analysis only if the copy-number screen is negative [2]; others detect both copy-number change and small variants from a single sequencing run. The scope of the small-variant step varies among centers: Sanger sequencing of all 79 exons with their flanking intronic sequence in some, exome or targeted panel sequencing in others. This standard testing pathway is described in detail elsewhere and is not revisited here [1,2]. In clinical series, approximately 2%–5% of patients still have no molecular diagnosis after such testing [3]. These two percentages are not contradictory: the size of the residual group depends on assay design and on the referral setting. This review concerns the patients who remain unresolved after such testing.

A patient should be regarded as molecularly unresolved when three conditions hold. First, the clinical phenotype is convincing. Where a muscle biopsy has been performed, absent or reduced dystrophin provides strong supporting evidence.

Second, a copy-number assay with validated exon-level resolution across the locus has been negative. Third, sequencing of the coding region with its flanking intronic sequence has been negative. Each condition matters. Without the first, the appropriate next step is to reconsider the diagnosis rather than to pursue rare *DMD* variant classes. Without the second or the third, an apparently unresolved case may reflect incomplete or unvalidated testing rather than a variant class the assays cannot detect. If a copy-number method does not cover every exon, as multiplex PCR does not, or has not been validated at single-exon resolution, it can report a true single-exon deletion as normal [1,2]. If an apparent single-exon deletion was dismissed without independent confirmation, it should be re-examined, since the apparent deletion may conceal a pathogenic small variant, as described below [1]. A negative sequencing result is less informative if the sequencing did not include the intronic sequence flanking each exon. Such cases should be resolved at the assay level before the patient is considered molecularly unresolved.

The first condition is more important than is usually recognized. Other muscular dystrophies can produce a clinically indistinguishable presentation: a man followed for years with a diagnosis of Duchenne muscular dystrophy was found instead to have *FKRP*-related limb-girdle muscular dystrophy when a second muscle biopsy showed normal dystrophin and sequencing identified biallelic *FKRP* variants [4]. Dystrophin immunostaining usually distinguishes dystrophinopathy from these conditions, but not always, since abnormal dystrophin has been described as a secondary phenomenon in sarcoglycanopathy [5]. In three situations — where dystrophin deficiency was sought but not demonstrated, where it is equivocal, or where the clinical and protein findings disagree — the exome or genome data already available should be reanalyzed systematically before rare *DMD* variant classes are pursued. In 101 unsolved neuromuscular families, reanalysis resolved 17; in five of these the causative variant was intronic and lay in a gene other than *DMD* [6].

Leaving the variant unidentified now has greater clinical consequences than in the past, because both mutation-specific therapy and variant-specific cascade testing require a defined variant [1,2]. Zhao and colleagues described a family in which the proband was diagnosed by biopsy but neither multiplex ligation-dependent probe amplification (MLPA) nor exome sequencing identified the variant; carrier testing was therefore unavailable, and a second affected child was subsequently born before combined long-read and RNA sequencing provided the diagnosis [3]. The sections that follow consider first why the assays failed in such a case and then what would be required to identify the variant.

DIAGNOSTIC BLIND SPOTS OF EXON-CENTRIC TESTING

The failure modes follow from the architecture of the locus rather than from poor practice (Table 1). *DMD* spans more than 2.22 Mb, of which over 99% is intronic; the coding sequence of the full-length muscle isoform totals 11,058 bases across 79 exons [7].

Dosage-based assays detect only unbalanced events, regardless of size. A balanced inversion or translocation that disrupts the gene produces no change in copy number, so it is undetectable by MLPA and by array comparative genomic hybridization whether it spans a few kilobases or most of the chromosome. An event that separates *DMD* exons from their promoter without deleting them is equally undetectable by these methods.

Probe-based methods have an artifact of their own. An MLPA kit provides only one probe per exon across the 79 ex-

Table 1. Diagnostic blind spots of exon-centric testing in dystrophinopathy

Blind spot	Mechanism	Variant class missed
Exon-centric capture	More than 99% of the 2.22 Mb locus is intronic, and intronic interrogation is limited to the sequence immediately flanking each exon	Deep intronic variants creating cryptic splice sites, disrupting splicing regulatory elements, or introducing polyadenylation signals
Dosage-based detection	MLPA and array CGH register only unbalanced change	Balanced inversions and translocations; events separating exons from the promoter without deleting them
Probe-binding artifact	A sequence variant beneath a single MLPA probe causes allele dropout by preventing hybridization	Small variants that mimic single-exon deletions, and true single-exon events dismissed as artifact
Short-read alignment	Reads covering a breakpoint inside a repetitive element match many near-identical copies and cannot be uniquely aligned	Structural variant breakpoints; complex rearrangements; intronic repeat expansions

CGH, comparative genomic hybridization; MLPA, multiplex ligation-dependent probe amplification.

ons of the full-length muscle isoform. A sequence variant under a probe-binding site can prevent the probe from hybridizing, and the resulting allele dropout is indistinguishable from a single-exon deletion. The EMQN guidelines therefore require any apparent single-exon deletion, and likewise any apparent single-exon duplication, to be confirmed by an independent method before it is accepted or dismissed [1]. Array comparative genomic hybridization is less susceptible to this artifact, because most exon-level events are covered by several oligonucleotide probes rather than one [1].

Coding-region sequencing reaches only the intronic sequence immediately flanking each exon. Deep intronic variants that create cryptic splice sites, disrupt splicing regulatory elements, or introduce premature polyadenylation signals all lie outside the interval captured by panel and exome assays [3,7].

Short-read data resolve breakpoints within repetitive sequence poorly. Short-read sequencing can infer structural variants from read-pair, split-read, and coverage signals, but that inference requires the reads to be assignable to a unique position in the genome. Breakpoints inside a repetitive element such as a LINE-1 retrotransposon may therefore be missed or poorly resolved, and large intronic repeat expansions may be missed or inaccurately sized. Long-read sequencing largely overcomes this limitation by spanning the repetitive sequence together with its unique flanking regions [3].

A negative standard workup is therefore not strong evidence against dystrophinopathy. The variant classes that the standard workup cannot detect are those that remain possible in an unresolved patient. Where the diagnosis is clinically convincing, such a result is better explained by the limitations of the assays than by a wrong diagnosis.

WHAT IS FOUND IN UNRESOLVED CASES

If the negative result reflects the assays rather than the diagnosis, the question that follows is which variant classes those assays failed to detect. Several studies now answer that question directly.

Waddell and colleagues studied 13 affected males with elevated creatine kinase, from seven families in which MLPA and exome sequencing had failed to yield a diagnosis, and identified a causative *DMD* variant in every family. Two families carried deep intronic single-nucleotide variants causing inclusion of a pseudoexon — an intronic sequence aberrantly spliced into the mature transcript. The remaining five harbored structural rearrangements: an insertion of approximately 118 kb of chromosome 8 sequence into intron 43, and four inversions, the largest spanning 119.8 Mb of the X chromosome [8]. Such an insertion is readily missed by exon-centric assays, which interrogate *DMD* sequence rather than sequence inserted from elsewhere in the genome.

Okubo and colleagues analyzed muscle transcripts from 20 Japanese patients who had dystrophin deficiency on biopsy but no exon variant on standard testing, and detected an abnormal *DMD* transcript in 19 of them. Exonization of intronic sequence accounted for 15 of these; the remainder comprised exon skipping, aberrantly spliced and polyadenylated transcripts, and transcription termination. The genomic causes were intronic single-nucleotide variants, chromosomal rearrangements, and, in one patient, a nucleotide repeat expansion that terminated transcription within intron 62 [9]. Such repeat expansions are not reliably detected by exon-centric testing.

Pan and colleagues independently applied a transcript-led strategy to 13 males with a clinical diagnosis of Duchenne or Becker muscular dystrophy but no definitive molecular result. Muscle RNA sequencing showed aberrant splicing or expression in 12 of them, and targeted reanalysis of DNA then identified the underlying deep intronic variants and complex rearrangements [10]. These two transcript-led studies therefore converged on the same principle: transcript analysis can direct the subsequent genomic search.

The largest series applied targeted long-read sequencing together with muscle RNA sequencing to 50 biopsy-confirmed Chinese patients in whom MLPA and exome sequencing had been negative, and reached a molecular diagnosis in all 50 [3]. Thirty of the 50 patients (60%) carried deep intronic single-nucleotide variants that led to pseudoexon inclusion or exon skipping. In the remaining 20 (40%) the variant was a complex structural rearrangement: 13 inversions, 5 translocations, and 2 insertions or deletions. Twenty-three of the variants (46%) had not previously been recorded in disease databases.

The same series also illustrates mechanisms that conventional testing cannot detect: small intronic inversions that activate pseudoexons, large repeat-rich insertions that introduce a polyadenylation signal and truncate the transcript, and balanced rearrangements that disrupt *DMD* without changing copy number [3].

Across the published unresolved cohorts, two categories recur most often: deep intronic splice-altering variants and complex structural variants. These studies go beyond what the guidelines anticipated. According to the EMQN guidelines, combined copy-number and coding-region analysis misses

only deep and mid-intronic splicing variants, and complex rearrangements are rare [1]. In contrast, the cohorts above suggest that structural variants form a proportion of the residual group comparable to that of deep intronic variants, at least among patients ascertained by a convincing dystrophinopathy phenotype and negative standard testing. Their relative frequencies, however, should not yet be treated as established. The only series large enough to give proportions is single-center, ethnically homogeneous and biopsy-confirmed [3], and ascertainment, referral pattern and population would all be expected to influence which variant classes are found. Both categories are sufficiently common that a workup addressing only one of them will leave a substantial proportion of patients molecularly unresolved.

MATCHING THE TECHNOLOGY TO THE VARIANT CLASS

The available modalities are complementary rather than hierarchical, and the choice follows from which variant class is suspected (Table 2).

Short-read genome sequencing covers intronic sequence and therefore captures deep intronic single-nucleotide variants inaccessible to exome sequencing. It has two limitations. The first is interpretive: intronic variants are numerous, and prediction alone rarely establishes pathogenicity. The second is technical: because of the alignment problem described above, repeat-embedded breakpoints may remain unresolved even when the event is detected.

Long-read sequencing, targeted or genome-wide, directly addresses this alignment limitation. It also detects deep intronic single-nucleotide variants: in the largest series, targeted long-read sequencing identified both the intronic variants and the structural variants [3]. Its distinctive advantage, however, is that it resolves structural variants and repeat expansions at nucleotide resolution and determines phase — whether two variants lie on the same allele — both of which matter for complex rearrangements. In the same series, 13 inversions of 1.9 kb to 95 Mb were mapped to the base pair and confirmed by Sanger sequencing [3]. A targeted assay is generally less costly and less computationally demanding, but it interrogates only the selected region: it cannot identify a causative variant in another gene, a limitation that matters most when the diagnosis itself is in question. Genome-wide long-read sequencing removes that restriction, at correspondingly greater cost. Both forms require genomic DNA of high integrity (a stricter requirement than for MLPA or short-read sequencing) and specialized bioinformatics for alignment and structural variant calling. These requirements, together with cost and limited availability outside specialized centers, remain obstacles to routine clinical adoption [3].

Optical genome mapping detects large structural rearrangements, including balanced inversions and translocations, at a resolution substantially higher than that of conventional karyotyping. A single case illustrates what it adds beyond karyotyping. A boy with clinically diagnosed Duchenne muscular dystrophy and negative standard testing was found on karyotyping to have a paracentric X-chromosomal inversion, but karyotyping could not determine whether the inversion involved *DMD*. Optical genome mapping localized the proximal breakpoint to *DMD* intron 41 and the distal breakpoint to an intergenic region 18.9 Mb away, so that the inversion splits

Table 2. Contribution of each modality after a negative standard workup

Modality	Resolves	Limitations	Sample requirement
Short-read genome sequencing	Deep intronic single-nucleotide variants; many structural variants	Repeat-embedded breakpoints at nucleotide resolution; functional consequence	Genomic DNA (blood)
Long-read sequencing (targeted or genome-wide)	Deep intronic single-nucleotide variants; greatest advantage for repeat-mediated, balanced, or complex rearrangements, repeat expansions, breakpoint resolution, and phasing	Functional consequence; a targeted assay interrogates only the selected region; high-integrity DNA, cost, limited availability, and bioinformatic demands	High-molecular-weight genomic DNA
Optical genome mapping	Large structural rearrangements, including balanced inversions and translocations	Single-nucleotide variants; functional consequence	Ultra-high-molecular-weight DNA
RNA sequencing / RT-PCR	Pseudoexon inclusion, exon skipping, intron retention, transcription termination	The underlying genomic lesion	Muscle RNA; blood not a reliable substitute
Dystrophin immunohistochemistry/ western blot	Pattern and extent of protein loss by domain	The genomic lesion	Muscle biopsy

the transcription unit and separates the exons beyond intron 41 from the promoter [11]. Optical genome mapping does not detect single-nucleotide variants, however, and therefore addresses only the structural category.

The three genomic methods above identify the variant; whether it alters the transcript is a separate question. Transcript analysis — RNA sequencing or targeted reverse transcription PCR (RT-PCR) — answers that question directly, showing pseudoexon inclusion, exon skipping, intron retention, or transcription termination. Dystrophin immunohistochemistry and western blot answer a different question. They show how much protein is lost and from which domains, but not what lesion caused the loss. Their contribution to the evidence is discussed below. For transcript analysis, muscle is the reference tissue, and blood is not a reliable substitute: only low-level illegitimate *DMD* transcription occurs in lymphocytes, and the splicing pattern there may differ from that in muscle [1]. Muscle, however, is not always available. Molecular testing now usually precedes biopsy, so some unresolved patients have never had one, and tissue stored years earlier may no longer yield usable RNA. Where no suitable material exists, two options remain. The first is a repeat biopsy taken specifically for transcript analysis; this may be considered once a candidate variant has been identified, but is considerably more difficult to justify as a speculative procedure in a child. The second is a surrogate tissue; several have been explored, but none has been validated against skeletal muscle [1].

RNA AS FUNCTIONAL EVIDENCE: WHEN IS IT INDISPENSABLE?

Identifying a candidate variant does not determine how much evidence is then needed to classify it as causative. In the published unresolved cohorts, DNA and RNA analyses were routinely performed together [3,8–10]. As a universal requirement, however, this does not fit every variant class: the need for transcript evidence depends on how much the genomic finding establishes by itself (Table 3).

A novel deep intronic single-nucleotide variant provides limited evidence on its own. A single base has changed within an intron that may exceed 100 kb; the same intron also contains many rare variants with no functional effect; and in silico tools predict an effect on splicing rather than demonstrate one. Transcript evidence supplies what the genomic position alone cannot establish: it shows the abnormal mRNA. Without transcript evidence, such a variant will usually remain of uncertain significance. For this variant class, RNA analysis is therefore effectively indispensable. The published series show this in practice: the genomic result alone did not establish the consequence, and transcript analysis was needed to do so [3,8–10].

A structural variant that does not cut the gene in two presents a similar problem. An inversion or an insertion that lies entirely within one intron leaves the promoter and all 79 exons in their normal order; a rearrangement whose breakpoints both lie outside the gene leaves it untouched. Such a variant is suspicious because of where it is, but its position does not show whether it changes the transcript: an intronic rearrangement may do nothing, or it may create a new splice site or a premature polyadenylation signal, and the DNA sequence alone cannot tell which. Transcript analysis is therefore usually decisive.

A rearrangement that directly disrupts coding exons, or separates them from the promoter, is itself direct evidence of gene disruption: the genomic architecture accounts for the absence of dystrophin without further inference. In this situation three findings may together be sufficient: breakpoints characterized at nucleotide level, concordance with the clinical phenotype, and a compatible pattern of dystrophin loss on protein stud-

Table 3. Evidentiary requirement according to variant class

Variant class	What the genomic finding establishes on its own	Role of transcript evidence
Novel deep intronic single-nucleotide variant predicted to create a cryptic splice site or activate a pseudoexon	Limited: the genomic position alone does not establish a functional effect, the interval contains many rare variants with no functional effect, and prediction is not demonstration	Effectively indispensable; without it the variant will usually remain of uncertain significance
Structural variant that does not cut the gene in two: inversion or insertion lying entirely within one intron, or a rearrangement whose breakpoints lie outside the gene	Little on its own: the variant is suspicious because of its position, but position does not show whether the transcript is changed	Usually decisive; without it the variant remains a candidate
Rearrangement directly disrupting coding exons, or separating them from the promoter	Direct: disruption of the coding exons, or their separation from the promoter, is in itself strong evidence of loss of function	Supportive rather than obligatory, where breakpoints, phenotype and dystrophin findings concur

ies. Transcript analysis is then supportive rather than obligatory. Where no suitable muscle RNA remains, insisting on it would only delay the diagnosis.

FROM DETECTION TO CLASSIFICATION

Classification is the step most often left implicit in reports of novel non-coding *DMD* variants. The original ACMG/AMP framework gives limited guidance on how predicted and experimentally observed splicing effects should be combined; the ClinGen Sequence Variant Interpretation (SVI) Splicing Subgroup has since published recommendations for how each should be weighted within that framework [12]. Two points are of particular relevance to a non-coding *DMD* candidate variant.

The first is that RNA evidence modifies the strength of PVS1 rather than entering as generic functional evidence. Where the observed aberrant transcript causes loss of function — by introducing a premature termination codon, or by removing clinically relevant protein sequence — the subgroup proposes that this evidence be captured by PVS1_Strength, a graded form of the loss-of-function criterion. The criterion is further annotated, for example as PVS1_Strength (RNA), to mark a call based on an assay rather than on prediction alone.

The second is that the same splicing evidence should not be counted again elsewhere. PS3 and BS3, the functional-evidence criteria, are reserved for well-established assays that measure an effect which the splicing assay itself does not capture. The in silico prediction that raised the suspicion is replaced by the experimentally derived weight rather than counted in addition to it. The evidence is overstated when a report describes an RNA result and then also applies a general functional criterion, or when it retains the in silico prediction code alongside the RNA-derived weight. Because later decisions are based on the classification rather than on the raw result, double-counting can affect downstream clinical interpretation.

Structural variants are less fully addressed by these recommendations. The ACMG/ClinGen technical standards score copy-number gains and losses [13], and the ClinGen splicing recommendations score splicing evidence. A balanced inversion or translocation changes no dosage, and it need not act through splicing, so neither document offers a framework for scoring it.

Where no framework applies, the evidence has to be assembled case by case, and the elements are the same as those set out in the previous section (Table 3). The genomic finding is considered first. A rearrangement that breaks the coding sequence, or separates the coding exons from the promoter, is strong evidence by itself; a rearrangement confined to one intron, or lying outside the gene, is not sufficient evidence on its own. The transcript result then supplies what the genomic finding lacks, and how much it is needed depends on that finding. Finally, the predicted consequence is compared with the dystrophin and clinical findings. The published cases show the range of practice: in the series of Waddell and colleagues, structural variants were confirmed by transcript analysis of muscle RNA [8], whereas the balanced inversion described above, which separates the exons beyond intron 41 from the promoter, was accepted as the diagnosis on the breakpoint and the phenotype alone, without transcript or protein studies [11].

The last of these elements, concordance with the dystrophin findings, applies to every variant class and is considered in more detail here. Dystrophin immunostaining shows how much protein is lost and from which domains; an aberrant transcript predicts what that pattern should be. If the transcript carries a premature termination codon and is degraded by nonsense-mediated decay, little protein is made, and staining for every domain should be uniformly absent or markedly reduced. If the transcript escapes decay and a truncated protein is made, staining should be lost only for the domains beyond the truncation point. In a patient who has already had a muscle biopsy, the prediction can be checked against the existing immunostaining without any further investigation. Agreement supports the candidate variant; disagreement means that the candidate should be re-examined.

CLINICAL CONSEQUENCES OF RESOLVING THE VARIANT

Molecular diagnosis is now a requirement for eligibility for several disease-modifying approaches: exon-skipping agents are prescribed according to exon amenability, and gene-transfer therapy requires a confirmed *DMD* mutation. An unresolved patient is therefore ineligible for the agents that require a defined mutation, and exon amenability cannot be assessed. Individual indications and age restrictions change over time and are not reviewed here. Beyond the approved agents, deep intronic variants that activate pseudoexons may themselves become targets for individualized antisense oligonucleotides designed to suppress the aberrant exon inclusion [14,15]. Such treatment is developmental rather than established, but defining the variant increasingly has therapeutic as well as di-

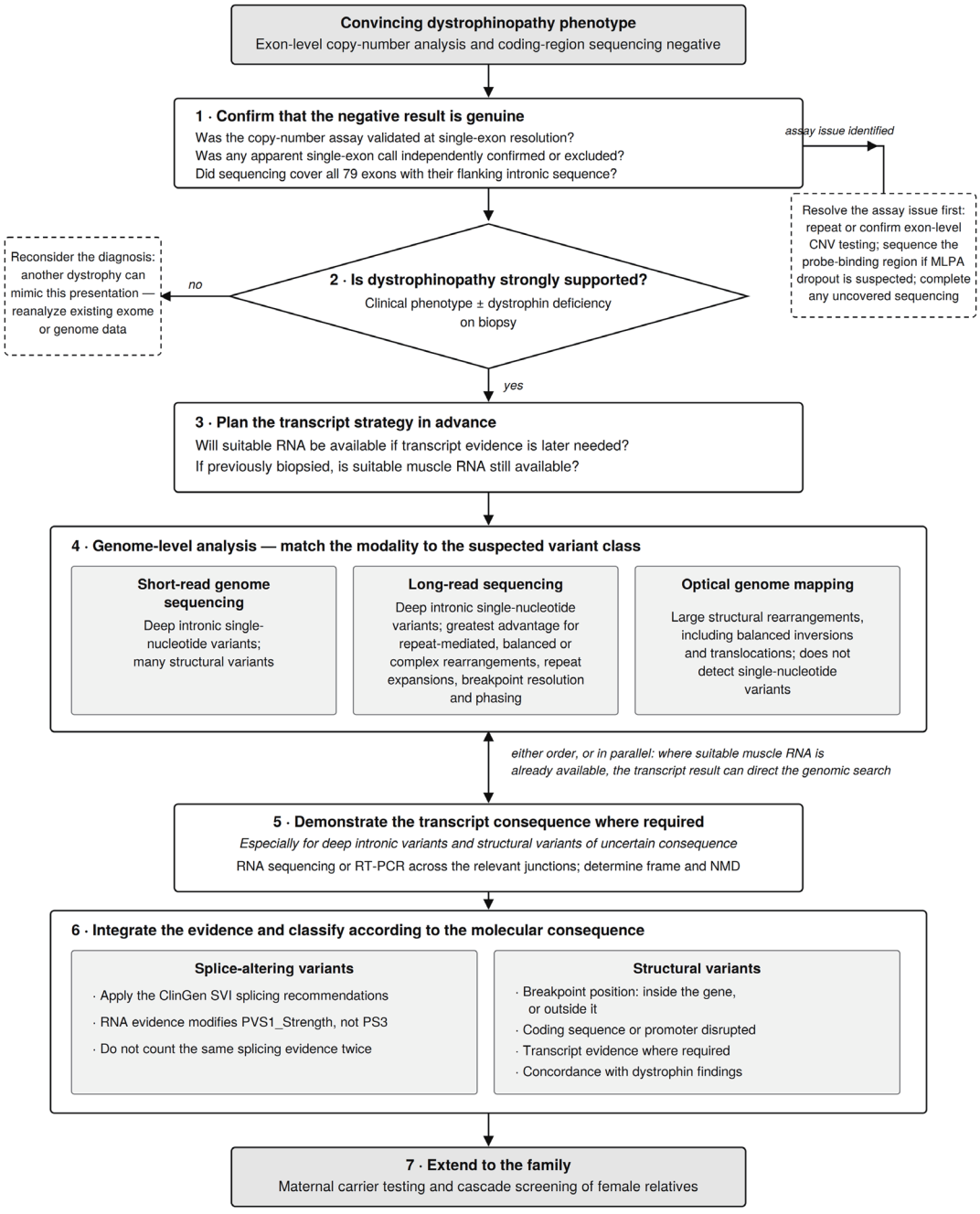


Fig. 1. Staged approach to the patient with a convincing dystrophinopathy phenotype in whom exon-level copy-number analysis and coding-region sequencing are negative. Steps 1 and 2 establish that the negative result reflects a variant the assays cannot detect rather than incomplete testing, and that dystrophinopathy remains the diagnosis under consideration; an assay issue at Step 1, or an unsupported diagnosis at Step 2, diverts the patient from this pathway (dashed boxes). Dystrophin deficiency on biopsy is supporting evidence at Step 2 rather than a prerequisite, since molecular testing is now usually requested before biopsy. Step 2 is needed because another muscular dystrophy can produce the same clinical presentation. Step 3 settles whether suitable RNA will be obtainable before a candidate variant emerges. Steps 4 and 5 are drawn in sequence but are not fixed in that order: where suitable muscle RNA is already available the transcript result can be obtained first and used to direct the genomic search, and the two analyses are often performed in parallel. Step 5 is conditional: it is needed where the genomic finding does not itself establish the consequence — usually for deep intronic variants and for structural variants of uncertain effect. Step 6 assembles the evidence and classifies it; the coding rules for splicing evidence, and the elements of evidence required for a structural variant, are given in the text. Step 7 extends the result to the family. NMD, nonsense-mediated decay; SVI, Sequence Variant Interpretation.

agnostic consequences.

Variant-specific carrier and cascade testing requires a defined familial variant; without one, only indirect approaches such as haplotype analysis remain, and these are feasible in only a few families [1]. Once the variant is defined, maternal carrier testing and cascade screening of female relatives become possible, allowing appropriate cardiac surveillance and informed reproductive counseling [1,16].

A POST-NEGATIVE APPROACH AND RESEARCH GAPS

Fig. 1 summarizes a staged approach. The first priority is to confirm that the negative result is genuine and that dystrophinopathy remains strongly supported; a patient whose testing was incomplete should be retested rather than investigated for rare variant classes. The availability of suitable RNA is determined in advance, so that transcript analysis remains possible when a candidate variant emerges. The genomic modality is then chosen according to the suspected variant class. Classification follows from the molecular consequence, and family testing proceeds once the variant is defined. Similar stepwise approaches incorporating RNA sequencing have recently been proposed [17].

Fig. 1 places genomic analysis before transcript analysis, but the order is not fixed: either analysis may be performed first. Where muscle RNA is already available, starting from the transcript is efficient: the aberrant splicing event defines the genomic region to be examined, and the search becomes targeted rather than exploratory. Both of the transcript-led studies cited above began in this way [9,10]. Where no muscle is available, or where a structural variant is suspected from the outset, the genomic analysis is the better starting point. In practice, the order depends on which material and which platforms are available, and the two analyses are often performed in parallel rather than in sequence.

Three questions remain open. The composition of the residual group in other populations is unknown: the published cohorts are few, and population differences are largely unexplored. Long-read sequencing and optical genome mapping have not been compared directly in this setting. The cost-effectiveness of a combined genomic and transcriptomic workup has not been formally examined. Leaving the case unsolved also has a cost: in the family described in the Introduction, a second affected child was born before the diagnosis was completed.

CONCLUSIONS

In the cohorts published so far — small, mostly biopsy-confirmed, and drawn from single populations — deep intronic splice-altering variants and complex structural variants account for most molecularly unresolved dystrophinopathies. It is not yet established whether the same distribution applies more widely, since cohort selection and population differences would both be expected to affect it. Short-read or long-read genome sequencing, with optical genome mapping for structural variants, can identify these variants, but pathogenicity must be established by evidence appropriate to the variant class. For a novel deep intronic variant, transcript evidence is effectively indispensable. For a structural variant that directly disrupts coding exons or separates them from the promoter, transcript evidence may be unnecessary if the breakpoint, phenotype and dystrophin findings concur. The remaining challenges are to define the composition of the residual group in other populations, to establish how long-read sequencing and optical genome mapping compare in this setting, and to establish the cost-effectiveness of this combined genomic and transcriptomic workup.

NOTES

Conflicts of interest

The author declares no conflicts of interest.

Use of artificial intelligence

During the preparation and revision of this manuscript, the author used Claude (Anthropic) to translate the author's draft into English and edit it, to draft revisions under the author's direction, including the response to reviewers, to render Figure 1 from the author's design, to help identify and verify references, and to format the manuscript according to the journal's instructions. The author decided the scientific content and the final wording, reviewed every passage prepared with the tool, verified all references against their original sources, and takes full responsibility for the content of the publication.

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Monogenic Causes of Short Stature in Children Initially Diagnosed with Idiopathic Short Stature

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Short stature is a common reason for referral to pediatric endocrinologists, yet its underlying cause often remains unidentified despite comprehensive clinical and biochemical evaluation. Recent advances in molecular genetic testing have revealed that a substantial proportion of children previously classified as having idiopathic short stature harbor pathogenic genetic variants. This review summarizes current genetic approaches to short stature and highlights major genes involved in growth plate regulation and the growth hormone–insulin-like growth factor 1 axis. Variants in genes such as SHOX, ACAN, NPR2, NPPC, IHH, and FGFR3 can disrupt growth plate chondrogenesis, whereas defects in GH1, GHR, IGF1, and IGF1R affect the GH–IGF-1 pathway. In addition, mild syndromic disorders, including those associated with PTPN11 variants, may present primarily as short stature. Phenotypic features, including body disproportion, bone age, biochemical findings, and family history, can provide important clues for selecting appropriate genetic tests. However, because many affected children lack distinctive clinical features, multigene panels and exome sequencing are increasingly important diagnostic tools. Establishing a molecular diagnosis may reduce unnecessary investigations, improve prognostic assessment, guide treatment decisions, and enable more individualized management. Genetic testing is therefore likely to become an increasingly integral component of the evaluation of children with unexplained short stature.

Key words: Short stature, Idiopathic short stature, Genetic testing, Next-generation sequencing, Growth disorders, High-Throughput Nucleotide Sequencing

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INTRODUCTION

Short stature (SS) stands as the leading reason children are referred to pediatric endocrinologists. It is conventionally defined as a height falling more than 2 standard deviations below the age-, sex-, and population-specific mean—a threshold that applies to approximately 2.3% of children [1]. Height is shaped by a range of genetic contributors, including chromosomal anomalies, pathogenic single-gene variants, and polygenic predisposition [2]. To pinpoint an underlying cause, children with SS are generally subjected to thorough clinical, biochemical, and imaging assessments. Nonetheless, a considerable number remain without a definitive diagnosis even after exhaustive evaluation [3]. This group spans both complex cases—marked by skeletal abnormalities, dysmorphic traits, neurodevelopmental delay, or congenital anomalies—and those whose short stature occurs in isolation, without any other notable clinical findings. An increasing body of research points to a genetic basis underlying many of these cases [4]. Monogenic etiologies of growth failure have been well documented for years, particularly among GH–IGF axis abnormalities, skeletal dysplasias, and syndromic disorders. Advances in molecular genetic techniques over the past three decades have markedly widened the scope of diagnostic evaluation, making genetic testing more accessible, affordable, and feasible for routine clinical use [5]. Early approaches centered on analyzing individual genes, but the advent of next-generation sequencing (NGS)—

encompassing exome sequencing (ES) and genome sequencing (GS)—has transformed genetic diagnosis across virtually every medical field. Notably, these technologies, previously confined largely to research applications, are now being incorporated with growing frequency into routine clinical care [6]. Against this backdrop, a considerable number of investigations have examined how effective genetic testing is at establishing a diagnosis in children presenting with short stature of unexplained origin [7,8]. This review aims to provide an overview of the genetic causes of short stature, with a particular focus on genes associated with growth disorders and their clinical phenotypes. In addition, we discuss the clinical implications of genetic testing in children with short stature to facilitate appropriate genetic evaluation and diagnosis (Fig. 1).

Exploring genetic approaches to short stature

Conventional diagnostic approaches to short stature in children have primarily emphasized hormonal abnormalities, chronic illness, and a limited set of genetic conditions. Earlier studies, however, suggest that routine extensive screening offers relatively low yield for identifying an underlying cause in children with short stature who otherwise appear asymptomatic [9]. Genetic causes of linear growth disorders identified to date are remarkably heterogeneous, involving a diverse array of cellular pathways; broadly speaking, these variants exert their effects by disrupting chondrogenesis at the growth plate [10].

When biochemical workup proves unremarkable and no distinctive phenotype is apparent, pinpointing the cause of short stature through history and physical examination alone becomes challenging. Under these circumstances, clinicians often turn to genetic testing—karyotyping to evaluate for Turner syndrome, chromosomal microarray to detect structural chromosomal abnormalities, targeted gene panels when specific genes or associated features are suspected, and ES for more extensive genetic evaluation. Beyond parental height measurements, evaluation of the extended family pedigree can assist in differentiating monogenic from oligogenic or polygenic etiologies, particularly in cases where one parent is also short-statured or where short stature follows an X-linked inheritance pattern among male relatives.

- When both parental heights fall in a low percentile and the patient's height deficit mirrors parental height (or predict-

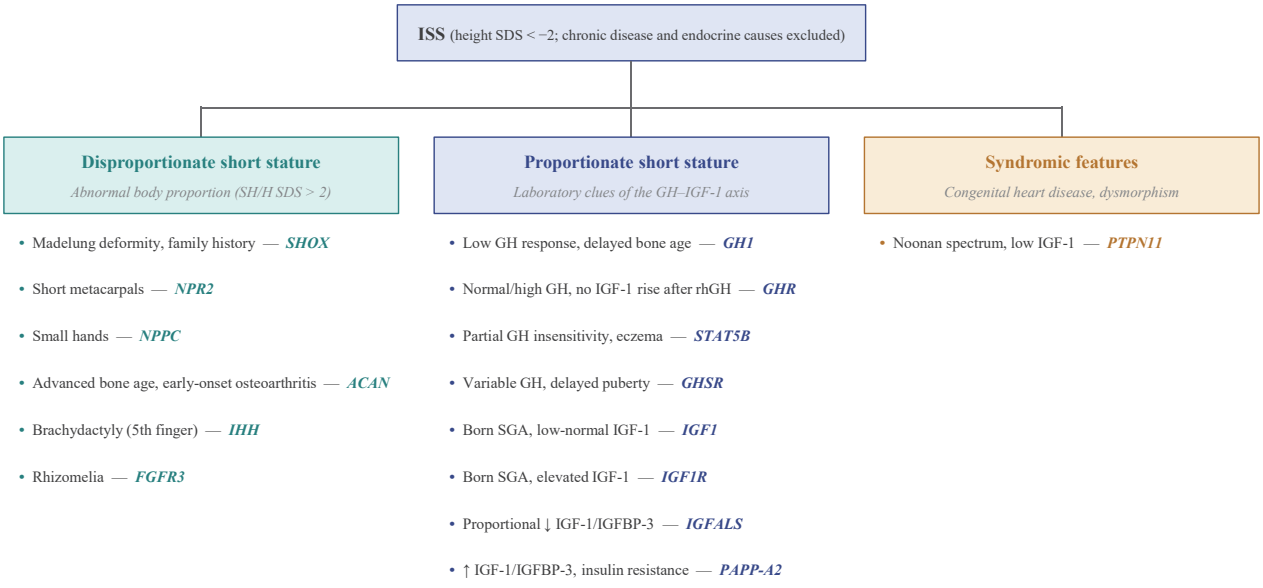


Fig. 1. Proposed diagnostic approach to the genetic causes of isolated short stature (ISS), based on associated clinical and laboratory clues. Children with ISS (height SDS <−2 after chronic systemic and endocrine causes are excluded) are grouped into three phenotypic categories that guide genetic testing. (Left) Disproportionate short stature (sitting height/height SDS >+2) suggests a growth plate disorder: (Middle) Proportionate short stature is evaluated with laboratory markers of the GH–IGF-1 axis: (Right) Syndromic features, such as congenital heart disease and dysmorphism, suggest a Noonan spectrum disorder. *ACAN*, aggrecan; *FGFR3*, fibroblast growth factor receptor 3; GH, growth hormone; *GHR*, GH receptor; *GHSR*, GH secretagogue receptor; IGF-1, insulin-like growth factor 1; *IGF1R*, IGF-1 receptor; *IGFALS*, IGF-binding protein acid-labile subunit; IGFBP-3, IGF-binding protein 3; *IHH*, Indian hedgehog; *NPPC*, natriuretic peptide precursor C; NPR2, natriuretic peptide receptor 2; *PAPPA2*, pregnancy-associated plasma protein A2; *PTPN11*, protein tyrosine phosphatase non-receptor type 11; rhGH, recombinant human GH; SDS, standard deviation score; SGA, small for gestational age; *SHOX*, short stature homeobox; SH/H, sitting height/height.

ed adult height (PAH) approximates midparental height (MPH)), familial short stature (FSS) is likely—though its genetic basis is often elusive, as it typically reflects the cumulative, incremental effects of multiple genes rather than a single variant. By contrast, a more pronounced deficit (< −2.5 SDS) despite similarly short parents raises suspicion for an underlying monogenic cause [11].

- A height deficit matching one parent's suggests autosomal dominant inheritance of the causal variant, often with multiple affected relatives across the extended family—as seen with *ACAN* or *SHOX* mutations [12,13].

- When both parents are of normal stature, yet the child has ISS, several possibilities merit consideration. Marked bone age delay, a family history of delayed puberty, and a PAH approximating MPH point toward CDGP. Otherwise, a genetic cause—potentially *de novo*, autosomal recessive, or X-linked (in boys)—should be considered [14].

Genes involved in growth plate regulation

Endochondral ossification is an intricate process occurring within the growth plate that drives bone elongation and the resulting increase in stature. It encompasses chondrocyte proliferation, hypertrophy, and senescence, along with synthesis of the cartilage matrix [15].

SHOX

SHOX, located in the pseudoautosomal region (PAR1) on the short arms of the X and Y chromosomes, regulates growth plate chondrogenesis and represents the most common monogenic cause of isolated short stature (~2.6% of nonsyndromic cases). Phenotype ranges from isolated short stature to Léri-Weill dyschondrosteosis in heterozygotes and Langer mesomelic dysplasia in homozygotes, typically due to gene or enhancer deletions. Disproportionate body segments (elevated sitting height/height ratio) are a key clinical clue warranting *SHOX* screening [16].

ACAN

ACAN encodes aggrecan, a key structural proteoglycan in growth plate and articular cartilage. Heterozygous mutations cause isolated short stature marked by advanced (rather than delayed) bone age, proportionate short stature, and growth impairment evident even prenatally. A blunted pubertal spurt and early growth cessation further compromise final height, often with body disproportion in adulthood. Additional features include brachydactyly, mild midface hypoplasia, and early-onset osteoarthritis affecting the knees by the fourth decade. Advanced bone age—contrasting with the delayed bone age typical of ISS—serves as a key diagnostic clue prompting *ACAN* testing [17].

NPR2 and NPPC

CNP and its receptor NPR-B (encoded by *NPPC* and *NPR2*) regulate endochondral ossification by stimulating chondrocyte proliferation, differentiation, and matrix synthesis, acting partly through inhibition of the *FGFR3* pathway. While biallelic *NPR2* loss-of-function causes acromesomelic dysplasia type Maroteaux, heterozygous *NPR2* mutations—first identified in ISS in 2013—account for 1.8%–13.6% of cases, producing variable short stature, abnormal body proportions, and nonspecific skeletal findings without a clear selection criterion for screening. Heterozygous *NPPC* mutations, more recently described in two families (six carriers), were associated with height SDS between -4.3 and -2.3 and a consistent small-hands phenotype [18,19].

IHH

IHH, expressed in prehypertrophic chondrocytes, is a key paracrine regulator of chondrocyte proliferation and differentiation; homozygous mutations cause acrocapitofemoral dysplasia, while heterozygous mutations cause brachydactyly type A1 (BDA1), though short stature is not a consistent BDA1 feature. In 2018, heterozygous *IHH* mutations were identified as a cause of growth impairment in 3.4% of an ISS cohort, without classical BDA1 findings. The predominant radiographic feature was shortening of the middle phalanx of the fifth finger (brachymesophalangia-V), present in 64.3% of affected hands—markedly higher than the 12.1% population prevalence. Affected individuals typically showed mild, progressively disproportionate short stature, with some born small for gestational age by birth length [20].

FGFR3

FGFR3 negatively regulates growth plate chondrogenesis; heterozygous gain-of-function mutations cause achondroplasia (the most common skeletal dysplasia, marked by rhizomelic disproportionate short stature) and the milder, more variable hypochondroplasia. While *FGFR3* alterations are rare among ISS-classified patients, some mild cases may go undiagnosed initially, and a previous recent study identified an *FG-*

FR3-activating mutation causing autosomal dominant proportionate short stature without other distinguishing features [21].

Genetic regulators of the GH-IGH1 axis regulation

Since GH and insulin-like growth factor 1 (IGF-1) were first identified as the primary regulators of longitudinal growth, early genetic studies of short stature focused on identifying defects within the GH–IGF-1–bone axis. GH, secreted by the pituitary gland, promotes bone elongation chiefly by regulating circulating and local IGF-1 synthesis. Growth failure can arise from defects at multiple points along this cascade, spanning from GH synthesis to IGF-1 action. Notably, affected individuals may lack the classical features of GH/IGF-1 deficiency or resistance, in which case they are often classified as having ISS.

GH1

GH1 defects—typically deletions or truncating mutations, though missense and promoter variants also occur—classically cause isolated GH deficiency (IGHD), presenting with proportionate short stature, low growth velocity, low IGF-1, and inadequate GH stimulation response; heterozygous/homozygous variants can also produce biologically inactive GH, phenotypically similar but with normal or elevated GH levels. However, milder *GH1* defects may lack these typical diagnostic features, as illustrated by a reported promoter mutation associated with ISS, leaving the underlying etiology undefined after standard workup [22].

GHR

GH acts via the GH receptor (*GHR*); homozygous or compound heterozygous *GHR* mutations cause complete GH insensitivity (Laron syndrome), marked by extreme short stature, normal/high GH, markedly low IGF-1 and IGFBP-3, and no IGF-1 rise after GH stimulation. Heterozygous *GHR* mutations produce a milder, more variable spectrum of GH insensitivity—only seven cases with a dominant-negative effect and recognizable partial GHI features have been reported, while broader ISS cohort studies report heterozygous *GHR* variants at 5%–15.5% frequency, though causality for many remains unproven. Low GHBP levels have been proposed as a marker of extracellular *GHR* domain defects in these patients. Given the subjective nature of clinical characterization, less deleterious heterozygous *GHR* mutations may underlie growth failure in a subset of ISS children with laboratory findings suggestive of partial GH insensitivity [23].

IGF1 and IGF1R

IGF-1, the principal growth factor in both prenatal and postnatal growth, signals through its cell-surface receptor *IGF1R*; homozygous *IGF1* deletions or loss-of-function mutations cause severe pre- and postnatal growth failure, microcephaly, and intellectual disability, with extremely low IGF-1 despite normal/high GH [24]. Heterozygous *IGF1* carriers show milder but significant short stature and reduced head circumference relative to unaffected relatives, with maternal transmission associated with greater height compromise, possibly reflecting placental dysfunction—as illustrated by a 2014 case of heterozygous *IGF1* deletion presenting as ISS with postnatal growth failure and low-normal IGF-1. Heterozygous *IGF1R* defects produce a comparable phenotype but with relatively elevated IGF-1 levels; although rare, presentation can be subtle, with short stature sometimes the sole clinical finding [25].

Other gene

PTPN11

PTPN11 encodes SHP2, a phosphatase central to several intracellular signaling pathways, including Ras/MAPK; activating heterozygous mutations cause Noonan syndrome (NS), featuring postnatal growth impairment, congenital heart disease, and facial dysmorphism, often with low IGF-1 reflecting partial GH insensitivity. As with other growth-related genes, phenotypic expression is highly variable—even within the same family—and craniofacial features evolve with age, making clinical diagnosis of NS challenging at times. Notably, pathogenic *PTPN11* variants have also been identified in children classified within ISS cohorts, underscoring the diagnostic overlap between mild NS and idiopathic short stature [26]. In addition to *PTPN11*, other genes involved in the RAS/MAPK signaling pathway, including *SOS1*, *RAF1*, and *KRAS*, contribute to growth impairment in patients with RASopathies. Furthermore, genetic defects affecting chromatin remodeling (e.g., *ARID1B* and *CHD7*), epigenetic regulation (e.g., *KMT2D* and *KDM6A*), DNA repair (e.g., *BLM*), and endosomal trafficking (e.g., *VPS33B* and *VIPAS39*) have been associated with short stature. Although these disorders often present with additional syndromic features, short stature may be an early or prominent clinical manifestation, highlighting the diverse molecular mechanisms underlying genetic growth disorders [27].

CONCLUSION

Childhood linear growth is governed by a complex array of

factors, and a substantial proportion of children classified as having ISS in fact harbor an underlying genetic cause.

Advances in molecular technology—particularly next-generation sequencing—are increasingly enabling identification of pathogenic variants even in mild phenotypes historically mistaken for ISS, despite the still-limited catalog of known causative genes. Establishing a genetic diagnosis carries practical value: it can spare children unnecessary testing and inform expected response to recombinant human GH (rhGH)—for instance, *SHOX*-deficient patients generally respond well to rhGH, whereas those with milder *GHR* defects tend to respond less robustly at standard doses. Emerging therapies may further personalize treatment, as the CNP analog vosoritide—already explored in achondroplasia—holds potential for children with *NPR2* or *NPPC* mutations. Because these conditions typically lack distinctive clinical features, candidate-gene testing alone is often inadequate, making multigene NGS panels or exome sequencing (prioritizing genes already linked to ISS) the preferred diagnostic strategy. A recent systematic review of 134 studies reported that the diagnostic yield of genetic testing in children with short stature varied according to the testing method, ranging from 4.7% for single-gene testing to 16.3% for chromosomal microarray, 21.6% for targeted gene panels, and 33.3% for exome sequencing. Notably, the diagnostic yield of exome sequencing was substantially higher in children with syndromic short stature (50.8%) than in those with isolated short stature (15.1%). Among children with isolated short stature, the most frequently identified genes included *PTPN11*, *ACAN*, *NPR2*, *COL2A1*, *SHOX*, *FGFR3*, *NF1*, *IGF1R*, *IHH*, and *COL11A1*, collectively accounting for 53.5% of genetically diagnosed cases. These findings highlight the importance of selecting appropriate genetic testing strategies based on clinical phenotypes [28].

According to the recent international guideline on genetic testing in children with short stature [29], genetic evaluation should be considered when clinical findings suggest an underlying genetic etiology. These include severe short stature (height <−3 SDS), disproportionate body proportions, skeletal abnormalities, dysmorphic features, neurodevelopmental disorders, and a family history suggestive of Mendelian inheritance. Genetic testing is also recommended in children born small for gestational age with persistent unexplained short stature when growth hormone treatment is being considered. Importantly, the absence of a family history does not exclude a genetic cause, as *de novo* variants may also contribute to growth disorders. Therefore, the selection of patients for genetic testing should be based on a comprehensive assessment of growth patterns, family history, physical findings, and associated clinical features rather than family history alone.

NOTES

Conflicts of interest

No potential conflict of interest relevant to this article was reported.

Ethical statement

Not applicable. This review article did not involve human participants or animals.

Use of artificial intelligence

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) to assist with English translation, language editing, and improving the clarity and readability of the manuscript. The tool was not used to generate scientific content or data. The authors carefully reviewed and verified the final manuscript and take full responsibility for its content.

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