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ARTICLE

Telethon Undiagnosed Disease Program: Structured approach to solving rare childhood-onset genetic diseases

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ABSTRACT

Purpose: Many children with severe genetic disorders remain undiagnosed despite advanced genomic technologies. Early diagnosis is vital for prognosis, genetic counseling, and targeted treatment development. This study aims to increase diagnostic rates in complex pediatric cases and foster research into disease mechanisms.

Methods: Launched in 2016, the Telethon Undiagnosed Diseases Program provides a structured, multicenter approach to rare disease diagnosis. Standardized case submission criteria ensured consistent clinical data collection. Children with severe, multisystemic disorders and prior negative genetic tests were eligible. After case approval, trio-based exome sequencing was performed, with regular reanalysis for unsolved cases until December 2024.

A list of authors of the Telethon Undiagnosed Disease Study group appears at the end of this article. Author Nicola Brunetti-Pierri is a GIMO editor and had no access to peer review as author.

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Results: Between June 2016 and December 2023, 1338 cases were submitted by 60 clinicians from 22 Italian centers; 1019 were accepted. A definitive genetic diagnosis was achieved in 49% of cases, implicating 330 genes. Most pathogenic variants (70.2%) were de novo, reflecting demographic trends, such as delayed parenthood. The remainder included autosomal recessive or X-linked variants, with homozygosity observed in 9% of patients.
Conclusion: The Telethon Undiagnosed Diseases Program significantly shortened the average diagnostic odyssey of ~8 years. Children born after 2016 benefited from faster diagnoses. This initiative offers a scalable, cost-effective model for improving diagnosis, guiding treatment, and supporting therapeutic innovation in rare pediatric diseases.

Introduction

Advancements in parallel sequencing, arrays, and bioinformatics have led to an unprecedented increase in the diagnosis and the identification of new Mendelian diseases.[1] This has, in turn, resulted in a better understanding of gene function and the development of targeted therapeutic approaches.[2] Severe pediatric-onset cases are mostly caused by single-gene pathogenic variants.[3] However, surprisingly, in the postgenomic era, the etiopathogenesis of a significant proportion of childhood-onset genetic disorders remains elusive.[4] Another major problem is specificity, with overdiagnosis due to misinterpretation of gene variants.[5] To overcome these problems and to provide a definitive diagnosis to several devastating childhood-onset diseases, we developed a strategy to optimize available resources to achieve diagnoses in the most severe undiagnosed diseases. The Telethon Foundation is a leading Italian charity that works to fight rare genetic diseases worldwide, with an excellent international reputation and a total investment of €660.3 million supporting 2960 research projects on genetic diseases over the last 30 years.[6] The Telethon Foundation has delivered results of global significance, such as the strategy that led to the Strimvelis and Libmeldly gene therapies for adenosine deaminase severe combined immunodeficiency (MIM #102700) and metachromatic leukodystrophy (MIM #250100), respectively.[7,8] In 2016, the foundation launched the Telethon Undiagnosed Diseases Program (TUDP), an initiative integrated with other undiagnosed diseases programs (UDPs) investigating rare diseases around the world, such as the US National Institutes of Health (NIH) UDP,[9] the Finding of Rare Disease Genes (FORGE) project in Canada,[10] the Initiative on Rare and Undiagnosed Diseases project in Japan,[11] the Undiagnosed Diseases Program-Western Australia,[12] and the Korean Undiagnosed Diseases Program,[13] all of which are part of the Undiagnosed Diseases Network International (UDNI). UDNI is a global network that established a consensus framework of principles, best practices, and governance to share scientific resources and expertise in undiagnosed diseases. The TUDP has its own specific focus, namely, the molecular resolution of severe undiagnosed pediatric cases that have already been extensively evaluated through routine genetic testing, including array comparative genomic hybridization (array CGH), targeted gene panel sequencing, and clinical exome sequencing (ES) made in singleton for 25% of cases. The TUDP prioritizes cases based on severe multisystem manifestations, neurologic involvement, and dysmorphic features. These eligibility criteria differ slightly from those of some of the other UDPs, and such differences might explain differences in terms of diagnostic approach, yield, and prevalence of identified pathogenic variants. Here, we describe the strategy used in the TUDP (Figure 1) and the results obtained to date.

Materials and Methods

The TUDP: National and international networks

The TUDP is coordinated by the Telethon Institute of Genetics and Medicine (TIGEM; www.tigem.it) in Pozzuoli, Italy, and a growing number of clinical genetics centers in Italy joined the program after a period of training. An online portal for the submission of undiagnosed patients was also created to receive individual cases from outside the network. In this instance, the patient was submitted by a physician and preassessed by one of the participating centers. In this first phase of the TUDP (TUDP 1.0 and TUDP 2.0), 22 clinical sites were included in the network and were involved in monthly clinical plenary meetings (CPMs) (Supplemental Table 1).

The TUDP is an active member of the UDNI and the European Commission-funded program "Solve-RD, solving the unsolved rare diseases" (http://solve-rd.eu/), which has also reanalyzed a number of phenotypically well-characterized genome/exome negative data sets from TUDP patients submitted through European Reference Networks.

Eligibility criteria and selection procedure

Most patients submitted to the TUDP presented with congenital anomalies, significant neurocognitive involvement, dysmorphic features, and/or multisystem

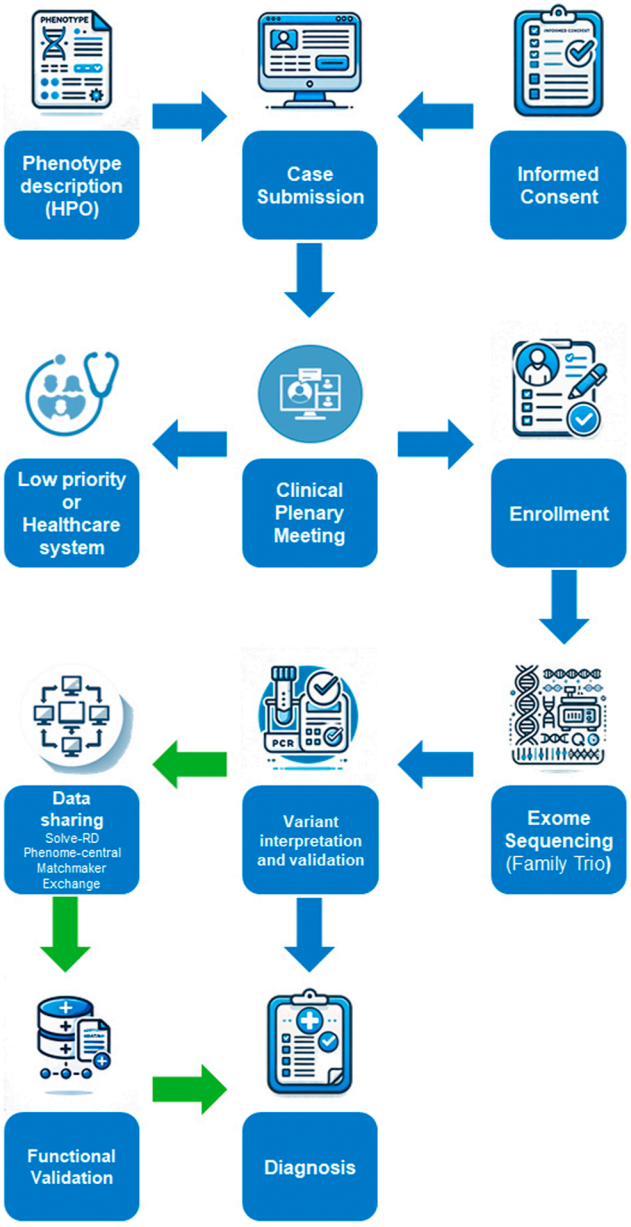

Figure 1 TUDP overview. The flow-chart illustrates steps of TUDP patient enrollment, including Human Phenotype Ontology (HPO) and informed consent acquisition, and monthly case discussion in clinical plenary meetings; samples processing by ES and data analysis on accepted families, leading to the generation of solved or unsolved reports. Arrows in green indicate an alternative path for unsolved cases, including data sharing, reanalysis and functional studies. ES, exome sequencing; TUDP, Telethon Undiagnosed Diseases Program.

manifestations. Requirements for enrollment were as follows: (1) proband age not above 18 years, (2) willingness of healthy biological parents to participate in the study, (3) complex phenotype, (4) complete set of phenotype data, (5) complete set of clearly negative genetic tests, including negative results from at least 180K array CGH, comprehensive gene panel sequencing (preferably clinical ES), and additional tests where indicated (eg, metabolic screening tests, Fragile X testing, or mitochondrial DNA testing). All cases were reviewed and filtered by a team of clinical geneticists from TUDP-affiliated centers, and filtered cases were then fully annotated with Human Phenotype Ontology (HPO) terms and presented in a standardized 5-slide PowerPoint format for discussion at the monthly CPM, attended by at least 1 member from each clinical center, TIGEM scientists, and the TUDP coordinator (Figure 1). During the CPM selection round, the completeness of previous testing was evaluated to categorize the presented cases as "undiagnosed." Cases were prioritized based on severity and specificity for monogenic diseases. About 250 families were annually enrolled. Those who did not meet the inclusion criteria (low priority) and showed evidence of a possible genetic condition were referred to the National Health Service for further investigation.

After acceptance at the CPM, the child's parents were asked to sign an informed consent form, and samples were sent to TIGEM for analysis. Quality controls were carried out promptly, and positive biological samples were processed. Approximately 8% to 10% of cases were lost at this stage.

Processing of personal data

A team from the Telethon Foundation assessed the ethical, legal, and financial aspects of informed consent and developed a model for data processing based on European Union General Data Protection Regulation compliance (https://gdpr.eu/). The informed consent developed and shared between participating centers listed a number of items, including (1) reasons for inclusion or exclusion from the study, (2) description of essential genetic tests, (3) name of person responsible for storing data and biological material, (4) explanation of how patients can withdraw from the study, (5) rules for data sharing and privacy, (6) expected results, (7) use of genetic diagnosis, (8) time frame for producing an initial report, (9) likelihood of a diagnosis, (10) possibility of unexpected results, including those unrelated to the condition under study, and (11) role of the Telethon Foundation in supporting patients after diagnosis. The informed consent form had to be signed at the time of enrollment and kept at the referral pediatric clinical center in accordance with the Declaration of Helsinki.

Sample handling and testing

All testing was centralized at the Telethon Foundation's in-house research institute, TIGEM, affiliated with the Vanvitelli University Hospital (Naples, Italy), providing economies of scale for clinical, genotyping, and bioinformatics analysis. DNA or RNA extraction, quality control, and quantification were performed. Different protocols were used as technologies were updated. For ES, enrichment was initially performed using SureSelect QXT Clinical Research Exome Kits (Agilent Technologies),

according to the manufacturer's instructions. A total of 178 trios were enriched with Clinical Research Exome v1 (CREv1, 54 Mb), 170 trios with Clinical Research Exome v2 (CREv2, 67 Mb), and all remaining trios (including repeats) with Human All Exon v7 (48.2 Mb) or v8 (41.6 Mb), which include mitochondrial probes. Negative cases with low coverage were systematically reanalyzed with the v8 enrichment. At baseline, sequencing was performed on a NextSeq500 system (Illumina) and, from 2019 onward, on an Illumina NovaSeq6000 system. Standard coverage for each family member was set at >95% coverage at >20×. For 25 families, short- and long-read genome sequencing (GS) was also performed.[14] A SurePrint Custom G3 Human CGH Microarray, 1 × 1M (Agilent Technologies) was used to confirm quantitative changes in exonic sequences of all diseases and candidate genes or to search for a second allelic variant. The bioinformatics pipeline and variant annotation protocol were set up as previously described and subsequently integrated with further improvements from TIGEM and Solve-RD.[15,16] An internal database was created to store sample information (personal data, genotypes, and phenotypes) and analysis output (retrieval of quality statistics, variant annotations). This database consists of about 8000 ES data sets and is part of the Network for Italian Genomes (www.nig.cineca.it). This network offers the advantage of comparing the frequency of variants from Italian patients because many variants have a different regional frequency distribution from that reported in international databases. In 2020 the TUDP pipeline was updated based on the new reference genome (GRCh38/hg38 December 2013; GeneBank #GCA_000001405.15).

Match Maker Exchange

The Match Maker Exchange platform (https://www.matchmakerexchange.org) was queried to connect clinicians and researchers around the world with an interest in the same gene/s. From the start of the project, deidentified patient phenotypic data from the TUDP were added to the PhenomeCentral web portal, a restricted access network for clinicians, researchers, and scientific consortia (including FORGE and the NIH UDP), to share phenotypic data with participating centers, and to find patients with similar phenotypes around the world and thus foster global collaborations. Patient data were subsequently shared using other tools, such as the Solve-RD Genome-Phenome Analysis Platform (GPAP), Decipher, and ClinVar.

Sanger sequencing

Sanger sequencing was performed to validate all confirmed pathogenic variants.

Sanger sequencing of the amplified fragment was performed using a BigDye v3.1 sequencing kit (Applied Biosystems) and a 3500 Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. The *RNU4-2* gene (HGNC:10193) was amplified by polymerase chain reaction using the following primers: 5'-GTTCCAACAACAAGAAACCTCC-3'; 5'-TCACGGAATACTCCTGAACAA-3'.

Results

Summary of TUDP cases

To identify possible pathogenic variants in undiagnosed cases, we recruited a total of 1308 families, low priority was assigned to approximately 10 % of cases (136), and 1172 families were accepted during the discussion at CPMs based on eligibility criteria. All enrolled patients had previously undergone array CGH and a targeted NGS panel related to the clinical suspicion at other centers. In some cases (about 25%), clinical ES was also carried out on singletons. ES was performed on 1019 TUDP families (a total of 1077 affected patients), who provided biological samples and informed consent (Table 1). About 13% of enrolled families did not undergo ES because patients either died, were diagnosed at another laboratory, or withdrew from the study. HPO terms for all analyzed patients were collected; the most frequent terms are listed in Supplemental Table 2. The average age at the time of recruitment was 9 years. For solved cases, the diagnostic odyssey time preceding TUDP inclusion was 8 years (±5.3 SD) (Figure 2A, Table 1).[17,18]

We diagnosed 48.8% of cases (497 families), a percentage higher than the reported diagnostic yields detected by similar programs (Table 1).[3] Pathogenic variants were found in 330 different genes (Supplemental Table 3), reflecting high genetic heterogeneity: 236 genes (71.5%) were involved in single cases (Table 1). Notably, 32 genes (9.7%) were found mutated in 3 or more families (Table 1, Figure 2B). Although most TUDP cases were diagnosed using trio ES, 8 cases (1.4%) were solved identifying copy-number or structural variants with the help of specific tools [*MECP2* (HGNC:6990), *EHMT1* (HGNC:24650)],[19,20] or single-exon-resolution molecular karyotyping [*AUTS2* (HGNC:14262), *SOX3* (HGNC:11199), *DEGS1* (HGNC:13709), *TBX6* (HGNC:11605)] or long-read GS [*NSD1* (HGNC:14234), *DEGS1*]. Two additional cases were diagnosed by short-read GS [*SPAST* (HGNC:11233), *AP1S2* (HGNC:560)] (Supplemental Table 4).

Spectrum of variants and mode of inheritance

A total of 58 compound heterozygous (11.5%), 41 homozygous (8.1%), 349 de novo (69.2%, including X-linked diseases), and 30 X-linked (5.9%, maternally inherited) pathogenic variants were identified in 504 solved TUDP cases, which included 7 cases with double diagnosis

Table 1 Demographic and diagnostic data from TUDP families

Total families	1019
Category	No.
TRIO	948
DUO	16
QUARTET	53
QUINTET	1
SEXTET	1
Diagnostic yield	No. (%)
Solved	497 (48.77%)
Unsolved	522 (51.22%)
Patient information	
Patient Age (average)	9 y
Male	614 (57%)
Female	463 (43%)
Total patients	1077
Mode of inheritance	**No. of Families (%)**
Autosomal dominant[a]	
De novo	319 + [4] (64.08%)
Inherited	12 (2.38%)
Autosomal recessive[a]	
Compound heterozygous	55 + [3] (11.51%)
Hom	41 (8.13%)
X-Linked[a]	
De Novo	25 + [1] (5.15%)
Maternal inheritance	30 (5.95%)
Two diagnoses[a]	
De Novo + X-linked	2 (0.39%)
Hom + De Novo	1 (0.19%)
Hom + Hom	2 (0.39%)
De Novo + De Novo	1 (0.19%)
AD (maternal + paternal)	1 (0.19%)
Gene occurrence	
Families per gene	No. of Gene (%)
1	236 (71.5%)
2	62 (18.8%)
3	17 (5.2%)
>3	15 (4.5%)
Total different gene	330
Reanalysis diagnostic yield	
	No. (%)
Solved	73 (17.18%)
Unsolved	352 (82.82%)
Total probands	425
Reanalysis outcome	
	No. (%)
Solve-RD collaboration	9 (12.32%)
New disease gene with TUDP contribution	12 (16.43%)
New disease gene discovered during TUDP	27 (36.98%)
Pipeline Update	25 (34.24%)

AD, autosomal dominant; *CNV*, copy number variation; *Hom*, homozygous; *SV*, structural variant; *TUDP*, Telethon Undiagnosed Disease Program.
[a]Single-nucleotide variant. Number in square brackets indicates SV and CNV.

(Table 1). Genotype-phenotype correlation also identified 14 pathogenic variants inherited from apparently healthy parents. Upon retrospective clinical evaluation, it was found that the parent who was heterozygous for the identified

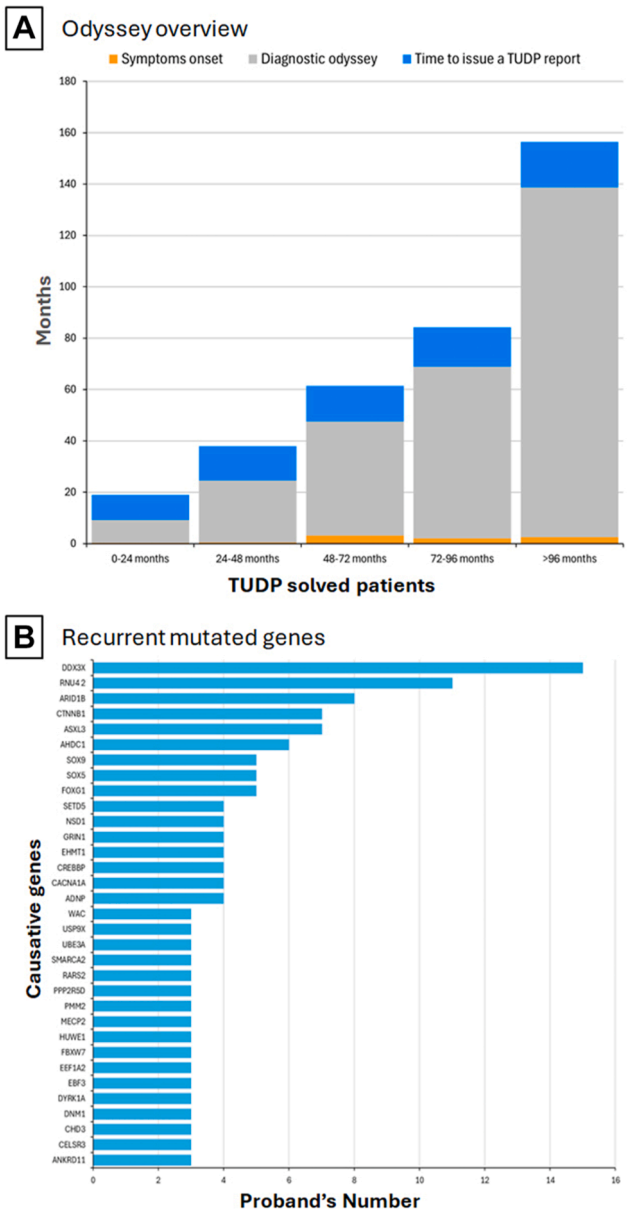

Figure 2 Patient diagnostic odyssey and most frequent causative genes. A. Odyssey overview. Distribution of patients' diagnostic odyssey across 5 age groups (months): 0-24, 24-48, 46-72, 72-96, and more than 96 months. The orange bar represents the interval from birth to the initial onset of clinical signs. The gray bar corresponds to the time before enrollment in the TUDP program without a molecular diagnosis, and the blue bar indicates the time from TUDP enrollment to a definitive molecular diagnosis. B. Recurrent mutated genes. The bar graph shows the list of causative genes that were identified in 3 or more families. TUDP, Telethon Undiagnosed Diseases Program.

variant was also mildly symptomatic. By far the most common type of pathogenic variant arose de novo in a single affected child with a negative family history. We found 183 de novo predicted loss-of-function protein-truncating variants (frameshift, nonsense) and 55 splice-site variants, whereas the remaining variants were protein-altering missense variants with loss- or gain-of-function

effects. Detailed information on solved TUDP cases (sex, age, genotype, and inheritance) is summarized in Supplemental Table 4. The prevalence of de novo variants was similar to that observed in the Deciphering Developmental Disorders study (69.2% vs 76%, respectively).[3]

We then compared parental age at the time of conception in the different classes of variants and found a more advanced paternal (36.38 years) and maternal (32.69 years) age for de novo variants compared with inherited pathogenic variants (34.80 years for the father and 30.37 years for the mother) ($P < .001$). This finding confirms the prediction of an increase in the occurrence of de novo variants in older parenthood.[21]

Matching for new genes and phenotypic expansion

In 33 families, we detected putative pathogenic variants in 30 novel candidate genes not previously associated with human disease. Match Maker Exchange platform allowed us to identify additional unrelated cases with a putative deleterious variant in the same gene and overlapping phenotypes. Sixteen of these genes were new discoveries identified with the contribution of the TUDP,[22-31] whereas the remaining 14 candidate genes are still being validated by in vitro and in vivo functional studies to support the pathogenicity, providing a definitive clinical diagnosis. However, most of the identified pathogenic variants were located in known disease genes (Supplemental Table 4). Among these, 31 original articles and 13 case reports illustrated phenotypic expansions identified by the TUDP. All the publications involving the TUDP are reported in Supplemental Table 5, including a recent study describing a therapeutic approach following diagnosis.[32] The contribution of HPO to the phenotype expansion was furtherly analyzed in the Supplemental Results section and Supplemental Figure 1.

ES reanalysis of unsolved cases

A systematic reanalysis was performed annually. As of December 2023, the reanalysis had only involved 425 cases that remained unsolved after the initial analysis. This increased the diagnostic yield by 17.2% (see Table 1). Raw trio sequencing data (FastQ) were reprocessed using the VarGenius analysis pipeline[16] based on the updated Genome Analysis Toolkit (GATK release 4.0.5). In addition, the remaining phenotypic and genomic datasets were processed using GPAP, an online tool for diagnosis and gene discovery in rare disease research developed as part of the Solve-RD project (https://solve-rd.eu).[33] Pathogenic variants were found in 73 reanalyzed patients, 9 cases (12.3%) were solved by the GPAP tool and 25 (34.2%) by the updated version of the VarGenius pipeline. In the remaining 39 reanalyzed cases (53,4 %), pathogenic variants were identified in genes associated with disease during the TUDP study (Table 1).[34-37]

Among these, we identified 11 probands with de novo variants in *RNU4-2*, recently described as causing a novel neurodevelopmental disorder (ReNU syndrome; MIM #620851).[36-38] Three variants were identified through whole-exome sequencing reanalysis, whereas the others were detected by Sanger sequencing screening of the entire unsolved cohort (see Supplemental Methods section).

Discussion

The TUDP is a multicenter program that aims to establish a sustainable standard for the diagnosis of rare and severe childhood-onset monogenic diseases that elude clinical and genetic recognition. We defined a strategy to achieve standardized clinical and genetic evaluation among all participating pediatricians and geneticists. The protocol provides for multiple levels of comparison and matching, with periodic reanalysis of unsolved cases and reports of clinical value to patients' families. The average age of accepted cases was 9 years; these patients had already spent an average of 8 years (±5.3 SD) in their diagnostic odyssey before being accepted into the TUDP, with peaks of up to a decade.[17,18]

For these families, the diagnosis has changed their lives, not only in terms of preconception counseling but, above all, in terms of giving a name to the disease and being part of a group of patients around the world who share the same desire to receive targeted therapy. The diagnostic odyssey has been significantly shortened since the launch of the TUDP. Individuals born from 2016 onward have been able to obtain a rapid and definitive diagnosis thanks to their immediate enrollment in this program. This can be contrasted with the diagnostic timelines for those born before the project's inception. Among the 497 solved cases, 151 had pathogenic variants in disease genes identified after the initiation of TUDP. In patients older than 8 years, 116 (76.8%) carried variants in these newly discovered genes, indicating that the diagnostic reporting time (approximately 18 months) was linked to the involvement of novel disease genes (Figure 2B).

The TUDP activities had the advantage of the Info_Rare portal, which was previously launched by the Telethon Foundation. Info_rare is a free online help service providing information to patients after a genetic diagnosis. Info_rare provides information on rare genetic disease centers, and diagnostic and referral centers for patient care, as well as news on scientific publications, ongoing genetic disease studies, and clinical trials. Patients with this support have sufficient information to join existing associations or to create new associations under the name of the gene responsible for the rare genetic disease.

The TUDP has achieved a genetic diagnosis in almost 49% of all enrolled patients. From 2016 to 2023, the diagnostic rate exhibited minimal fluctuations, as demonstrated in Supplemental Figure 2. This stability may be attributable to a number of factors, including the systematic

updating of bioinformatics pipelines and the reanalysis of unsolved cases. Additionally, the continuous identification of new disease genes has contributed to this stability. The TUDP program has played a significant role in this effort. The output of the study so far includes not only new diagnoses for patients but also 74 publications, the identification of 16 new disease genes, and the creation of specific calls for scientists to study rare genetic diseases (Supplemental Table 5).

Diagnosis rates for undiagnosed conditions vary widely between studies conducted around the world. In general, diagnostic rates tend to be higher in children than in adults, a difference that may be partly due to the greater multifactorial contribution in late-onset diseases (polygenic, immune, infectious, environmental, etc).[39] Recruitment of families after clinician-led preselection and routine genetic testing (eg, array CGH and clinical exome testing) resulted in a cohort that was depleted of most known syndromes and large structural variants. These criteria are common to other UDPs and reduce the apparent diagnostic yield compared with first-line testing and distort factors influencing the likelihood of receiving a diagnosis. Therefore, the diagnostic yield of this study is a conservative estimate, and a yield greater than 75% would be expected if trio GS were offered as a first-line strategy.[40-42] Other studies support the conservative nature of the reported diagnostic yields in preselected cohorts after routine screening.[42-44]

Considerable variation in diagnostic yield, ranging from 26% to 36%, was observed in the first programs for undiagnosed childhood diseases.[45,46] Detection rates may vary as result of technical, analytical, and knowledge differences. Technical differences are mainly due to exome enrichment and sequencing technologies, analytical differences to the bioinformatics pipeline, and the increasing number of new disease genes observed in recent years may also explain the higher diagnostic rate of UDPs in recent years.[47] Assuming that all phases were performed to optimal standards, the higher success rate of the TUDP (about 49%) compared with most-reported data from other programs could be the effect of both selection criteria and data reanalysis. All patients enrolled in the TUDP were severe, <18 years of age, were analyzed in trio, and had received a negative array CGH result. We believe that these selection criteria enriched our cohort of monogenic diseases. Only trio sequencing allows the identification of undescribed de novo pathogenic variants, which were those most commonly found in this study (70%). Although expected, such a high prevalence of de novo pathogenic variants as a consequence of increasing parental age at conception was striking. Aging increases the frequency of germline variants, and the cultural and social tendency to delay parenthood is likely to have several social implications in Western populations.[21] In contrast, the reduction of parental consanguinity in Western demographics will make homozygosity rarer and more limited to cases from countries in the Middle East, North Africa, and South Asia.[48] The inclusion of adult patients and adult-onset disorders in other programs, such as the NIH UDP, may explain their lower diagnostic rate.[49]

Despite the high diagnostic yield achieved by the TUDP, a significant proportion of patients remain undiagnosed. In about 1.4% of our cases, the genetic defect was identified by additive studies based on tools for structural variant calling from mapped paired-end sequencing reads, the use of high-resolution array CGH with single-exon coverage of all known disease-causing genes, specifically developed within the TUDP, and the use of long-read GS technology. This result is consistent with the emerging evidence that still elusive cases may be in part solved with a similar combined approach.[19,20,33] The hypothesized genetic defects underlying these cases include deep intronic splice variants, regulatory element variants, elusive complex structural changes, mosaicism, and polygenic disorders. All of these could be solved in the next few years with the introduction of future technologies, such as those providing end-to-end chromosome sequencing and full-length single-cell RNA sequencing. However, the "dark matter" of unsolved cases may have an additional explanation: the lack of answers may be primarily due to a lack of knowledge. The real number of human disease genes is certainly higher (at least 6000-13,000) than those currently described, and the majority remain unknown because there are too few affected cases, and their phenotype is too unspecific to establish causality.[50] These cases remain unsolved because no other patients with the same variant have been identified. If this hypothesis is correct, then the most cost-effective strategy will be to increase the number of patients who are trio-sequenced and to store the data for reuse. According to OMIM statistics, 150-200 new single-gene disorders have been identified annually in recent years. As this trend accelerates, the number of known disease genes will grow even faster. Indeed, having access to such a large repository of DNA and next-generation sequencing data from undiagnosed patients allowed us to rapidly resolve a significant number of cases, both through reanalysis of ES data and targeted analyses. A striking example is the recent discovery that the *RNU4-2* gene that has been recognized as one of the most common causes of syndromic neurodevelopmental disorders[36,37] and, in our case series, the second most frequently mutated gene after *DDX3X* (HGNC:2745) (Figure 2B).[38]

This study has implications for both clinical practice and health policy, ending the diagnostic odysseys for patients and providing correct preconception information. For example, extended preconception carrier screening will not effectively prevent most cases caused by de novo pathogenic variants. The information provided by this study should be used by national health systems to improve the accuracy and cost-effectiveness of diagnosis of rare pediatric diseases using our protocol. Unsolved TUDP cases represent a resource for the discovery of unknown mechanisms underlying genetic diseases by the next phase of the TUDP (TUDP 3.0) that includes further phenotypic and genetic analysis of unsolved TUDP cases using optical genome mapping, long-read GS, and RNA studies. Reported findings point to an increase of at least 5% to 10% of diagnosed cases,[51] but more conservative estimates suggest that a proportion of these variants could

also be detected by conventional short-read sequencing, if coverage-related loss of detection and algorithm-driven loss of accuracy could be avoided.

Data Availability

All data presented in this study are available upon request.

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Ethics Declaration

Informed consent was obtained from all participants to study or from their legal representatives. The study was approved by the Ethics Committee of the Federico II University Hospital in Naples, Italy (ID number 8635/19).

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Conflict of Interest

Andrea Ballabio is cofounder and shareholder of Casma Therapeutics and advisory board member of Avilar and Amplify Therapeutics.

Additional Information

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